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**Evaluation of risks from creation of novel RNA
molecules in genetically engineered wheat plants
and recommendations for risk assessment**

An expert opinion of Professor Jack A. Heinemann, PhD
28 August 2012

On the 12th July 2012 I was contacted by the Safe Food Institute with a request to provide an expert scientific opinion on “GM wheat research being conducted by the CSIRO.” Included in this correspondence was the additional request that: “If you do believe that there are risks, can you please provide recommendations for any studies that would provide information to confirm or exclude any risks to human health.”

The following is my expert opinion about whether plants genetically modified to produce an RNA interference (RNAi or co-suppression) effect may create a risk for human health or the environment.

I am a molecular biologist. I have been an academic at the University of Canterbury since 1994. Prior to that, I was employed by the US National Institutes of Health. My doctorate was conferred by the University of Oregon at Eugene (1989) and my Bachelor of Science (with honours) degree from the University of Wisconsin, Madison (1985). I am involved in risk assessment research and participate in risk assessment through evaluation of assessments provided to regulatory bodies and through the development of international guidance documents for risk assessment. I have over 100 scholarly works published on the topic of molecular biology, genetics, risk assessment and other scientific matters within my expertise. I publish in leading international journals and my work has been recognised by prestigious professional organisations for its excellence.

I will provide a short overview of how dsRNA-mediated silencing (via RNAi, cosuppression and other similar pathways) works and how it is manipulated through genetic engineering. More extensive coverage with relevance to risk assessment can be found in Appendix 1 of Heinemann (2009). I will focus on the risk pathways that I believe are plausible and relevant. Potentially critical technical details of the actual constructs used in the wheat that were being trialled were not available to me. I understand that these details are not in the public domain¹. *This analysis therefore is based on the potential for modifications that could cause harm from the viewpoint of a scientist attempting to minimise type II errors, i.e., those that would falsely find no risk when one indeed exists.*

¹ A search of Commonwealth Scientific and Industrial Research Organization (CSIRO) patents in the US and Europe revealed two related patents on the use of dsRNA to control gene expression in plants (US 8,183,217B2, EP 2 333 061 A1), but neither of these listed specific DNA sequences for SE genes.

Background:

The change intended to be introduced into the genetically modified (GM) wheat through genetic engineering was the production of novel RNA molecules that 'turn off' the expression of genes; these are called regulatory RNAs (a type of non-coding RNA). The vast majority of existing commercial GM plants (e.g., herbicide tolerant or insecticide traits) are not intended to make RNA molecules that are involved in gene regulation. This type of modification is therefore rare and has not benefited from extensive or validated safety testing procedures. Therefore my focus and special interest is on the RNA level changes.

Definitions

Non-coding RNA is all RNA in a cell that is not directly used as a co-factor to order amino acids during protein synthesis (i.e., not mRNA).

dsRNA is double-stranded RNA. The strands are held together by hydrogen bonds between nucleotides in a way analogous to DNA.

There is a temptation to think of genetic engineering as "tinkering with DNA". All commercialised GM plants at this time are created through *in vitro* DNA modification. However, not all of them are created with the intention to produce a new protein. A growing minority are designed to change their RNA content. The reason for this is the finding that RNA, specifically double-stranded RNA (dsRNA), is an important regulator of gene expression (Appendix 1 of Heinemann, 2009).

Those who have studied molecular biology know that RNA is an intermediate molecule used in the cellular reactions of translation to synthesise proteins. The most familiar form of RNA is mRNA, the single-stranded messenger. Nevertheless, types of dsRNAs have been known for a long time. For example transfer RNA (tRNA), which is also usually taught in the first encounter with molecular biology, has dsRNA regions. However, it is only in the last 10-15 years that small dsRNA molecules have become known for their role in regulating gene expression (Hutvagner and Simard, 2008).

dsRNAs are variously called siRNA (short inhibitory RNA), miRNA (microRNA) or microRNA-like 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 caused by gene silencing: disrupting the connection between genes and the production of the proteins specified by genes².

Definitions

siRNA is a processed linear dsRNA molecule where the two strands are held together through inter-molecular base-pairs.

miRNA is processed single-stranded RNA with a dsRNA region held together through intra-molecular base-pairs. miRNA-like molecules derive from siRNA.

RNAi, PTGS and TGS refer to gene silencing brought about through production of or exposure to dsRNA molecules. The gene silencing is caused by inhibition of translation, mRNA degradation (Figure 2), or (TGS) methylation of DNA (and modification of histones).

² For an excellent animation, see <http://www.nature.com/nrg/multimedia/rnai/animation/index.html>.

dsRNAs form when both strands of a DNA molecule are transcribed to synthesise complementary RNA molecules (which then bind together in the same way as DNA), or when stretches of intra-molecular complementarity create stem-loop structures (Figure 1). 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 (Figure 2), or to target and chemically modify DNA sequences by addition of methyl groups and cause modification of DNA-associated histone proteins. This process 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 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).

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 (Denli and Hannon, 2003, Weld et al., 2001). The new RNA sequence may be created when the DNA strand not normally used as a co-factor for transcription is used as such (perhaps because the insert had a cryptic promoter activity or inserted near a promoter). The resulting single-stranded RNA may bind to the target mRNA to create regions of linear dsRNA that can be processed into siRNA (Figure 1). Another possibility is that the insert contributes to the formation of a stem-loop (also called “hairpin”, “short-hairpin” (sh) and “pan-handle” RNA)³, from which the “stem” may be processed into an miRNA-like molecule (Figure 1).

dsRNAs are remarkably stable in the environment. 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). 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 Jr., 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).

The concern I was asked to comment on is whether siRNAs designed to silence the *SEI* and *SEII* genes (the SBE I and SBE II proteins) of wheat and barley might have off-target effects, particularly when these novel dsRNA molecules enter the human food supply. From what is known about the biochemistry of dsRNA-mediated silencing, and the chemistry of RNA, it is clear that genetically engineered changes at the RNA level can have important implications on both

³ E.g., see language in CSIRO patent US 8,183,217B2.

the GM wheat (as intended, and otherwise) and other organisms exposed to the wheat.

Definitions

Off-target effects are those that result in unintended silencing of other (not targeted) genes.

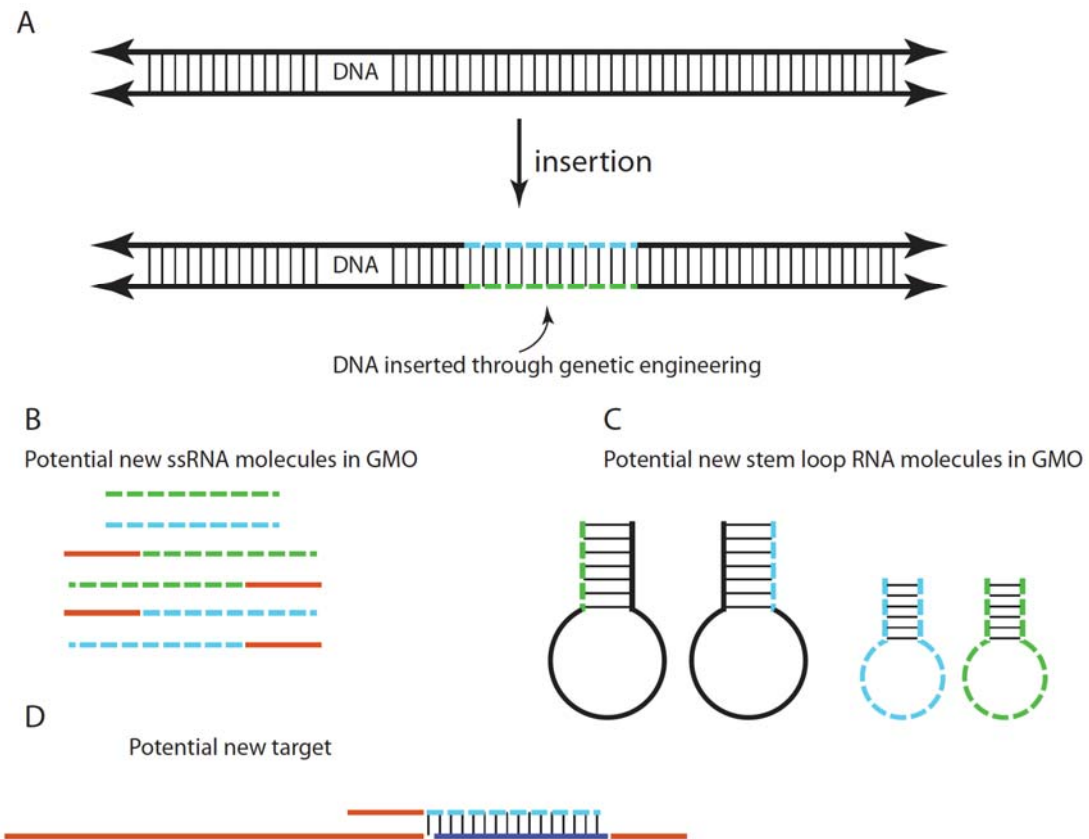


Figure 1: source of new dsRNA molecules from genetic engineering.

(A) Regardless of the source of the DNA inserted (dashed blue and green lines in the black double stranded DNA molecule) into a genome by genetic engineering, it creates new sequences. 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 by the engineering process, or 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 off-target (red line with purple target section) gene silencing in the GMO or in organisms that eat the GMO.

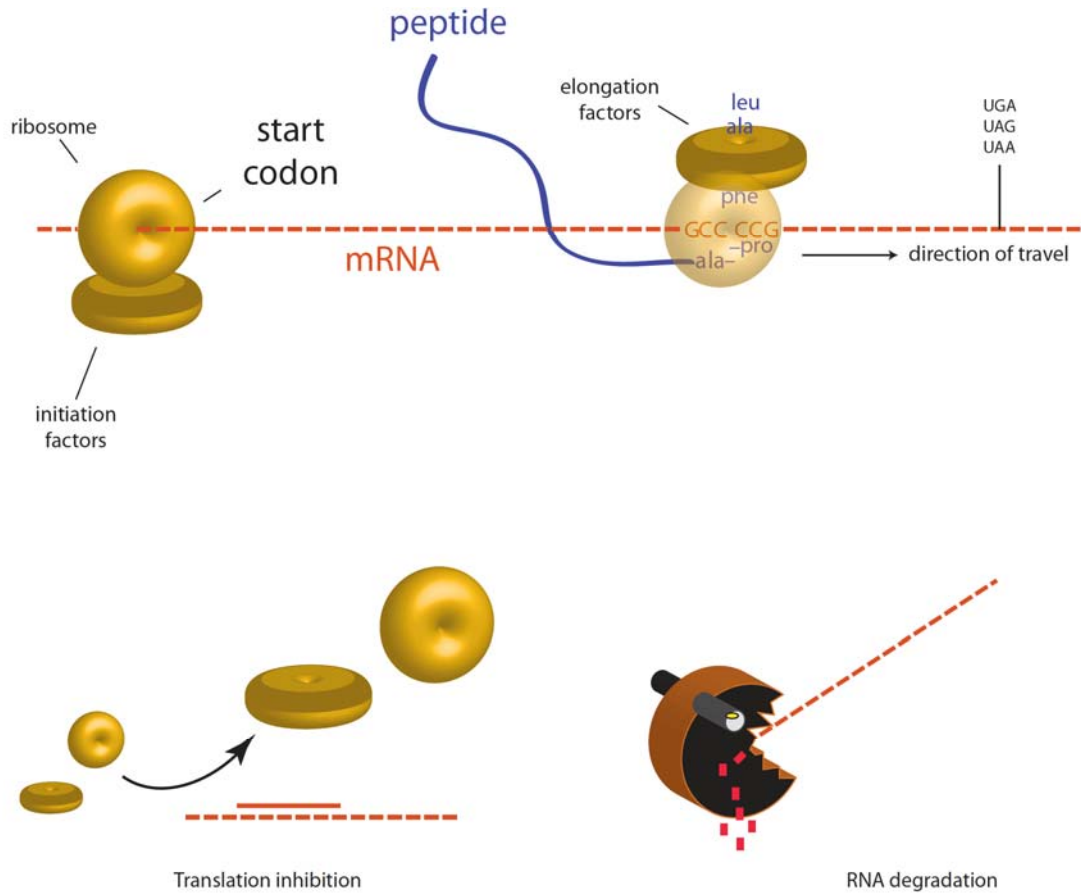


Figure 2: translation and the two pathways of cytoplasmic dsRNA-mediated silencing.

Translation of proteins (top) is the process of linking individual amino acids (the subunits of proteins) into a polymer, called a peptide (blue ribbon). The central enzymatic activity is provided by the ribosome, a multi-protein and RNA complex (brown), which uses the mRNA (red) as a co-factor and a particular sequence, the start codon, as the place to begin protein synthesis. Specific dsRNAs are processed through a complex biochemical pathway to create short single stands that in an associate nucleoprotein complex block translation by direct occlusion of the ribosome (bottom left) or by recruiting enzymes that depolymerise the mRNA (bottom right).

- I. It is reasonable for the plant-generated novel RNA molecules to be considered relevant to a human health risk assessment.
 - (i) Plant-derived microRNA precursors have been detected in human blood. This demonstrates that they can survive digestion and be taken up via the gastrointestinal tract (Zhang et al., 2012b). Plant-derived microRNAs are chemically and structurally similar to siRNA constructs intended to be produced in the wheat and thus their characteristics are predictive of the characteristics of the siRNA constructs. *There is strong evidence that siRNAs produced in the wheat will transfer to humans through food.*
 - (ii) Those dsRNAs that have been shown to transmit via food are stable through cooking and at pH 2.0 for at least 6 hours (Zhang et al., 2012b). *There is strong evidence that siRNAs produced in the wheat will remain in a form that can transmit to humans even when the wheat has been cooked or processed for use in food.*
 - (iii) These plant-derived dsRNA molecules silenced an endogenous gene in human tissue culture cells, and *in vivo* in mice liver, small intestine, and lung (Zhang et al., 2012b). *There is strong evidence that once transmitted, siRNA produced in wheat would have the biological capacity to cause an effect.*

Therefore, it is possible that novel dsRNA molecules created through the genetic engineering of the wheat, or made at concentrations unique to this wheat, may be stable through storage, cooking and processing when used as a human food, and then transmit through food or inhalation to humans and have the potential to cause adverse effects. These effects may be through acute exposure leading to a cytoplasmic RNAi effect (Figure 2) or a nuclear TGS effect following exposure. Notably, little is known about the number of off-target effects that are possible. For example, a TGS effect arising from “chromatin remodeling events...may represent a novel mode of off-target effect” (p. 2994 Hawkins et al., 2009) and considerably extend the range of off-target effects.

Wheat is one of the humanity’s largest sources of calories and nutrition. It contributes 530 kcal/capita/day and 16 g of protein/capita/day to the world food supply (FAOSTAT, 2007)⁴. This is up from 410 kcal/capita/day and 13 g of protein/capita/day in 1961 (first year of FAOSTAT statistics), and steady with rice as a protein and calorie source (about 20% of dietary calories not counting alcohol)⁵. Australia is the 9th largest producer of wheat and contributes an estimated 3% to world production. Australia is consistently one of the top 5 exporting nations, contributing by weight 11% to world trade in wheat and wheat flour, and 13% in barley (FAOSTAT, 2009). Without question, changes to wheat and barley in Australia – either good or bad – are important to both Australians and to the rest of humanity.

- II. It is reasonable for the plant-generated novel RNA molecules to be considered relevant to an environmental risk assessment.
 - (i) Specific siRNAs can be toxic and the toxicity can be transmitted through food to animals of environmental relevance. This was demonstrated when

⁴ FAOSTAT is a database of the United Nations Food and Agriculture Organisation.

⁵ Barley contributed only 7 kcal/capita/day and 0.2 g protein/capita/day in 2007.

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 into siRNA that silenced one or more genes essential for life, or essential for detoxifying natural plant toxins (i.e., gossypol in cotton). *There is strong evidence that siRNAs produced in the wheat will transfer to recipient organisms in the environment.*

This risk is known to the Commonwealth Scientific and Industrial Research Organization (CSIRO), the GM wheat developer, because it holds a patent to transmit dsRNA through food to arthropods and to cause gene silencing in the animals⁶. 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.”

- (ii) Unintended secondary dsRNAs that might be generated *in planta* or in animals consuming the plant cannot be anticipated but may well exist. These secondary dsRNAs may have gene regulatory activities and thus act like siRNA. This means that dsRNAs created by the genetic engineering of wheat may cause the production of additional unintended or unanticipated dsRNA molecules in *both* the genetically engineered wheat and in any organism that consumes the wheat. *Any of these unintended secondary dsRNA molecules could be the cause of an adverse effect.*

Importantly as well, RNAi can be transmitted across plant tissues regardless of where the interference is initially generated (Jorgensen, 2002, Klahre et al., 2002, Yoo et al., 2004). This means that the introduced siRNAs may not be confined to the intended tissue (e.g., endosperm) and that the entire plant needs to be used for testing toxicity to indicator birds, mammals, insects and nematodes. *There is evidence that unintended secondary siRNAs potentially could be produced either in the wheat as an unavoidable outcome of the modification, or in organisms exposed to wheat tissues during cultivation or storage.*

- (iii) It is relevant and noteworthy that other regulatory agencies in Australia describe the pathways through which RNAi arises as poorly understood (FSANZ, 2009). In such cases, any risk assessment would have a high uncertainty as to the level of risk especially from unanticipated effects.

Wheat crops cover 29% of Australia’s agricultural area, which is 53% of Australia’s surface. Wheat and barley combined cover 37% of agricultural land at about 17.6 million hectares (FAOSTAT, 2009). A change in these crops with unanticipated environmental effects could have large scale consequences.

Introduction:

The Australian CSIRO has constructed genetically modified wheat varieties intended to not express *SEI* only in the endosperm because of an induced RNA interference. Barley varieties have been produced that are intended to express neither *SEI* nor *SEII* in the endosperm. The RNAi is intended to be created

⁶ EP 2 333 061 A1

through the introduction of transgenes constructed to produce substrates for the endogenous dsRNA processing pathways in plants. To my understanding, these constructs involve tandem repeats of two sequences, with the second sequence being in the opposite orientation (i.e., inverted repeat) to the first. This allows for intra-molecular base-pairing and encourages the formation of short-hairpin dsRNA (Figure 3). The repeated sequences are presumably exonic sequences from *SEI* and *SEII*, respectively, separated by intron 3 of *SEI*. The actual sequences used are not known to me (see above). The constructs are intended to be processed through canonical splicing pathways to remove intron 3 and increase the efficiency of processing the resulting dsRNA into siRNA.

SE genes encode the SBE starch branching enzymes. These enzymes create α -1-6 linkages through cleavage of α -1-4 linkages on linear chains of glucose. Similar genes are found in many other organisms, including humans. The similar activity to *SEI* in humans is encoded by *GBE*, the glucan (1,4- α -), branching enzyme 1.

Definitions

Intron is a region in an mRNA molecule that is removed prior to translation through reactions called splicing.

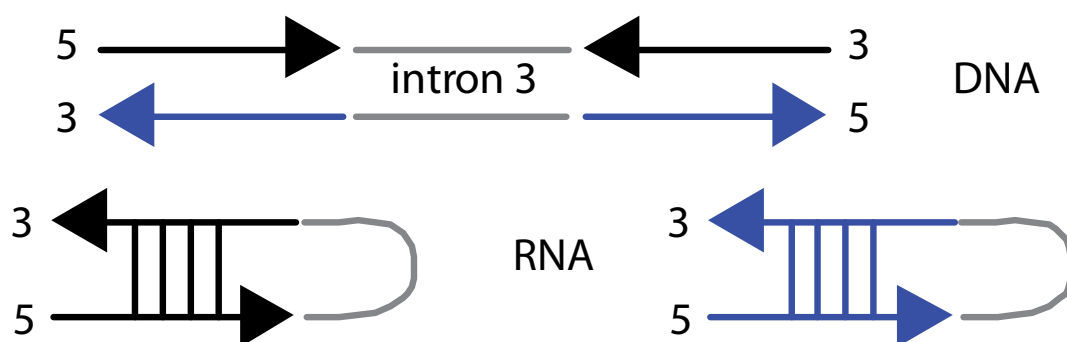


Figure 3. Hypothetical structures of regulatory RNA molecules in GM wheat.

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 *SEI*.

I make a preliminary consideration of whether siRNAs generated to silence *SEI* might have silencing activity in humans. Note that this is a conservative initial appraisal for several reasons.

First, the actual siRNA sequences were not available. Thus, I consider whether any siRNA sequences could have an effect, not whether all possible siRNAs would have an effect.

Second, unintended secondary siRNAs that might be generated *in planta* or in humans cannot be anticipated but may well exist. For example, it was secondary dsRNAs that were the effective agents in establishing RNAi in insect pests who

ate GM corn plants designed to produce primary dsRNA molecules (Baum et al., 2007), and it was pre-mature microRNA of rice plants modified by human cells that caused silencing of genes in human tissue culture cells (Zhang et al., 2012b).

Moreover, siRNAs designed against the coding region of a target may also find unintended binding targets in the introns of pre-mature RNA in the nucleus (Seinen et al., 2011). Although introns may normally be removed, there are many known exceptions and removal can differ between tissues and time of development, so introns remain potential targets of siRNAs.

Third, bioinformatic tools are not definitive for predicting the effectiveness of siRNAs at causing an RNAi effect, particularly for ruling out an effect. As a biosafety scientist, I am interested in minimising type II errors (those that result in false negative identification) rather than minimising type I errors (those that result in a false positive identification). Any possible siRNA identified through bioinformatics techniques is a candidate for further testing, but bioinformatics is not a substitute for other kinds of testing.

Fourth, not all available bioinformatics techniques were used to create this initial appraisal. A more refined list of candidates might be found using even more sophisticated and specialist techniques (Birmingham et al., 2006). However, these techniques were sufficient to identify possible unintended targets.

Finally, the bioinformatics parameters used below have not all come from a study of off-target effects in humans. Humans may be more or less reactive to the same siRNAs. Since I am a biosafety scientist, my concern is to minimise type II errors and thus I assume that humans are at least as responsive as any research organism until proven otherwise. The literature has a bias toward reporting siRNAs with large effects rather than systematically cataloguing all effects. This contributes to a perception of fewer than actual unintended effects. Since again I am minimizing type II errors, I assume that effects may be small but biologically relevant unless proven otherwise.

I consider as relevant dsRNA-mediated off-target effects that result in RNA cleavage, translational inhibition (co-suppression), or transcriptional silencing (TGS).

Methods:

GBE and *SEI* sequences were accessed from the NCBI database⁷. The sequences were compared for matches. Areas with a high density of identity were then evaluated for potential to be targeted by the same siRNA.

Critical assumptions:

Information for this report was taken from the Office of the Gene Technology Regulator's publication DIR093 "Limited and controlled release of wheat and barley genetically modified for altered grain starch composition."⁸ That report provided the name of genes targeted for silencing and the origin in general terms of the sequences that were used to construct the event that is expected to express the intended siRNAs.

⁷ NCBI Reference Sequence: NM_000158.3; GenBank: AF525764.1

⁸ <http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/dir093>

Sequence analysis was performed using publicly available accessions on the NCBI database. The DNA sequences of the named genes may or may not be exactly the same as in the wheat varieties used by CSIRO. However, this is the best possible source of information barring access to the actual commercial-in-confidence sequences. Absent for this analysis were the DNA sequences used by CSIRO to construct the siRNAs. This proprietary information is necessary to conduct a definitive bioinformatics analysis for risk assessment. To compensate for this, all possible known sequences from the named source genes as available in NCBI database were used.

Therefore, this analysis provides an estimation of the plausibility that the GM wheat event could cause an adverse effect via the expression of these novel dsRNA molecules. The results neither imply that all possible targets of interest have been identified, nor that all targets identified are certain to be silenced. For these final hypotheses to be tested, the actual sequences would be required. It would be incorrect to assume that if targets discussed below were not affected, that the constructs were safe for use. Likewise, it would be incorrect to conclude that sequence similarity between actual siRNAs and any targets below will necessarily cause the targets to be silenced. Moreover, no bioinformatics-based analysis will be capable of making a definitive finding of safety. However, it can provide evidence that assists in focusing experimental testing for potential adverse effects and in that way contribute to the risk assessment and reduce uncertainties built into regulatory decisions.

Evaluation criteria:

1. Perfect sequence matches indicate high probability of RNAi (that is ~21/21 matches).
2. Short matches can cause off-target effects (Lin et al., 2005). "In conclusion, 15 nucleotides, and perhaps as few as 11 contiguous nucleotides, of sequence identity are sufficient to direct silencing of nontargeted transcripts and therefore...off-target gene regulation can occur as a result of degradation of mRNA transcripts with partial identity to the siRNA sequence" (p. 636 Jackson et al., 2003).
3. Approaching or exceeding 95% identity over 40 nucleotides is predicted to cause RNAi (Rual et al., 2007).
4. Short (≥ 7 contiguous⁹) identical matches in the 3' untranslated region (UTR) of mRNA can be more determinative than number of matches overall (Birmingham et al., 2006).

Results:

The *SEI* sequence used in this evaluation was originally submitted to GenBank by the CSIRO. It includes the coding sequence for *SEI* and a preceding pseudogene. Since I do not know what sequence was used by CSIRO, the entire submitted sequence was included in this analysis.

⁹ These researchers found that surrounding sequences, perhaps through their effect on 2° structure of the mRNA, accentuated the strength of the siRNAs. "The context dependent silencing mediated by partial complementation between a siRNA and its unintended targets makes it more difficult to predict the off-target effect of a given siRNA" (p. 4534 of Lin et al, 2005).

The human *GBE* and plant *SEI* genes are very similar (Appendix 1). There is enough identity throughout the compared sequences to prevent ruling out possible silencing of the human *GBE* by siRNAs generated against *SEI*. For example, in the region of nucleotides 987-1024 of the *GBE* open reading frame, and 21363-21400 within the open reading frame of *SEI*, there are 32 matches (86% identity), with a stretch of one mismatch in a run of 21 contiguous bases, and 16 out of 16 contiguous bases making perfect matches. In the region of nucleotides 1694-1730 of the *GBE* open reading frame, and 22076-22112 within the open reading frame of *SEI*, there are 37 matches out of 40 contiguous nucleotides (93% identity), made up of 14 matches in 14 nucleotides, 5 matches in 7, 16 matches in 16 and a final 2 matches out of 3 contiguous nucleotides (Figure 4).

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Query  1687  CTACCTTGAAAAGTGCATTGCTTATGCAGAGAGCCATGATCAGGCAT  1733
      | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Sbjct  22069  ATATACGGAAAAGTGCATTGCATATGCTGAGAGCCATGATCAGGTAT  22115

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Figure 4. High density identity relationship between *SEI* and *GBE*.

Shown are nucleotides 1687-1733 of *GBE* (“query”) and 22069-22115 of *SEI* (“subject”). Vertical lines indicate identity.

SEI was also compared to the entire human genome (Appendix 2). Multiple matches of ≥ 21 contiguous nucleotides were found (see, e.g., horizontal red arrows indicating sequence matches on pages 6/770, 11/770, 15-16/770, 31/770, 38-39/770, 43/770 and 62-65/770)¹⁰. Thus, there may potentially be other unintended silencing effects depending on the siRNAs used in the GM wheat.

Short sequences of perfect identity between an siRNA and the 3′ UTR region of unintended mRNAs are also predictive for creating an RNAi effect. Sequence matches from the mRNA of *SEI* were thus compared to the 3′ UTR region of *GBE* (Table 1). Except for one entry in Table 1, the match with the 3′ UTR region of *GBE* is to a sequence 5′ of the *SEI* start and may not be a source of CSIRO siRNAs. Again, this is not certain given the ambiguity of the source sequence for the siRNAs. However, one sequence match to the 3′ UTR region of *GBE* is in the predicted mRNA of *SEI* in intron 13 (row one, Table 1). This may be a source of off-target effects should the siRNAs migrate to the nucleus (Robb et al., 2005).

Plausible risk pathways:

Following from the rationale that siRNA produced by genetic engineering, or secondary dsRNAs that are caused by the modification, may be biologically relevant to human health and the environment, I consider pathways through which potential adverse effects might arise.

¹⁰ Note that they were not characterized for being known open reading frames.

Production of intended siRNA molecules may both cause intended gene silencing and have off target effects, i.e., may silence genes other than those intended. Unanticipated off-target adverse effects can be difficult to detect.

Off-target effects are common, difficult to predict, but not inevitable. They arise from matches between the intended siRNA and sequences in other genes (Jackson et al., 2003, Lin et al., 2005, Seinen et al., 2011, Semizarov et al., 2003). Some unintended effects of siRNAs kill cells (Fedorov et al., 2006), but lesser and still concerning adverse effects can require more sensitive techniques to reveal (Zhang et al., 2012a).

Off-target effects can also arise from imperfect matches between the intended siRNA and sequences in other genes, particularly when these matches are in the 3' UTR of an RNA (Birmingham et al., 2006).

In addition, off-target effects can arise from either perfect or imperfect matches between secondarily derived dsRNAs and sequences in other genes (Baum et al., 2007, Gordon and Waterhouse, 2007, Zhang et al., 2012b).

Any off-target effect may cause silencing in either another gene of wheat, or in an organism consuming the wheat or exposed in some other way (possibly through inhalation, although this exposure pathway has not yet been tested to my knowledge). Any off-target effect that caused the generation of additional secondary dsRNAs would potentially create even more unanticipated off-target effects.

Table 1. Highlighted matches between 3' UTR region of *GBE* and potential siRNAs of *SEI*.

Nucleotide range		Perfect match length	Overall identity (%)
<i>GBE</i>	<i>SEI</i>		
2796	24642	11	82
2465	17647	11	100
2632	11339	11	79
2632	11925	11	100
2647	12745	11	100
2688	8819	11	100
2700	11063	15	100
2807	10211	11	81
2813	15854	11	100
2961	11580	11	100
3073	9243	11	76

Scientific studies I would recommend to precede a field trial:

With the plausible risk pathways understood, what kinds of scientific studies or assurances should have been, in my opinion, undertaken to address concerned members of the public?

- I. Bioinformatics studies to identify any likely unintended targets of the intended siRNAs in humans and species used as indicators of key ecological functions or which are protected. These studies would have looked for perfect matches or similar sequences in the coding region and introns (Seinen et al., 2011), and perfect matches in seed regions of 3' UTRs, of RNAs derived from whole genome sequences, where available.
- II. When a whole genome sequence of sufficient confidence is not available for a species, more specific laboratory experiments might need to be conducted.
- III. Experimental verification of the intended siRNAs (both sequence and structure) in wheat and demonstration that silencing is by a known dsRNA-mediated pathway, determining any role for either or both cytoplasmic (Figure 2) or nuclear (TGS) silencing pathways.
- IV. Any potential off-target effect identified through the bioinformatics analysis should either cause the siRNA to be rejected and another sought, if possible, or be further evaluated by tissue culture studies (human or animal cells), for example as done by Zhang et al (2012b), long-term (e.g., at least two year long) feeding studies (non-human), or inhalation studies (non-human) testing for potential silencing of identified unintended genes. Animal studies cannot substitute for use of human tissue culture studies in a human health risk assessment (Burchard et al., 2009).
- V. For any siRNA not found to cause an adverse effect on animals, further testing should be conducted to exclude the *in planta* production of secondary dsRNA molecules with other off-target effects, especially before any purposeful potential exposure to humans. This could be done through a semi-targeted qualitative profiling of small RNA molecules using high throughput sequencing in a comparative assessment between the GM and conventional parent (Heinemann et al., 2011), a similar comparative profiling exercise from (human, animal) tissue cultures either exposed or not to the intended siRNAs, and/or proper animal/insect feeding and inhalation studies if not already conducted above.

Conclusions:

- (1) There are extensive similarities between the plant *SEI* gene and the human *GBE* gene. The bioinformatics analysis cannot rule out unintended cross reactivity between siRNAs, designed to silence *SEI*, and *GBE*.
- (2) There are extensive similarities between *SEI* (including its introns) and other genes in the human genome. The bioinformatic analysis cannot rule out unintended cross reactivity between siRNAs, designed to silence *SEI*, and other genes.
- (3) In plants, siRNAs can be systemically transmitted. It would not be possible without experimental confirmation to ensure the absence of the siRNAs in tissue other than endosperm.
- (4) An RNAi effect can result in the generation of unintended secondary siRNAs. These may extend the potential for unintended cross reactivity with *GBE* or other human genes.

(5) It would not be possible to exclude unintended silencing effects without proper genetic testing. Unintended activities are species-specific (Burchard et al., 2009), so testing should be conducted in animals, but also animals with established patches of human cell tissue, and using relevant human tissue culture cells.

Respectfully yours



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References¹¹:

Ahlenstiel, C. L., Lim, H. G. W., Cooper, D. A., Ishida, T., Kelleher, A. D. and Suzuki, K. (2012). Direct evidence of nuclear Argonaute distribution during transcriptional silencing links the actin cytoskeleton to nuclear RNAi machinery in human cells. *Nucl. Acids Res.* *40*, 1579-1595.

Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., *et al.* (2007). Control of coleopteran insect pests through RNA interference. *Nat Biotechnol* *25*, 1322-1326.

Birmingham, A., Anderson, E. M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., Baskerville, S., Maksimova, E., Robinson, K., Karpilow, J., *et al.* (2006). 3[prime] UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Meth* *3*, 199-204.

Burchard, J., Jackson, A. L., Malkov, V., Needham, R. H. V., Tan, Y., Bartz, S. R., Dai, H., Sachs, A. B. and Linsley, P. S. (2009). MicroRNA-like off-target transcript regulation by siRNAs is species specific. *RNA* *15*, 308-315.

Cogoni, C. and Macino, G. (2000). Post-transcriptional gene silencing across kingdoms. *Curr. Opin. Genet. Develop.* *10*, 638-643.

Denli, A. M. and Hannon, G. J. (2003). RNAi: an ever-growing puzzle. *Trends Biochem. Sci.* *28*, 196-201.

Fedorov, Y., Anderson, E. M., Birmingham, A., Reynolds, A., Karpilow, J., Robinson, K., Leake, D., Marshall, W. S. and Khvorova, A. (2006). Off-target effects by siRNA can induce toxic phenotype. *RNA* *12*, 1188-1196.

FSANZ (2009). Application A1018 Food Derived From High Oleic Acid Soybean Line DP-305423-1 Supporting Document 1. Food Standards Australia New Zealand.

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- Gordon, K. H. J. and Waterhouse, P. M. (2007). RNAi for insect-proof plants. *Nat Biotechnol* 25, 1231-1232.
- Grewal, S. I. S. and Elgin, S. C. R. (2007). Transcription and RNA interference in the formation of heterochromatin. *Nature* 447, 399-406.
- Hawkins, P. G., Santoso, S., Adams, C., Anest, V. and Morris, K. V. (2009). Promoter targeted small RNAs induce long-term transcriptional gene silencing in human cells. *Nucl. Acids Res.* 37, 2984-2995.
- Heinemann, J. A. (2009). Hope not Hype. The future of agriculture guided by the International Assessment of Agricultural Knowledge, Science and Technology for Development (Penang, Third World Network).
- Heinemann, J. A., Kurenbach, B. and Quist, D. (2011). Molecular profiling — a tool for addressing emerging gaps in the comparative risk assessment of GMOs. *Env. Int.* 37, 1285-1293.
- Hutvágner, G. and Simard, M. J. (2008). Argonaute proteins: key players in RNA silencing. *Nat Rev. Mol. Cell Biol.* 9, 22-32.
- Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P. S. (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21, 635-637.
- Jorgensen, R. A. (2002). RNA traffics information systemically in plants. *Proc. Natl. Acad. Sci. USA* 99, 11561-11563.
- Klahre, U., Crete, P., Leuenberger, S. A., Iglesias, V. A. and Meins, F., Jr. (2002). High molecular weight RNAs and small interfering RNAs induce systemic posttranscriptional gene silencing in plants. *Proc. Natl. Acad. Sci. USA* 99, 11981-11986.
- Lin, X., Ruan, X., Anderson, M. G., McDowell, J. A., Kroeger, P. E., Fesik, S. W. and Shen, Y. (2005). siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. *Nucl. Acids Res.* 33, 4527-4535.
- Mao, Y.-B., Cai, W.-J., Wang, J.-W., Hong, G.-J., Tao, X.-Y., Wang, L.-J., Huang, Y.-P. and Chen, X.-Y. (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat. Biotechnol.* 25, 1307-1313.
- Mello, C. C. and Conte Jr., D. (2004). Revealing the world of RNA interference. *Nature* 432, 338-342.
- Reyes-Turcu, F. E. and Grewal, S. I. S. (2012). Different means, same end — heterochromatin formation by RNAi and RNAi-independent RNA processing factors in fission yeast. *Curr. Opin. Genet. Dev.* 22, 156-163.

Robb, G. B., Brown, K. M., Khurana, J. and Rana, T. M. (2005). Specific and potent RNAi in the nucleus of human cells. *Nat. Struct. Mol. Biol.* *12*, 133-7.

Rual, J.-F., Klitgord, N. and Achaz, G. (2007). Novel insights into RNAi off-target effects using *C. elegans* paralogs. *BMC Genomics* *8*, 106.

Seinen, E., Burgerhof, J. G. M., Jansen, R. C. and Sibon, O. C. M. (2011). RNAi-induced off-target effects in *Drosophila melanogaster*: frequencies and solutions. *Brief Func Genomics* *10*, 206-214.

Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D. N. and Fesik, S. W. (2003). Specificity of short interfering RNA determined through gene expression signatures. *Proc. Natl. Acad. Sci. USA* *100*, 6347-6352.

Tabara, H., Grishok, A. and C. Mello, C. (1998). REVERSE GENETICS:RNAi in *C. elegans*: Soaking in the Genome Sequence. *Science* *282*, 430-431.

Weld, R., Heinemann, J. and Eady, C. (2001). Transient GFP expression in *Nicotiana plumbaginifolia* suspension cells: the role of gene silencing, cell death and T-DNA loss. *Pl. Mol. Biol.* *45*, 377-385.

Yoo, B.-C., Kragler, F., Varkonyi-Gasic, E., Haywood, V., Archer-Evans, S., Lee, Y. M., Lough, T. J. and Lucas, W. J. (2004). A systemic small RNA signaling system in plants. *Pl. Cell* *16*, 1979-2000.

Zhang, G., Guo, B., Wu, H., Tang, T., Zhang, B.-T., Zheng, L., He, Y., Yang, Z., Pan, X., Chow, H., *et al.* (2012a). A delivery system targeting bone formation surfaces to facilitate RNAi-based anabolic therapy. *Nat Med* *18*, 307-314.

Zhang, H. and Zhu, J.-K. (2012). Seeing the forest for the trees: a wide perspective on RNA-directed DNA methylation. *Genes Dev.* *26*, 1769-1773.

Zhang, L., Hou, D., Chen, X., Li, D., Zhu, L., Zhang, Y., Li, J., Bian, Z., Liang, X., Cai, X., *et al.* (2012b). Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Res* *22*, 107-126.