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A comparative evaluation of the regulation of GM crops or products containing dsRNA and suggested improvements to risk assessments

Jack A. Heinemann [a,b,*], Sarah Zanon Agapito-Tenfen [b,c], Judy A. Carman [d,e]

[a] School of Biological Sciences, University of Canterbury, Christchurch, New Zealand
[b] Centre for Integrated Research in Biosafety, University of Canterbury, Christchurch, New Zealand
[c] Crop Science Department, Federal University of Santa Catarina, Florianópolis, Brazil
[d] Health and the Environment, School of the Environment, Flinders University, Australia
[e] Institute of Health and Environmental Research, Adelaide, South Australia, Australia

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ABSTRACT

Changing the nature, kind and quantity of particular regulatory-RNA molecules through genetic engineering can create biosafety risks. While some genetically modified organisms (GMOs) are intended to produce new regulatory-RNA molecules, these may also arise in other GMOs not intended to express them. To characterise, assess and then mitigate the potential adverse effects arising from changes to RNA requires changing current approaches to food or environmental risk assessments of GMOs. We document risk assessment advice offered to government regulators in Australia, New Zealand and Brazil during official risk evaluations of GM plants for use as human food or for release into the environment (whether for field trials or commercial release), how the regulator considered those risks, and what that experience teaches us about the GMO risk assessment framework. We also suggest improvements to the process.

1. Introduction

All commercialized genetically modified (GM) plants are currently created through *in vitro* DNA modification. Most are designed to create a new protein. However, a growing minority are designed to change their RNA content in order to regulate gene expression (Table 1). This is because RNA, specifically double-stranded RNA (dsRNA), is now known to be an important regulator of gene expression (Appendix 1 of Heinemann, 2009). In fact, in the future, GM products are likely to arise from only *in vitro* RNA modification rather than from *in vitro* DNA modification (Heinemann, 2009).

RNA is an intermediate molecule used in cellular reactions of protein synthesis. The most familiar form of RNA is mRNA, the single-stranded messenger. However, it is only just over a decade since the biochemistry of small dsRNA molecule has begun to be studied. This form can function as a gene regulator (Hutvágner and Simard, 2008).

dsRNAs include siRNA (short-inhibitory RNA), miRNA (microRNA), shRNA (short-hairpin RNA) and so on and are foundation substrates in biochemical pathways that cause RNAi (RNA interference), PTGS (co-suppression, post-transcriptional gene silencing) and TGS (transcriptional gene silencing). In short, RNAi, PTGS and TGS are what occur when the connection between genes and the production of the proteins specified by genes is disrupted.

dsRNAs form when both strands of a DNA molecule are transcribed to synthesize complementary RNA molecules (which then bind together in the same way as strands of DNA), or when stretches of intramolecular complementarity create stem-loop structures. A long dsRNA molecule (e.g., pre-mature miRNA) is processed into a shorter dsRNA (e.g., miRNA) and then one strand is retained – the guide strand – to direct protein complexes to target mRNA molecules and prevent their translation (cytoplasmic pathways), or to target and chemically modify DNA sequences by addition of methyl groups and cause modification of DNA-associated histone proteins (the nuclear pathway). The nuclear pathway is known to inhibit transcription and to seed heterochromatin formation (Ahlenstiel et al., 2012; Grewal and Elgin, 2007; Reyes-Turcu and Grewal, 2012; Zhang and Zhu, 2012).

Once a silencing effect is initiated, the effect may be inherited. The biochemistry of this process varies depending on the organism and remains an area of active research with many unknown aspects. Nevertheless, it is known for example that human cells can maintain the modifications necessary for TGS, creating actual or potential epigenetic inheritance within tissues and organisms (Hawkins et al., 2009). In some cases the dsRNA pathways induce RNA-dependent DNA methylation and chromatin changes (TGS) that persist through reproduction or cell division, and in other cases the cytoplasmic pathways remain active in descendents (Cogoni and Macino, 2000).

Unintended gene silencing is a common outcome of the genetic engineering process. Indeed, most cells initially engineered using *in vitro* nucleic acid techniques ultimately "silence" the gene inserted because of the engineering-associated production of dsRNA (Carthew

* Corresponding author at: School of Biological Sciences, University of Canterbury, Christchurch, New Zealand. Tel.: +64 3 364 2500.
E-mail address: jack.heinemann@canterbury.ac.nz (J.A. Heinemann).

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Table 1
Various GM crops with intended RNA changes in the food approval pipeline.

Product	Status	Ref/application code
Flavr Savr Tomato	Withdrawn from market	(Sanders and Hiatt, 2005)
High oleic acid soybean lines G94-1, G94-19 and G168[b]	FSANZ[a] approved (2000) withdrawn from market	A387
New Leaf Y and new leaf plus potatoes[c]	FSANZ approved (2001) withdrawn from production	A383 and A384
High oleic acid soybean line DP-305423-1	FSANZ approved (2010)	A1018
Herbicide-tolerant, high oleic acid soybean line MON87705	FSANZ approved (2011)	A1049
Golden mosaic virus resistant pinto bean	Brazil approved (2011)	(Tollefson, 2011)
papaya ringspot virus resistant papaya	USA (1996), Canada (2003) and Japan (2011)	USDA[d] GMO Compass[e]
altered grain starch wheat	OGTR[a] approved for field trials and human feeding study (2009)	DIR093[f]

[a] Food Standards Australia/New Zealand (FSANZ) http://www.foodstandards.gov.au/consumerinformation/gmfoods/gmcurrentapplication1030.cfm; Office of the Gene Technology Regulator (OGTR, Australia).
[b] "Withdrawn from [FSANZ] Standard 1.5.2 in 2011 because never commercialized."
[c] The way the virus protein gene used as a transgene causes resistance to the potato viruses (Y and PLRV) was unknown at time of approval. However, it is well known now that gene duplications (which occur when the virus infects the GM plant) cause silencing of both copies of the gene through RNAi.
[d] http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Japan%20approved%20GM%20papaya_Tokyo_Japan_12-19-2011.pdf.
[e] http://www.gmo-compass.org/eng/database/plants/59.papaya.html.
[f] http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/dir093.

and Sontheimer, 2009; Hannon, 2002; Weld et al., 2001). The new RNA sequence may be created when the DNA strand that is not normally used as a co-factor (or "template") for transcription is used as such. The resulting single-stranded RNA may bind to the target mRNA to create regions of linear dsRNA that can be processed into siRNA (Fig. 1). Another possibility is that the insert contributes to the formation of a stem-loop, from which the "stem" may be processed into an miRNA-like molecule (Fig. 1).

dsRNAs are remarkably stable in the environment; a property perhaps overlooked based on the relative instability of single stranded species of RNA (Parrott et al., 2010). Insects and worms that feed on plants that make dsRNA can take in the dsRNA through their digestive system, where it remains intact (Gordon and Waterhouse, 2007; Mao et al., 2007). RNAi has been induced through oral exposure in several insect pests (Chen et al., 2010; Whyard et al., 2009) and oral exposure to dsRNA has been shown to reduce the lethal effects of the Israeli Acute Paralysis Virus on honey bees (Maori et al., 2009). Worms can absorb dsRNA through their skin when dsRNA is suspended in liquid (Cogoni and Macino, 2000; Tabara et al., 1998). Once taken up, the dsRNA can circulate throughout the body and alter gene expression in the animal (Mello and Conte, 2004). In some cases, the dsRNA taken up is further amplified or causes a secondary reaction that leads to more and different dsRNAs ("secondary" dsRNAs) with unpredictable targets (Baum et al., 2007; Gordon and Waterhouse, 2007). They also readily transfer to mammals through food where they can circulate in blood and alter gene expression in organs (Hirschi, 2012; Zhang et al., 2012a).

The stability and transmissibility of dsRNAs suggest the potential for existence of exposure routes that are relevant to human and environmental risk assessments of genetically engineered/modified (GM) organisms. As the great majority of existing GMOs in the environment or human food have been modified to introduce one or more

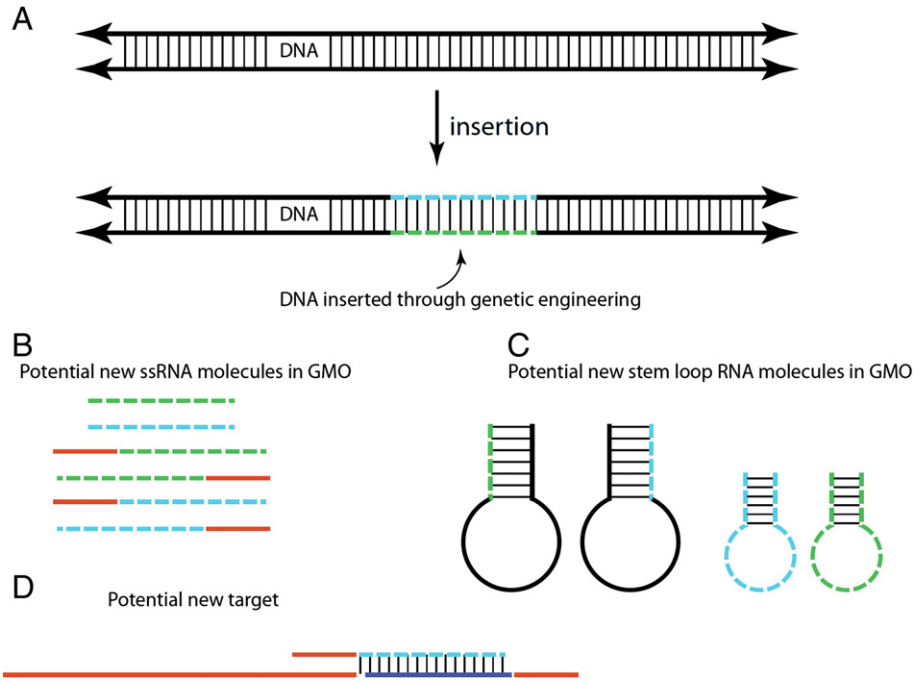

Fig. 1. Source of new dsRNA molecules from genetic engineering. (A) DNA inserted (dashed blue and green lines in the black double stranded DNA molecule) into a genome by genetic engineering creates new sequences regardless of the source of the DNA. The DNA used will create new sequences because it will be bordered (boundary between dashed and solid lines) by different sequences than in the source genome or it may be sourced from a genome that has no or few sequence matches. (B) Transcription will produce new RNA molecules (red and dashed blue and green lines) that might be able to form dsRNA because of complementarity or (C) because of internal base-pairing causing stem-loop structures to form (base-pairing illustrated with thin black connecting lines). (D) This may lead to intended and unintended off-target (red line with purple target section) gene silencing in the GMO or in organisms that eat the GMO.

additional proteins, there has been no formal international guidance on the risks specific to GMOs that introduce a new dsRNA, much less the development and testing of validated safety assurance procedures specific to dsRNA. The topic is gaining attention as evidenced by recent conferences and reviews (CERA, 2011; Parrott et al., 2010), but what is emerging is an *ad hoc* treatment of the various products that intentionally create novel dsRNA molecules, with most (perhaps all so far) regulators not considering the potential for adverse effects, particularly any unintended adverse effects of the dsRNA.

We examine the history of risk assessment of GMOs producing dsRNA, with a focus on the regulatory contexts of Australia, New Zealand and Brazil. Australia and New Zealand have different regulators for food and the environment whereas Brazil has one regulator that performs both functions. We show similarities in the approach by these three countries to considering the risks of dsRNA. As new information becomes available, these regulatory procedures will no doubt evolve. The reason for this analysis is to both create a historical record of the emergence of this risk and for this risk to serve as another case study in how 'early warnings' may be incorporated into risk assessments at the cutting edge of technology.

1.1. Risk assessment

Risk assessments are required on GM plants prior to use as food or release into the environment in many countries (Paoletti et al., 2008). The Codex Alimentarius Commission, a joint UN Food and Agriculture and World Health Organization, provides international guidance on conducting such risk assessments for human foods (Codex, 2003a, 2003b, 2008). This body is recognized by many countries as the appropriate body for issuing guidance on food (e.g., Brent et al., 2003). Codex promotes trade harmonization by limiting the range of potential objections to transboundary movement of GM-based products (Millstone and van Zwanenberg, 2002; Paoletti et al., 2008). The closest equivalent of the Codex on the environmental risk assessment of GMOs is the Secretariat to the Convention on Biodiversity which provide guidance in accordance with Annex III of the Cartagena Protocol on Biosafety (AHTEG, 2010).

A comparative assessment can be a guiding framework for risk assessment for food and the environment. This may be changing with the introduction of GMOs designed to have altered nutritional characteristics or which contain pharmaceutical or industrial products (Heinemann, 2007; Quist et al., 2013). As such, assumptions made either explicitly or implicitly in the context of substantial equivalence are due for review (TBT, 2012).

1.2. RNA and generally recognized as safe (GRAS)

Unless the dsRNA made by the GM plant is intended to act as a pesticide, the RNA itself is rarely formally considered in a risk assessment. This is surprising because Codex guidance draws special attention to the characterization of novel RNAs, stating: "Information should be provided on any expressed substances in the recombinant-DNA plant; this should include: A) the gene product(s) (e.g. a protein or an untranslated RNA)" (paragraph 32 of Codex, 2003a).

When unexpected RNAs derived from mRNA were detected by independent researchers in one of the first significant commercial GM soybean varieties (Rang et al., 2005; Windels et al., 2001), the concern raised was that it may be used to create different forms of protein rather than the RNA being a risk *per se*. In response, the developer of the GM soy said that RNA "is generally recognized as safe (GRAS)", and thus "the presence of…secondary RNA transcripts themselves raises no safety concern" (p. 5 Monsanto, 2002).

Importantly, those views have evolved and the developer has acknowledged the value of assessing the risks of at least some novel RNA molecules. However, a limited amount of research on those risks has been undertaken. Thus "the current peer-reviewed literature lacks published studies specifically assessing the safety of consuming endogenous longer dsRNAs, siRNAs or miRNAs in human food or animal feed" (p. 354 Ivashuta et al., 2009).

Also the approach taken by Ivashuta et al. (2009, p. 354) which was to produce a "documented history of safe consumption for small RNAs in order to demonstrate the safety of the RNA molecules involved in this form of gene suppression in plants" (p. 354 Ivashuta et al., 2009) does not establish the safety of novel small RNAs and sequence-determined risks. "Neither overall amounts of small RNA molecules, nor the presence of benign small RNAs in conventional plants are sufficient as evidence that all novel small RNAs will be safe in the food chain or environment" (p. 1291 Heinemann et al., 2011). Earlier literature also failed to recognize the need for sequence-determined effects (Parrott et al., 2010). These sequence-determined activities cannot be considered GRAS in general terms without specific supporting evidence.

RNA is a common part of food and an inherent part of all organisms. Thus, as a chemical, it is generally regarded as safe within the limits of its normal concentrations, that is, perhaps not as a meal all on its own. This is consistent with how it is considered by the Environmental Protection Agency (EPA) of New Zealand (previously called the Environmental Risk Management Authority) (for more details on this, see Heinemann, 2009).

Like DNA, what makes RNA an issue for risk assessment is that it has a nucleotide sequence and that sequence delimits its particular biochemical activities. These sequence-determined activities cannot be considered GRAS. Of particular interest is when any particular sequence leads to a defined range of matches with RNA molecules in humans or other animals. That range can be described as:

- Perfect sequence matches of approximately 21 nucleotides long;
- Approaching or exceeding 95% identity over 40 nucleotides (Rual et al., 2007);
- Short ($\geq$7 contiguous) identical matches in the 3′ untranslated region (UTR) of mRNA, which can be more determinative than the number of matches overall (Birmingham et al., 2006).

1.3. Assessing exposure

In order for dsRNAs produced in plants to cause adverse effects in humans and animals, there must be a route through which humans and animals are exposed. The most likely exposure routes are ingestion and inhalation (e.g., from wheat flour used commercially and in home kitchens), but future formulations of agents incorporating dsRNA and designed to be absorbed may in time increase the relevance of contact exposure.

1.3.1. Human

Some microRNAs of plant origin have been detected in the blood of Chinese people, demonstrating that dsRNAs can survive digestion and be taken up via the gastrointestinal tract (Zhang et al., 2012a). These plant-derived dsRNA molecules silenced an endogenous gene in human tissue culture cells, and in mouse liver, small intestine, and lung (Zhang et al., 2012a).

A survey of existing transcriptomic data of small RNA molecules from human blood and tissue sources, farm animals and insects confirmed that regulatory RNAs from plants can be found in animals, including humans (Zhang et al., 2012b). Interestingly, the transcriptomic survey data found some dsRNAs from plants more frequently than predicted from their level of expression in plants.

Evidence is lacking concerning the causes of preferential transmission or retention of plant dsRNAs in animals, and there is some doubt whether accumulation in animal blood and tissues of plant dsRNAs from dietary exposure is a universal mechanism or, at least in some cases, an artifact of the sequencing process. However, no robust evidence suggests that the exposure of humans and animals can be disregarded. Furthermore, the authors of the transcriptome surveys did not collect

samples from humans or mammals, and thus cannot be assured that the underlying source studies drawn upon were equivalent to the human and mouse study described above. The survey study is therefore not able to challenge the findings of the human and mouse study.

However, the survey study did provide evidence that not all dsRNAs may be equally prone to dietary transmission or retention (Zhang et al., 2012b). The selective packaging of dsRNA molecules into microvesicles would protect and transport the dsRNAs to target tissues (Jiang et al., 2012). Neither study, however, provided a way to predict which dsRNA molecules would be preferentially transmitted, retained or remain active, and under what circumstances that may occur.

1.3.2. The environment

Specific siRNAs can be toxic and the toxicity can be transmitted through food to animals of environmental relevance. This was demonstrated when GM maize and cotton plants were engineered to express novel siRNAs that were intended to be toxic to target insects (Baum et al., 2007; Gordon and Waterhouse, 2007; Mao et al., 2007). The toxicity was due to the dsRNA being transmitted from plant tissues to the insects by ingestion, and then being further processed in the animal into an siRNA that silenced one or more genes essential for life, or essential for detoxifying natural plant toxins (i.e., gossypol in cotton). Others have used direct feeding of dsRNA or dsRNA in liposomes as insecticides (Chen et al., 2010; Whyard et al., 2009).

As with the human studies discussed above, there is evidence of selective uptake of miRNAs from food. A feeding study of insects found that some small RNAs that were less abundant in the plant were more abundant in the insects that fed on the plant (Zhang et al., 2012b). It also found that insects which fed on dicots seemed to accumulate a miRNA that was more suggestive of a monocot origin. As a meta-analysis of small RNA datasets, this study could not confirm the purity of diets or exposure routes for animals (e.g., ingestion, inhalation, soaking through skin). The authors suggested that many or most detections of plant miRNAs in animals occurred via contamination from non-dietary sources. While this is important speculation, contamination does not sufficiently explain all the results. The contamination source proposed by the authors was from the mixture of plant and animal small RNA pools combined during multiplex sequencing. This can occur because of the capacity of the DNA sequencers to run reactions in parallel. However, that conclusion seems at odds with regular detection by others of miRNAs of plant origin that are not the most abundant plant miRNAs and assumes that all datasets assembled by others would have had the same mixture of plant and animal libraries used by the authors of the transcriptomic survey (Zhang et al., 2012b).

2. Examples of how GM crops containing dsRNA are regulated

2.1. Example 1: Food Standards Australia New Zealand (FSANZ)

The regulatory framework for GM crops in Australia and New Zealand consists of a shared food safety regulator called FSANZ. Under the Food Safety Act, FSANZ must approve as safe all foods derived from GMOs, following an assessment based on "internationally recognized scientific, risk-based methods" (p. 411 Brent et al., 2003). FSANZ uses information provided by the developer of the GMO, but also the scientific literature, advice from independent scientists and the evaluations of regulators from other countries (Brent et al., 2003; Hansard, 2008).

The Centre for Integrated Research in Biosafety (INBI) has argued within the regulatory framework of Australia/New Zealand that as part of the legislated requirement for safety testing of GMOs, any potential novel dsRNA molecules should be described and then evaluated for causing physiological effects in the GM plant or on any consumer of the plant, be that insects, wildlife or humans (Heinemann, 2009; Heinemann et al., 2011). Based on the same data as used by the safety regulator, INBI recommended that the kind, quantity and effects of any unintended dsRNAs produced in the GMO should be described (where possible), and then be included in a final evaluation of whether the food is "as safe as" (p. 410 Brent et al., 2003) the non-GM counterpart. INBI scientists predicted that dsRNA could be *transmitted to humans through food*, and that dsRNA would be sufficiently resistant to cooking and normal stomach pHs to potentially be taken up by cells or circulated through blood. If this were the case, there would be the potential to cause unintended and possibly adverse gene silencing in humans (Heinemann et al., 2011).

FSANZ, however, has regularly dismissed INBI's recommendation to describe and evaluate dsRNA unique to, or produced at unique amounts in, GM food. FSANZ has argued that 1) dsRNA does not transmit to humans through food; 2) dsRNA would be unstable in cooking or during digestion; and 3) the techniques that might be used to find dsRNAs are not routinely used in safety studies.

For example, in INBI's (then called NZIGE) first submission to FSANZ on an application called A524 (application for Roundup Ready wheat) in 2004, INBI called attention to the potential for dsRNA to transmit from GM plants to humans through food. INBI was referring to the *unintended production of novel dsRNA molecules* in its submission because the GM wheat being considered by FSANZ was not engineered to purposefully produce these molecules. Nevertheless, silencing effects are commonly caused through the genetic engineering process and the concerns were relevant. FSANZ never replied to INBI because the applicant withdrew the application prior to FSANZ issuing a decision on the product.

In January of 2005 and also in June of 2006, INBI again corresponded with FSANZ on the potential for dsRNA to cause adverse effects, and the plausibility of food as an exposure route, in its series of submissions on application A549 (approval for GM high lysine corn LY038). Through this exchange FSANZ made clear its reasoning on dsRNA.

INBI (NZIGE):	"The creation of novel RNA molecules by insertion of DNA into the maize genome could create species of RNA that are harmful to humans, possibly through food." "An adequate molecular characterization of all novel RNA molecules, that may pose a risk to consumers, is missing along with microarray analysis of the transcriptome of the LY038 line. There is published evidence that genetic components of the LY038 event produce novel RNA molecules. There is also evidence in animal studies that some small RNA molecules can be transmitted through food, causing lasting, sometimes heritable, effects on consumers and their children." "In order to make an assessment of global changes in the transcriptome, and specific changes cause by the insertion(s) of I-DNA, the Authority should require microarray descriptions capable of detecting novel RNA species in the modified plant, with the RNA source being the plant grown under a variety of relevant field conditions. The microarray should comprehensively represent the genomes of the cultivar of maize modified and unmodified, and *any novel RNA species should be tested against the human genome for RNAi activity* [emphasis added]." "Microarray descriptions should be capable of detecting novel RNA species in the modified plant, with the RNA source being the plant grown under a variety of relevant field conditions. The microarray should comprehensively represent the genomes of the cultivar of maize modified and unmodified. Since LY038 may be found in food, variant RNAs should be screen using a microarray for the human genome."
FSANZ:	"The rationale behind this recommendation is presented in the NZIGE submission in Section 1.3. *This section presents a summary of the biological properties of RNA that is generally accurate*. However, the scientific evidence *does not support the theory that RNA molecules in food can be transmitted to mammalian cells and exert effects on endogenous genes*. RNA is rapidly degraded even in intact cells. *Following harvest, processing, cooking and digestion, it is unlikely that intact RNA would remain. Even if it did, it is very unlikely that it would enter human cells and be able to exert effects on endogenous genes* [emphasis added]. What little is known about transcription levels of genes across entire plant genomes indicates that gene transcription may vary considerably even between closely related plants (Bruce et al., 2001; Guo et al., 2003; Umezawa et al., 2004). This high level of differential expression is thought to be due to a number of factors including environmental conditions and genotype. For this reason, analysis of changes in the transcriptome, while interesting, would not indicate whether these changes are within the range of natural variation nor would it provide any further information on the safety of the food" (FSANZ, 2006).

FSANZ drew several assumption-based lines of reasoning at the time to argue that existing evidence was sufficient to dismiss relevant exposure routes. For example, FSANZ did not draw on scientific evidence when it said that dsRNA would be degraded in the stomach, all dsRNA would be equally prone to degradation, none would be subject to recruitment, all would be passed through ingestion (and not also inhalation), and that plant-derived dsRNAs were incapable of being taken up by human cells. In doing so, it avoided having to consider the possibility of adverse effects of dsRNA because it did not recognize a route of exposure. Critically, FSANZ ignored sequence-determined risks when it referred to natural variation in transcription.

INBI continued to alert FSANZ both to the use of assumption-based reasoning and to the scientific plausibility of the exposure routes in its subsequent submission on application A1018 (2009), where a GM soybean was intended to produce a novel dsRNA. In that case, rather than respond to the expressed safety concerns, FSANZ focused exclusively on available profiling techniques for detecting the molecules (FSANZ, 2009a). The response failed to address the substantive issue of the exposure route.

From Table 1 it is clear that FSANZ has approved for use as human food at least 5 GM products (described in applications A383, A384, A387, A1018, A1049) with modifications intended to produce novel forms or concentrations of dsRNA. The first approval we could find occurred in 2000. These approvals were made despite FSANZ's acknowledgement that there was scientific uncertainty about how the modification caused the trait. For instance, in its approval of virus-resistant potato (application A384) FSANZ said: "The exact mechanism by which the viral protection occurs is unknown" (p. 8). Little had changed by the time FSANZ approved GM soybeans in application A1018: "The Applicant speculates that suppression of expression of the endogenous *gm-fad2-1* gene is mediated via co-suppression in which the introduced fragment leads to an overabundant production of sense mRNA which in turn leads to production of dsRNA *via a pathway that is still not understood*" (emphasis added to p. 12). To which INBI responded that: "Under such circumstances where the biochemistry of the modification itself is considered to be *speculation and is not understood*, it is difficult to understand how FSANZ has achieved confidence that the Applicant could report all unintended effects of the modification."

INBI was able to make scientifically credible submissions on the biology, biochemistry and chemistry of RNA. This was acknowledged by FSANZ, who stated: "the NZIGE submission…presents a summary of the biological properties of RNA that is generally accurate". INBI created an exposure scenario and potential adverse effects based on its knowledge of nucleic acid chemistry, the biochemistry of silencing pathways and extensive expertise in the biochemistry of horizontal gene transfer. Subsequently, the predictions about exposure routes and potential for food-transmitted dsRNA to alter gene expression in humans and animals were systematically confirmed (Hirschi, 2012; Zhang et al., 2012a). Here are various statements made by FSANZ on the topic of acknowledging the risk of transmission of dsRNA from GM plants being considered for approval for use as food and contrasting evidence-based statements from the scientific literature:

FSANZ (2006)	"However, the scientific evidence does not support the theory that RNA molecules in food can be transmitted to mammalian cells and exert effects on endogenous genes".
Zhang et al. (2012a)	"Further *in vitro* and *in vivo* analysis demonstrated for the first time that food-derived exogenous plant MIR168a can pass through the mouse gastrointestinal (GI) track and enter the circulation and various organs especially the liver where it cross-kingdomly regulates mouse LDLRAP1 protein expression and physiological condition." "Functional studies *in vitro* and *in vivo* demonstrated that MIR168a could bind to the human/mouse low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA, inhibit LDLRAP1 expression in liver, and consequently decrease LDL removal from mouse plasma. These findings demonstrate that exogenous plant miRNAs in food can regulate the expression of target genes in mammals." "plant MIR168a and MIR156a were detected in various mouse tissues, including liver, small intestine, and lung"
Zhang et al. (2012b)	"Of 83 animal [small]RNA public datasets used for analysis, 63 (including 5 datasets from human and mouse cultured cell lines) had at least one sequence that had perfect identity to a known plant miRNA"
FSANZ (2006)	"RNA is rapidly degraded even in intact cells. Following harvest, processing, cooking and digestion, it is unlikely that intact RNA would remain".
Zhang et al. (2012a)	"Interestingly, plant miRNA were stable in cooked foods". "To mimic GI tract environment, the effect of acidification on the stability of plant miRNAs and mammalian miRNAs was examined. Total RNA isolated from rice or mouse liver was adjusted to pH 2.0 and kept at 37°C for several hours…acidification did not significantly affect the yield and quality of miRNAs. The majority of plant miRNAs and mammalian miRNAs can survive under acidic condition for at least 6 h."

This comparison of assumptions used by FSANZ and quotes from the recent literature exposes the weakness of assumption-based reasoning in risk assessment.

2.2. Example 2: Office of the Gene Technology Regulator (OGTR) Australia

The OGTR is Australia's regulator for field trials and commercial releases of GM plants into the environment (Fox et al., 2006). The OGTR has issued 10 licences for field trials of GM wheat since 2007 (OGTR, 2012a). Traits being tested range from abiotic stress tolerance to altered grain starch and nutritional characteristics. Of these, we focus primarily on licenses DIR093 and DIR112 issued to the Commonwealth Scientific and Industrial Research Organisations (CSIRO) to field-test wheat with altered grain starch composition and to use some of the wheat to feed human volunteers to determine if the wheat had certain commercially-desired effects in the volunteers.

The DIR093 decision concerns the genetically modified wheat varieties that use dsRNA to silence the gene *SEI* in the endosperm of the plant. *SEI* encodes a starch branching enzyme. Barley varieties were also developed that were intended to silence two genes called *SEI* and *SEII* that encode for branching enzymes in the endosperm. The RNAi was created through the introduction of recombinant DNA molecules, or transgenes, that were constructed to produce substrates for the endogenous dsRNA processing pathways in plants.

These constructs involve tandem repeats of two sequences, with the second sequence being in the opposite orientation (i.e., an inverted repeat) to the first. This allows for intra-molecular base-pairing and encourages the formation of short-hairpin dsRNA (Fig. 2). The repeated sequences are presumably exonic sequences from *SEI* and *SEII*, respectively, separated by intron 3 of *SEI*. The constructs were intended to be processed through canonical splicing pathways to remove intron 3 and increase the efficiency of processing the resulting dsRNA into siRNA.

According to the OGTR: "The partial sequences used in the constructs were isolated from wheat, and non-GM barley contains homologues of the introduced wheat genes; the regulatory sequences are also widespread in the environment" (p. 38 OGTR, 2009). While this is impossible to independently verify because the sequence of the transgene was protected as confidential commercial information (OGTR, 2009), it is unlikely to be correct at the RNA level for three reasons. First, the sequence at the RNA level is unique to the GM plant because there is no RNA in the non-GM plant that has both the matching and inverted repeat on the same strand. Second, and importantly, presumably no dsRNA molecule of this type exists in non-GM wheat. Third, there is recognition by the OGTR that the transformation process may lead to incorporation of 'vector' sequences (OGTR, 2009). These are DNA molecules that have never been part of the wheat genome. So while many parts of this sequence may exist in places in the wheat genome, it is inaccurate to conclude

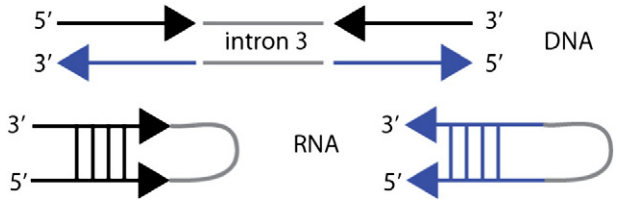

Fig. 2. Hypothetical structures of regulatory RNA molecules in GM wheat of DIR093. Arrows indicate repeated sequences and their orientation. The numbers 5′ and 3′ indicate strand polarity. The grey line labeled intron 3 is the third intron of the wheat gene *SEI*.

that there is history of RNA molecules of this particular sequence, structure or function in our food.

There is no evidence in DIR093 that the risk assessment process considered the risk that the dsRNA may transmit to animals or people (see Table 6 of OGTR, 2009). At the time that the decision was written, the potential for the dsRNA to transmit to insects and nematodes was well known (Baum et al., 2007; Cogoni and Macino, 2000; Gordon and Waterhouse, 2007; Mao et al., 2007; Tabara et al., 1998). In fact, the CSIRO holds a fundamental patent on the technique for expressing dsRNA in GMOs for the purpose of transmitting the dsRNA to target pests, with the aim of affecting the biology of those pests (Whyard et al., 2011). Indeed, in its patent application, the CSIRO makes claim to a process for delivering dsRNA through "feeding a transgenic organism expressing the dsRNA to the arthropod. The transgenic organism is selected from, but not limited to, the group consisting of: plants, yeast, fungi, algae, bacteria or another arthropod expressing the dsRNA."

Because it did not consider the risks of the dsRNA transmitting to animals and people who ate the GM wheat, *ipso facto*, the OGTR did not consider which genes may be silenced by any such transmission. It therefore may not have considered that animals and humans have similar sequences within their mRNAs to those present in the GM plants. Nor did it consider the possible consequences of partial or complete silencing of unintended target genes in animals and people.

In fact, the OGTR stated that there was no identified risk from the dsRNA in these GM wheat varieties. The OGTR defines risk as: "A *risk* is only identified when a hazard is considered to have some chance of causing harm. Events that do not lead to an adverse outcome, or could not reasonably occur, do not represent an identified risk and do not advance any further in the risk assessment process" (p. 2 OGTR, 2009). Thus it can be concluded on the basis of this information that a risk assessment was not done on the dsRNA and experiments testing specific risk hypotheses were not required by the regulator. Indeed the regulator was quite specific about not requiring any risk assessment for animals or humans eating the GM wheat, stating on page 32 of DIR093: "The potential for allergic reactions in people, or toxicity in people and other organisms as a result of exposure to GM plants with altered grain starch composition as a result of the introduced RNAi constructs is *not an identified risk* and will not be assessed further", and issuing similar conclusions on environmental risks on page 33. In drawing these conclusions, the OGTR only considered the effect of altered grain composition, and not the sequence-determined potential effects of the dsRNA. Perhaps for this reason, the OGTR permitted the CSIRO to undertake animal and human feeding studies to investigate whether the GM wheat had the anticipated commercially attractive benefits without first requiring the CSIRO to look for any adverse health effects.

Furthermore, in a license issued under DIR112 for a different trait in wheat created through the use of RNAi, the OGTR again said that there was no identified risk arising from the dsRNA made by the wheat. However, by the time this license was approved, experimental evidence of the exposure route to humans was available. The OGTR document was cognisant of this, stating: "As discussed in Risk Scenario 5, RNAi constructs (via siRNAs) can give rise to off-target silencing effects within the plant, leading to changes other than the intended effects. In addition, a recent publication [(Zhang et al., 2012a)] has reported evidence that natural plant miRNAs can be absorbed by mammals through food intake, and have the potential to modulate gene expression in animals" (paragraph 120 OGTR, 2012b).

The OGTR justified its position using assumption-based reasoning: "Even if novel small RNAs are taken up by people or animals, to have any effect a number of conditions would have to be met: the siRNA-containing wheat would need to constitute a large proportion of the diet, the siRNA would need to be expressed at high levels in the wheat material consumed, match a target sequence of a human or animal gene and be taken up by specific human and animal cells expressing that gene. Lastly, it is likely that even if the siRNAs were acquired through food intake and did affect the expression of mammalian genes, such an effect would be transient as was reported by" (Zhang et al., 2012a) (paragraph 123 OGTR, 2012b).

These assumptions remain to be tested. So far, there is no evidence to quantify what *proportion of the diet* is required to reveal important effects. Consequently, there is no understanding of how much exposure could be considered to be safe. Furthermore, recurring dietary exposure to the GM wheat could result in "transient" effects that were more or less constantly present and hence *would* be relevant to a risk assessment.

In theory, very few molecules of siRNA are needed to cause a therapeutically relevant effect, and possibly fewer still to cause some effect even if complete silencing is not the outcome of exposure to an siRNA. First, there is evidence of a *sequence-independent toxic effect* of dsRNA. This kind of toxic effect is length dependent, e.g., molecules over 30 base-pairs in length (Bass, 2001; Elbashir et al., 2001). Second, sequence-determined risks can be primed in some organisms by an initially small number of dsRNA molecules. "It has been suggested that one siRNA can cleave as many as ten cognate mRNAs. This catalytic nature of mRNA targeting by siRNAs…suggest[s] that a potent siRNA will effectively function at much lower concentrations without saturating the endogenous miRNA machinery. It has been estimated that, it may take only about 1,000 siRNA molecules/cell to silence gene expression efficiently (an estimate derived from the frequencies of individual endogenous miRNAs in cells). Quantitative information about the numbers of siRNAs required for efficient gene silencing would be important for establishing safe dosing regimen for RNAi drugs and to avoid potential toxicity" (p. 598 Seyhan, 2011). Furthermore, bees fed daily on dsRNA directed at a bee virus demonstrated resistance to the virus (Maori et al., 2009), suggesting that regular exposure through food can have potent physiological and immunological consequences.

Moreover, RNAi can cause heritable changes (through epigenetic transmission) that may result in persistent changes either within cells or entire tissues of people, and be heritable through reproduction in some animals and other organisms (Cogoni and Macino, 2000; Cortessis et al., 2012; Lejeune and Allshire, 2011).

Neither sequence-independent nor sequence-determined off-target risks formed part of the risk assessment described in the OGTR documents, despite the OGTR's Scenario 5 recognising their existence. This omission occurred even though *unintended secondary dsRNAs* may be generated in the GMO (Dillin, 2003; Pak and Fire, 2007; Sijen et al., 2007) or in animals exposed to the GMO (Baum et al., 2007; Gordon and Waterhouse, 2007) and neither the identity of the secondary dsRNAs nor their consequences can be predicted. 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 through inhalation or consumption. With the anticipated development of biopesticides and other agents containing dsRNAs intended to transverse epidermal layers of plants or target insects (Monsanto; Zhang et al., 2013), contact exposure may also have to be considered.

2.3. Example 3: golden mosaic virus resistant pinto bean approval by Comissão Técnica Nacional de Biossegurança (CTNBio) Brazil

CTNBio is a consulting and deliberating multidisciplinary collegiate body that establishes safety technical norms for the authorization of research-related activities and the commercial use of GMOs and their by-products, based on Biosafety Law 11.105/2005 and their normatives (e.g. Normative Resolution nº 5 regarding risk assessments rules). In its deliberations, CTNBio uses information given to it from the developer of the technology as well as submissions sent by independent scientists and the community (Ordinance no. 373, article 2nd).

Independent scientists raised safety questions to this body during the decision making process for approval in Brazil of a GM variety of pinto bean (*Phaseolus vulgaris*) event 5.1. The bean was made virus resistant through induced RNAi (Aragão and Faria, 2009).

In this example, we will focus our discussion on the scientific arguments presented by researchers at the Federal University of Santa Catarina (UFSC) (Agapito-Tenfen and Nodari, 2011) that were submitted to CTNBio and CTNBio's technical report (CTNBio, 2011).

The transgenic pinto bean was genetically modified using particle bombardment, which introduced an insert of about 50 kbp into the bean genome (Aragão and Faria, 2010b). From this insert an intron-hairpin construction (i.e., a *rep* cassette) was transcribed to induce post-transcriptional gene silencing against the *AC1* gene of the Bean Gold Mosaic virus (Bonfim et al., 2007). Similar to the wheat example above, the hairpin RNA mimics a miRNA. In this case, the dsRNA is capable of silencing the viral mRNA for the replication protein. However, the mechanism by which the viral protection occurs in this specific event is unknown (see page 12 of Aragão and Faria, 2010b). Similar to the case of FSANZ, CTNBio has accepted uncertainties about the underlying biochemistry of the trait in their decision to grant approval.

The Brazilian Agricultural Research Corporation (Embrapa) claimed confidentiality about the details of the DNA sequence and associated molecular characterizations of the product (see page 12 of Aragão and Faria, 2010b). This was agreed by CTNBio (see pages 1 and 6 of CTNBio, 2011). As with the case described in Example 2 above, an independent evaluation of the actual sequences used in the GM pinto beans was impossible. In addition, there appear to be more details granted confidentiality than just the DNA sequence, further complicating attempts to provide the regulator with external opinions (Supplementary Data).

The Embrapa 5.1 event that was assessed has truncated copies of the *rep* cassette, including one copy in the anti-sense orientation, and plant genome sequences incorporated adjacent to the *rep* cassette (Aragão, 2011; Aragão and Faria, 2010a). The researchers at UFSC argued that truncated forms of the *rep* cassette, as well as unexpected adjacent sequences, could generate dsRNA molecules that could lead to adverse off-target effects in the bean or other organisms eating the bean.

The detection of dsRNAs in raw and cooked plant organs (seed and leaves) was only tested using *northern blotting* which is based on probe affinity hybridization. This technique is not quantitative or as discriminating as, for example, quantitative real time PCR (q-RT-PCR) or high-throughput sequencing of the small RNA pool (Heinemann et al., 2011). Northern blotting should have been used in conjunction with q-RT-PCR which can detect targets at even lower concentrations with high stringency because it uses small primers (e.g., around 15bp). The techniques complement one another because rearrangements that might produce false negative PCR results may be detected by northern blotting, and PCR is more sensitive. Moreover, the CTNBio risk assessment did not report on the possibility of unintended RNAs being transcribed from additional inserts in the form of truncated copies, which were detected, nor did it require confirmation of the sequence and structure of the intended and anticipated dsRNA molecules.

To address these concerns, the UFSC researchers suggested that the intended dsRNA molecules should be confirmed and quantified, and safety testing for adverse effects should include feeding studies.

The safety assessment did include a rat feeding study, but for various reasons this was not considered to be satisfactory for testing the specific hypothesis of an adverse effect arising from the dsRNA. In that study, Wistar rats were fed for 35 days. One group of 10 rats was fed raw transgenic pinto beans; a second group of ten rats was fed on conventional raw pinto beans; a third (control) group of ten rats was fed a casein-rich diet; and a fourth (control) group of four rats was fed a non-protein diet. However, only 3 rats per group were killed for the morphological, histological and biochemical analyses. The proponent did not perform any immunological analyses of pregnant rats or any second-generation rats as requested by CTNBio Normative Resolution nº 5 Annex III. They argued that "there was no scientific basis" to do so "since no alteration in animal weight gain was observed" (p. 106 Aragão and Faria, 2010b).

Moreover, raw beans caused the death of many rats starting 20 days after the start of the experiment but the exact number of dead animals was not disclosed (Aragão and Faria, 2010b). It is well known that anti-nutritional factors common in beans, as for soybeans, are removed by cooking (Gupta, 1987), and these effects could have obscured any more subtle toxic or other potential effects of the dsRNA.

In another study, rats were fed orally with a solution of 6 mg of total RNA extracted from leaves. Not only were few details of this experiment provided, such as the number of rats used or length of the study, but the use of total RNA from leaves as feed would not appropriately address possible health effects deriving from the new dsRNA molecules, especially in humans, as humans do not eat pinto bean leaves. This approach fails to address important variables. First, in extracting the entire transcriptome, there is no assurance that the different physical forms of the RNA (large vs. small, single-stranded vs. double-stranded transcripts) will be retained in the sample with equal efficiency. Second, the extraction procedure might remove other factors specifically associated with miRNA-like molecules, such as argonaut proteins or microvesicles, that might be relevant to their protection during digestion and or transport following ingestion. Third, the concentration and kind of dsRNAs in leaves might be different to beans. Finally, the feeding studies used were not equivalent to a safety assessment for humans because the use of leaves and uncooked beans did not take into account the "potential effects of food processing, including home preparation" (p. 18 Codex, 2003a) because humans do not eat the leaves or uncooked beans.

In this example we introduce an additional biosafety consideration beyond human food safety and effects on beneficial environmental organisms.

In order for the GM bean to be effective, any viruses exposed to the transgenic plant must be reliably contained and neutralized by the RNAi effect in order for the trait to be effective. If the effect of the RNAi response is inconsistent or weak, enough viruses may replicate to generate random variants that overcome or counter dsRNA-mediated silencing (Lafforgue et al., 2011). For example, a variant with a mutation in the *AC1* gene that reduced the number of matches with the guide RNA might then arise by selection on the GM bean.

That is, in this case the dsRNA is similar to the insect toxin expressed by "Bt" plants in that it has an intended target effect on a pest/pathogen population. As a pest resistance trait, the bean creates a selective pressure on the virus population. If that pressure is too weak, it might undermine pathogen management. Insecticidal plants are approved for use in the context of a pest management strategy to maintain the efficacy of the trait. The management strategy for Bt plants, e.g., the use of a high-dose coupled with a nonGM refuge, is meant to both maintain the efficacy of the product and to prevent the GM plant from undermining the use of *Bacillus thuringiensis* as a pesticide in non-GM farms (Heinemann, 2007).

Since backcrossing into inbred lines is a normal part of the commercial process of developing a GM plant, the stability of the expression of the intended trait should be part of the risk assessment process. This is especially true given that Embrapa's event 5.1 demonstrated variability in susceptibility levels. Embrapa reported that from 10 to 36% of F1 individuals, depending on the genetic background of the plant, were virus susceptible (Aragão and Faria, 2010b). The developer had hypothesized that the presence of susceptible phenotypes in these hemizygous plants can be explained by the concept of 'gene-dosage' (Aragão and Faria, 2010b). That is, the hemizygous plants may express a smaller amount of the intended dsRNA molecules than homozygous plants.

However, UFSC researchers have argued that 'gene-dosage' alone does not explain the difference in susceptibility levels. For instance, in Table V.17 of Aragão and Faria (2010b) data indicate a variety of susceptibilities for hemizygotes only. These plants would all have the same number of transgenes. The UFSC researchers then proposed three hypotheses that could explain the results obtained for the F1 plants: (i) instability or truncation of the insert; (ii) environment x gene interactions; or (iii) virus-mediated transgene silencing or resistance to silencing (Noris et al., 2004; Taliansky et al., 2004). Testing these hypotheses would have provided the regulator with the biochemical explanation of the varying levels of resistance and informed a risk management plan. However, CTNBio did not require the developer to address the varying susceptibility levels. Interestingly, the regulator appeared unaware of this variability because they concluded that the segregation pattern was what they expected and that the observed phenotypes were normal in all crosses made (CTNBio, 2011).

3. Generalising from the examples

Although a few products based on dsRNA-mediated silencing have been approved, the commercialization history of these products is spotty. Flavr Savr Tomato, New Leaf Potatoes and the G series of high oleic acid soybeans were withdrawn from market shortly after release (FSANZ, 2009b; Monsanto, 2001). The exceptions are papaya and pinto beans which have been consumed on a relatively small basis.

Given that few people would be exposed to artificial siRNAs, and exposed in low amounts through consuming currently approved products, it is not surprising that regulators from different countries have not established common, validated assessment procedures for these molecules (ACNFP, 2012; Lusser et al., 2011). A validation process establishes both the relevance and reliability of a test. Validation usually involves establishing the test definition, assessing the within- and between-laboratory variation in the results, the transferability of the test between laboratories, the predictive capacity of the test, how applicable the test is to the situation and how well the test conforms to certain standards (Hartung et al., 2004). Regulation of traits based on dsRNA in GMOs is therefore currently based on ad hoc standards and acceptance of unpublished studies conducted by GMO developers even though the approval of the first human food based on dsRNA-mediated silencing occurred nearly 20 years ago. The regulatory community is only now actively debating how these molecules should be assessed.

There are also discordant statements about expected standards appearing in the literature. For example, The US Environmental Protection Agency (EPA) considers dsRNAs intended as pesticides to be biopesticides and they are considered on a case-by-case basis (letter from US EPA to JAH, personal files). However, some have reported that the US EPA requires that dsRNA for use as a biopesticide must have fewer than 20 matching nucleotides in a row to any unintended target gene to ensure the absence of off-target effects (BBSRC, 2012; Maori et al., 2009). However, we could not verify this with the US EPA, who said: "The EPA's Office of Pesticide Programs has not issued any specific regulatory guidance on dsRNA and honey bees" (letter from US EPA to JAH, personal files).

While the absence of validation of scientific procedures has often been used by regulators to resist incorporating new scientific findings into the safety assessment of GM products (Heinemann et al., 2011), the absence of validated procedures for widely used approaches in current safety testing does not seem to have been equally problematic.

Since there are no internationally agreed and validated procedures for excluding either exposure routes or potential adverse effects of particular dsRNA molecules that may be produced as a result of genetic engineering, whether intended or otherwise, for the foreseeable future all GMOs intended for release (as a field trial or to unregulated status) or food should be submitted to a battery of testing for unknown dsRNAs and unintended effects of dsRNAs. The testing should provide empirical evidence capable of delivering confidence for any claims of the absence of any unintended dsRNAs or of an unintended effects of any dsRNAs.

We hope to fill some of the void on this topic by suggesting a testing regime in Section 4.

3.1. Common assumption-based regulatory approaches

GMO risk assessment has a history of contention and contested practices reflecting an underlying landscape of scientific uncertainty and lack of consensus (e.g. Dolezel et al., 2006, 2011; Séralini et al., 2009, 2012). Products based on dsRNA techniques have been placed in the commercial marketplace before a complete understanding of the biochemistry has been established (see Example 1), even before the basis of the trait was understood to function via RNAi. For example, it was not until years after approval and subsequent withdrawal of the Flavr Savr Tomato that the owner of the crop determined that the engineered characteristics were actually based on RNAi (Sanders and Hiatt, 2005). In fact, approvals remain in place on most dsRNA-based traits without, to our knowledge, any peer-reviewed published evidence of the existence of the intended dsRNA molecules and confirmation of the cause of silencing. This may occur due to market forces and innovation policies placing incentives on the early commercialization of technology, resulting in products that outpace the development of safety-assuring science and periods of reflection by the scientific community.

That dsRNA could cause silencing in humans was considered surprising just 12 years ago, because of assumptions that sequence-determined silencing should not work in humans (Bass, 2001). Such assumptions had legacy effects in risk assessment well after it was shown that initial failures to demonstrate RNAi in humans were due to using dsRNA molecules that were too long and induced a sequence non-specific interferon response and general cessation of translation (Bass, 2001; Elbashir et al., 2001).

dsRNAs developed for use as drugs in medicine have also floundered. Despite their huge potential and an initial rush to get them to clinical testing, they have failed to work because they cannot be delivered at effective concentrations (Seyhan, 2011). Failure to achieve a man-made delivery system does not imply that all dsRNAs are safe because not all dsRNAs are equally efficiently taken up or stable (see Section 1.3.1), and the effects of some may be enough to cause harm at concentrations lower than needed to cause the intended trait (Zhao et al., 2001).

Assumption-based deflection of risk is not unique to GMOs or dsRNA. For example, scientific conflict on the appropriateness of the safety testing of the now withdrawn drug VIOXX arose early in the drug's lifetime but was not taken seriously until harm became evident (Box 1). The assumptions behind the VIOXX approval and assumptions highlighted in the examples above demonstrate how regulatory bodies, rather than requiring evidence that a product is safe before

Box 1
Other assumption-based failures.

(1) VIOXX (also known as rofecoxib) is the trade name for an anti-inflammatory drug that was popular among those who suffer from arthritis. The drug was approved by the US Food and Drug Administration (FDA) and sold from 1999. By the time it was withdrawn from the market in 2004, it was estimated to have caused 139,000 heart attacks and killed 26,000 people (Michaels, 2005; Wadman, 2005).
The delay in identifying the risk was caused by the manufacturer not publishing its safety data until 18 months after the drug was commercialized (Topol, 2004). Independent scientists re-analyzed the published data, found a worrying trend in them and recommended that the drug be re-evaluated for its cardiovascular effects (Mukherjee et al., 2001). "Given the remarkable exposure and popularity of this new class of medications, we believe that it is mandatory to conduct a trial specifically assessing cardiovascular risk and benefit of these agents. Until then, we urge caution in prescribing these agents to patients at risk for cardiovascular morbidity" (p. 958 Mukherjee et al., 2001).
This recommendation was dismissed by the manufacturer, whose scientists instead argued that the drug used as a control (naproxen) was impressively and unexpectedly cardioprotective (Michaels, 2005; Topol, 2004). The FDA failed to require independent testing (Michaels, 2005; Topol, 2004). If the recommended study had been conducted shortly after it was recommended in 2001, the drug might have been off the market 3 years earlier, or still on the market but sold only to a more limited range of patients.

(2) In 1995, one of the UK's leading laboratories on transmissible spongiform encephalopathies (TSE, also known as prion diseases), rushed into publication a preliminary study with relevance to the question of whether the infectious agent could transmit from cattle to humans (Anonymous, 1995).
The study used mice that were genetically modified to express a human version of a protein that changes in conformation when humans develop Creutzfeldt–Jacob disease (CJD). These mice were then exposed to the bovine version of the protein derived from animals with bovine spongiform encephalopathy (BSE). There was no statistically significant increase in TSE in these mice, suggesting that the agent causing BSE was not transmissible to humans from cattle (Collinge et al., 1995).
The journal published a news item in the same issue wherein it reported a conflict between the authors and other scientists, the latter claiming that the results were neither informative for the conclusion nor responsibly published even when qualified as preliminary (Anonymous, 1995). The essence of the concern was that the disease can have an incubation period between exposure and symptoms; the animals may not have been old enough to show symptoms at the time of publication (Hope, 1995); and the incubation period for cross-species disease was already known to be longer still. Critically important, and recognized by the authors at the time, was the possibility that their results could be false negatives because of interference by the mouse version of the protein, stating: "Interference with human prion propagation by mouse PrP may also be relevant" (p. 782 Collinge et al., 1995). The relevant control group of mice expressing only the human form of the protein had been started much later and results were not available at the time they chose to publish.
Questions remain about why the article was published when it was based on preliminary data, and why it was published by such a prestigious journal at a key time in the government's deliberation on the safety of UK beef, rather than waiting a few months for a complete set of experiments to be finished. It later would be shown that the infectious particle in beef could transmit to humans (and to GM mice), with the Collinge group among those credited with providing convincing evidence (Almond and Pattison, 1997). In an obscure addendum to the 1995 article appearing again in *Nature*, the group announced "We also included the interim results of challenge of mice expressing only human prion protein…with BSE; these mice had remained disease-free 264 days post-inoculation. We have now found that from 489 days onwards, some of these…mice developed a prion disease" (p. 526 Collinge et al., 1997).
According to Google Scholar (22 October 2011), there are 173 citations of the 1995 *Nature* article, 971 to the 1997 *Nature* article credited with making the connection between food-born prions and the form of CJD (new variant CJD (vCJD)), but only 1 citation to the 1997 *Nature* addendum linking the preliminary (and false negative) 1995 results to the findings of infectivity confirmed in 1997. By 2009, at least 164 deaths were attributed to vCJD (Cummings, 2010). How many more people ate tainted UK beef for longer because of this study is not possible to determine. However, at least one highly prominent UK neuroscientist at the time reversed his previous advice to the public to avoid UK beef (Anonymous, 1995).

allowing it to enter the marketplace, now tend to require proof of harm to withdraw the product from the marketplace.

Of the three government regulators described in the examples above, one is a food regulator (FSANZ), one is an environmental safety regulator (OGTR) and one has dual roles (CTNbio). Yet all used *a priori* assumptions that they did not need to do a risk assessment of novel dsRNA molecules, rather than requiring experimental evidence that these molecules caused no adverse effects. In addition, a recent review paper has also used *a priori* assumptions that did not capture sequence-determined risks (Parrott et al., 2010) whereas a recent conference that included industry participation did consider sequence-determined risks when they acknowledged that the potential for off-target effects was due to potential pairing between siRNA and unintended transcripts of non-target genes (CERA, 2011). In contrast to our findings, the conference concluded that existing safety evaluation protocols were adequate for identifying all adverse effects from dsRNA. However, the conference proceedings did not cite the high-profile literature on secondary dsRNAs (Pak and Fire, 2007; Sijen et al., 2007) and the conference predated the confirmation that dsRNAs could be transmitted through food (Hirschi, 2012; Jiang et al., 2012; Zhang et al., 2012a). These significant omissions may have led to their different conclusions about safety testing protocols.

There are two ways to apply assumption-based reasoning, or "arguments of ignorance" (Cummings, 2010), under scientific uncertainty. The first way forms the basis of the examples in Section 2. The second way is to avoid harm. When used to avoid harm, assumption-based reasoning is internationally sanctioned as the precautionary principal/approach. This approach sees the burden of proof remaining with the developer and the regulator before a potential harm can be shifted to society. Precaution under uncertainty has a high international normative standard of application, being recorded in the Rio Declaration as "Where there are threats of *serious or irreversible damage*, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental

degradation" (emphasis added). So does chronic low-level morbidity count as serious? And even if the damage caused by an effect can be fully healed, does that make any suffering at the time reversible? There is a considerable amount of disagreement in the scientific community on these sorts of normative judgements.

3.2. Facing uncertainty in the risk assessment of dsRNA-based GM traits

There are scientifically accessible and demonstrated techniques to address any absence of evidence about whether the existence of unintended dsRNA molecules or unintended genomic modifications arise from the use of novel siRNAs (Heinemann et al., 2011). However, each of these techniques have 'blind spots', including limits of detection that may be too high to ensure that not finding an siRNA is biologically meaningful. Thus, other tests may still be warranted, such as *in vitro* testing using tissue culture cells and proper animal experiments that encompass all relevant exposure routes.

The first step in a risk assessment is hazard identification. When this step fails, then the risk assessment fundamentally falters. The examples in Section 2 describe not just how a risk was recognized and then systematically denied, but in many cases a refusal to acknowledge that any risk existed at all.

4. Recommended risk assessment

While it is clear that the regulatory framework for assessing the risks of GM plants is evolving and responding to new information, it is also clear that there is disagreement on when or how rapidly an observed biological phenomenon relevant to a risk assessment necessitates a regulator asking for experimental evidence to address potential adverse effects. This has created a vacuum for the risk assessment of dsRNAs unique to or at unique concentrations in GMOs. To help fill this vacuum, we consider the kinds of scientific studies or assurances that could be undertaken to evaluate the safety of these products.

4.1. Sequential approach

(1) Bioinformatics should be used to identify any likely, unintended targets in humans and other critical organisms (e.g., plants, fungi, animals, microbes) of the siRNAs intentionally produced by the GM plant (Fig. 3). Such organisms should include species that are used as indicators of key ecological functions or which are protected. These studies would look for perfect sequence matches or similar sequences both within outside of coding regions, e.g., introns (Seinen et al., 2011), and perfect matches in seed regions of 3′ UTRs, of RNAs derived from whole genome sequences, where available. The algorithms must be able to identify short sequences of identity between the intended siRNA and any potential target. If the comparison is restricted to an estimate of overall similarity between the gene intended to be silenced and other genes, then the short but biologically meaningful matches may be overlooked (Birmingham et al., 2006; Chalk and Sonnhammer, 2008; Scacheri et al., 2004). The siRNAs chosen from this analysis would be those that are least likely to create off-target effects.

(2) All new intended and unintended dsRNA molecules should be identified in the GM product (Heinemann et al., 2011). Ideally, this would be done through a semi-targeted qualitative profiling of small RNA molecules using next generation sequencing in a comparative assessment between the GM and conventional parent (Heinemann et al., 2011).
The silencing pathway should be experimentally verified. The experiments should be able to distinguish between a role for cytoplasmic or nuclear (TGS) silencing pathways, and the contribution of each to silencing. This is because the different pathways may produce different off-target effects. This may require examining changes at the DNA level, such as changes in methylation.
If unintended novel dsRNAs are identified in the GM product, bioinformatics should again be used to gauge the possibility of any off-target effects from these dsRNAs.

(3) If no likely adverse effects are identified after step 2, then testing should be conducted on animal and human cells in tissue culture,

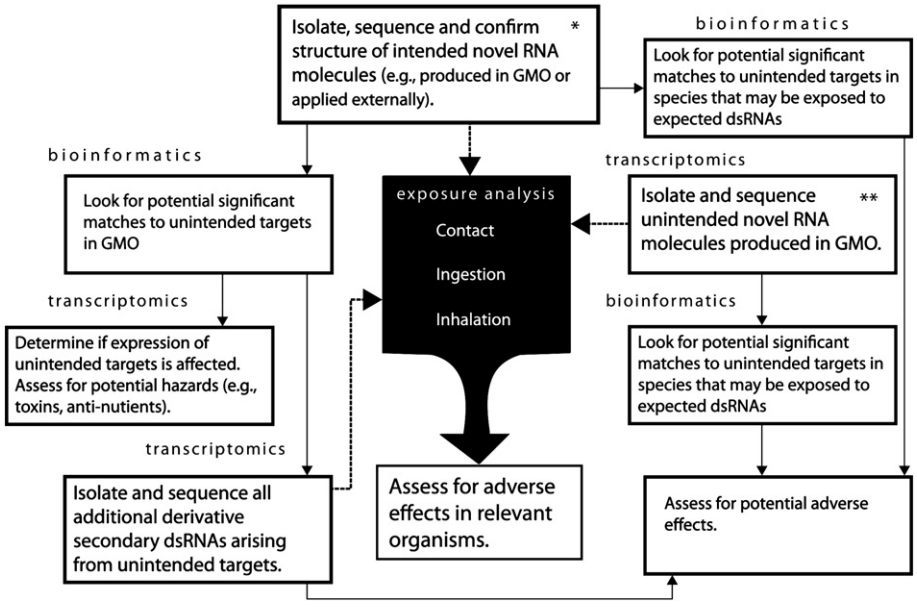

Fig. 3. Sequential approach to assessing the potential for adverse effects arising from dsRNA-initiated modifications to organisms. Bioinformatics is used to capture known hypothetical targets of both intended and unintended dsRNAs so that potential adverse effects can be assessed. Transcriptomics is used to verify and characterise all relevant changes at the transcriptome level. Exposure analysis is used to design the appropriate kinds of organism-level tests for adverse effects. (*) Starting point for intentional introduction of dsRNAs; (**) starting point for unintended changes to the transcriptome. Bioinformatics assessments are inferences or judgments made based on predictions. Assessments made following exposure are based on data from experiments.

for example as done by Zhang et al. (2012a). dsRNAs extracted from the GM product or from test animals exposed to the product should be provided to the cells to monitor changes in gene expression. Uptake by the cells should be confirmed. If no adverse effects are found in these assays, using a variety of different tissue-specific cell types, then animal transcriptome or proteome changes should be monitored. If the intent is to assess human safety outcomes, then animals with embedded patches of human cells in the gastrointestinal tract would be preferred (Xiang et al., 2006).

The use of semi-targeted qualitative profiling of small RNA molecules from the tissue culture cells, patches of human cells and animals tested could also determine whether the effect of the siRNAs was transferable. It would also reveal any generation of unintended novel RNAs (Heinemann et al., 2011).

Although dsRNAs are intended to silence genes, they can also activate genes, for example, when they silence a gene repressor. Therefore, the GM product also should be evaluated for compositional changes, anti-nutrients and new immunomodulating proteins as would be done for any GM product with intentional changes to the proteome.

(4) To specifically test for adverse effects on humans, more dedicated testing would be needed. Long-term testing should be conducted on at least two different animal species to fully test for any unintended effects of the dsRNA and its associated GM product (e.g., a GM plant). At least one of these animal species should be a mammal, so that it has a comparable biology to humans. The testing process should include all expected exposure routes, e.g., ingestion, inhalation, which is a potential exposure route (Renner et al., 2012), and contact via the skin. Feeding studies should use both the intended dsRNA molecules and the edible portion of the GM product.

The animal studies should include studies to investigate whether there are any toxic effects such as any damage to liver, kidneys, or any other organ, any increased risk of any reproductive problems (including to see if any dsRNA-related changes are inherited) over at least two generations, and any increased risk of cancer and any increased risk of an immune response to the GM product, such as an allergic reaction.

When investigating toxic effects it is important that: (a) a control group of animals, fed the non-GM parental organism, is included for comparison; (b) there are enough animals in each group to derive a statistically significant assurance of either harm or safety, e.g., 25 male and 25 female animals per dietary group for toxicity studies (for specific guidance of minimum numbers of animals for various tests, see OECD guidance documents TG451, TG452 and TG453 and discussion in enHealth, 2012); (c) the animals are fed from the ages of just-weaned and for at least six months (and preferably for the lifespan of the animal species, particularly if carcinogenic outcomes and/or life-long consequences of consuming dsRNA are being evaluated); (d) sub-groups of animals are fed various doses of the GM plant, including high doses; (e) full biochemistry and hematology analyses on blood are done on every animal as a minimum requirement (Séralini et al., 2009, 2012), with additional testing informed by earlier experiments; and (f) a full autopsy is conducted on the animals at the end of the experiment, which includes histology on all major organs.

Depending on the outcome of the experiments, further testing may be required. It is noteworthy that dsRNAs intended for use as human therapeutics or vaccines would probably require full clinical testing. Therefore, it might be worthwhile to adopt similar testing for unintended effects of dsRNAs taken up through food or exposure to food products (Box 2).

5. Summary

While some GMOs have been designed to make new dsRNA molecules, in other GMOs such molecules may occur as a side-effect of the genetic engineering process. Still others may make naturally-occurring dsRNA molecules in higher or lower quantities than before. Some dsRNA molecules can have profound physiological effects on the organism that makes them. Physiological effects are the intended outcomes of exposure to dsRNA incorporated into food sources for invertebrates; biopesticides and other topically applied products, and could be the cause of off-target effects and adverse effects in non-target organisms. “A daunting outcome is raised, that each [dsRNA] formulation might have its own risks” (p. 514 Aronin, 2006).

Two separate studies have now provided evidence for miRNAs of plant origin in the circulatory system or organs of humans or mammals (Zhang et al., 2012a, 2012b). In addition, there is experimental evidence demonstrating that some dsRNA molecules can be transmitted through food or other means and can affect those organisms through alterations in gene expression (Zhang et al., 2012a).

Box 2
Should clinical testing be routine?

Shortly after the discovery of RNAi, new pharmaceuticals and vaccines based on dsRNA molecules were proposed, with some rushed into testing (Brisibe et al., 2003; Hirschi, 2012; Seyhan, 2011). Interestingly, any dsRNA intended to silence a gene for medical reasons requires full clinical safety and efficacy trials whereas any unintended silencing by a food-borne dsRNA requires no testing in some jurisdictions. Regulators concerned by this difference in standards based on the intended use, rather than unintended risk, of the product, may consider further testing beyond what is described in Section 4.

If animal studies fail to find any adverse effects and also demonstrate any putative benefit, clinical testing on humans could then be undertaken before the GM product comes into the human food supply, using the standard phases of a clinical trial process (Carman, 2004). In Phase I, initial studies are done on a small number of volunteers to determine if there are any adverse effects, before studies are done to determine if the GM product has any beneficial health effects in Phase II. The part of the product that people eat should be used in these studies.

In Phase I of the clinical trial process, where adverse effects are investigated, the study should investigate whether there is: (1) any uptake of the dsRNA into people, (2) any silencing of any genes in people, (3) any toxic effects such as any damage to liver, kidneys, or any other organ, or (4) any increased risk of an immune response to the GM product, such as an allergic reaction.

When investigating toxic effects it is important that: (a) a control group of people, fed the isogenic or near isogenic non-GM parental organism, is included for comparison; (b) there are enough people in each group to derive a statistically significant assurance of either harm or safety, e.g. 25 males and 25 females per dietary group; (c) people are fed for at least six months; (d) sub-groups of volunteers are fed with various doses of the GM plant, including high doses; and (e) full biochemistry and hematology analyses on blood are done on every participant as a minimum requirement.

Production of intended dsRNA molecules may also have off-target effects due to silencing genes other than those intended. Unanticipated off-target adverse effects can be difficult to detect and they are not possible to reliably predict using bioinformatics techniques.

Regulatory bodies are not adequately assessing the risks of dsRNA-producing GM products.

As a result, we recommend a process to properly assess the safety of dsRNA-producing GM organisms before they are released or commercialized (Fig. 3). This process includes the following: (1) bioinformatics to identify any likely, unintended targets of the dsRNA in humans and other key organisms; (2) experimental procedures that would identify all new intended and unintended dsRNA molecules in the GM product; (3) testing animal and human cells in tissue culture for a response to intended and unintended dsRNAs from the product; (4) long-term testing on animals; and possibly (5) clinical trials on human volunteers.

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