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DRAFT

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The following is provided at the request of the Safe Food Foundation. It is my 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.

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.

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 domain. *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.*

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 very new and has not benefited from extensive or validated safety testing procedures. Therefore my focus and special interest is on the RNA level changes.

FOR COMMENT

dsRNA-mediated silencing is a biochemical pathway of which dsRNA molecules are the starting substrates. Processed derivatives are used to target protein complexes to mRNAs (which are used as a template for protein assembly) with identical or similar sequences.

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, actually double-stranded RNA (dsRNA), is an important regulator of gene expression (for review see 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 (Hutvágner 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 genes[1].

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 can arise from siRNA.

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 DNA sequences and cause their modification by addition of methyl groups, known to inhibit transcription (Grewal and Elgin, 2007).

New dsRNA molecules are commonly created by the genetic engineering process. Indeed, most cells initially engineered using *in vitro* nucleic acid techniques ultimately "silence" the gene inserted because they cause the 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, from which the "stem" may be processed into an miRNA-like molecule (Figure 1).

[1] For an excellent animation, see http://www.nature.com/nrg/multimedia/rnai/animation/index.html.

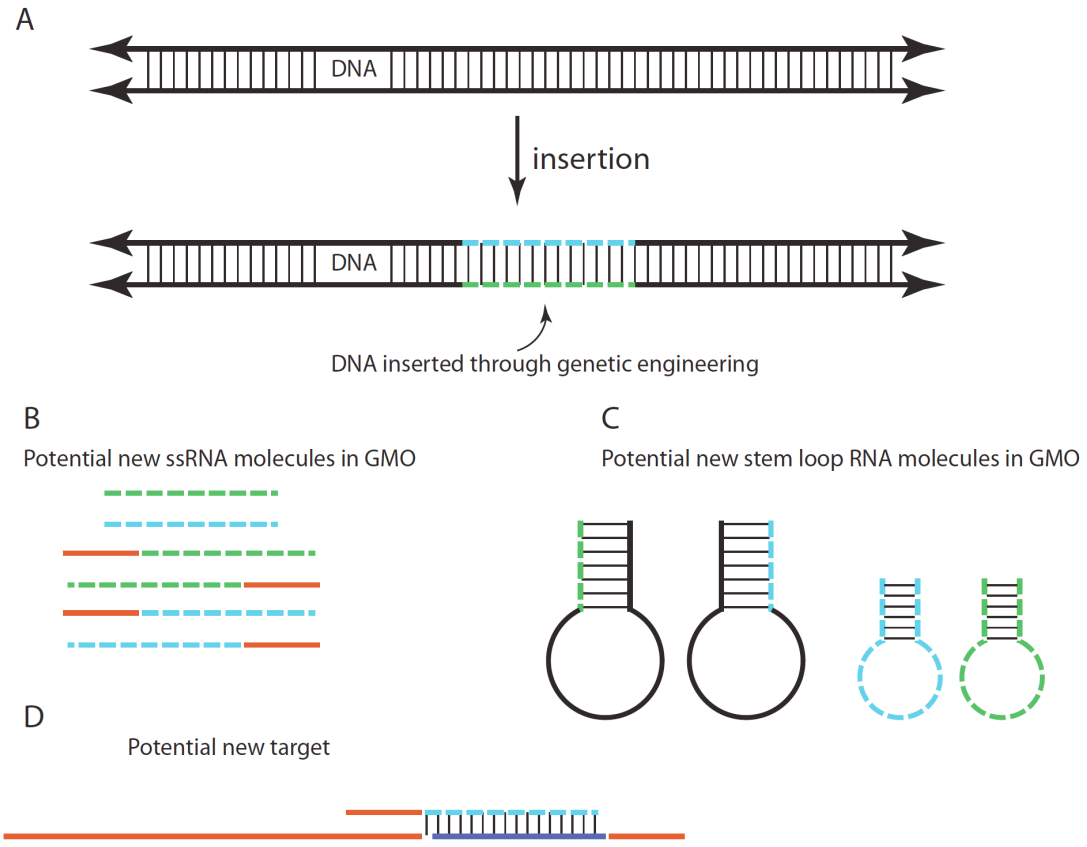

Figure 1: source of new dsRNA molecules from genetic engineering.
(A) Regardless of the source of the DNA inserted (dashed blue and green lines in the black double stranded DNA molecule) into a genome by genetic engineering, it creates new sequences. The DNA used will create new sequences because it 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.

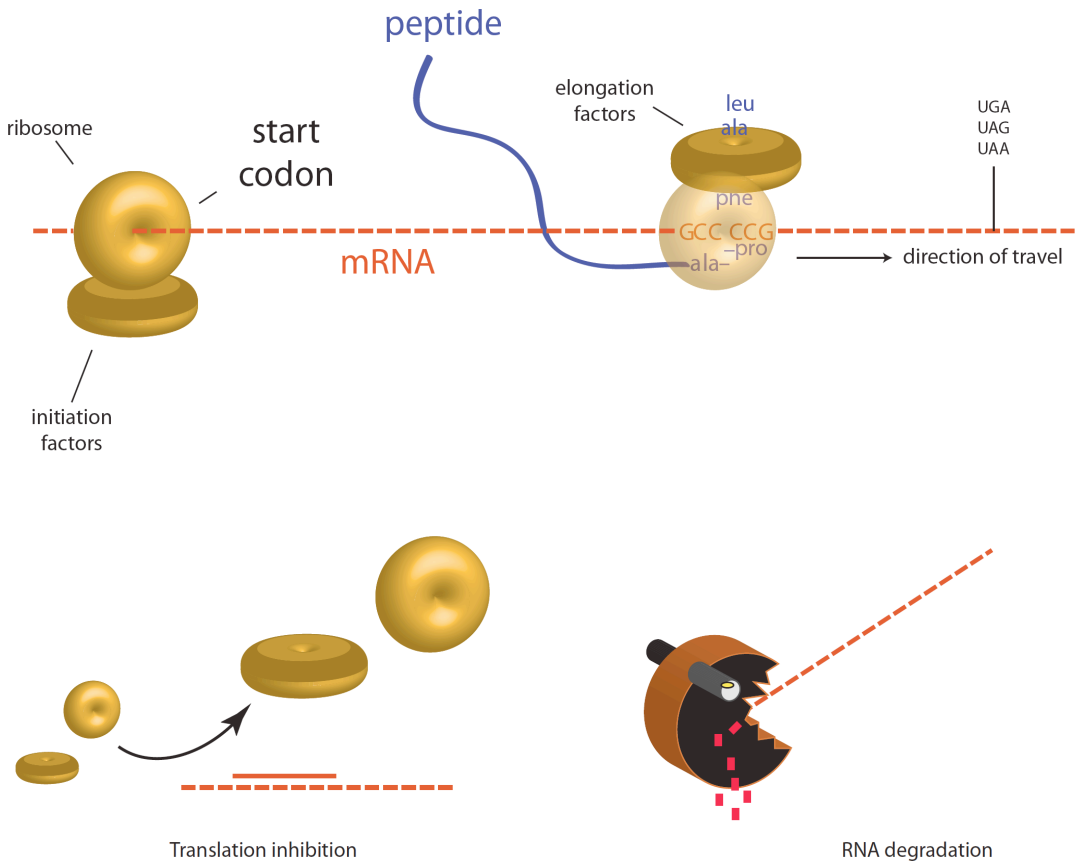

Figure 2: translation and the two pathways of cytoplasmic dsRNA-mediated silencing.
Translation of proteins (top) is the process of linking individual amino acids (the subunits of proteins) into a polymer, called a peptide (blue ribbon). The central enzymatic activity is provided by the ribosome, a multi-protein 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 enzyme that depolymerise the mRNA (bottom right).

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, or methylation of the gene (TGS).

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

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 silence a human 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 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.

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)[2]. 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 about 20% of dietary calories (not counting alcohol) and protein[3]. Australia is the 9th largest producer of wheat and contributes an estimated 3% to world production. Australia is consistently one 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 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.*

(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.

[2] FAOSTAT is a database of the United Nations Food and Agriculture Organisation.
[3] Barley contributed only 7 kcal/capita/day and 0.2 g protein/capita/day in 2007.

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 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 us, because they are claimed as Commercial in Confidence. 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.

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

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-alpha-), branching enzyme 1.

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 insecticidal dsRNA (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). Therefore, any possible siRNA identified through bioinformatic techniques is a candidate for further testing.

Fourth, not all available bioinformatic techniques were used to create this initial appraisal. More plausible 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 bioinformatic 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 again feeds into 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.

Methods:

GBE and SEI sequences were accessed from the NCBI database[4]. 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.

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

4. Short (≥7 contiguous[5]) 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.

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).

SEI was also compared to the entire human genome (Appendix 2). Multiple matches of ≥21 contiguous nucleotides were found. (They were not characterized for being known open reading frames.) 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.

[5] 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).

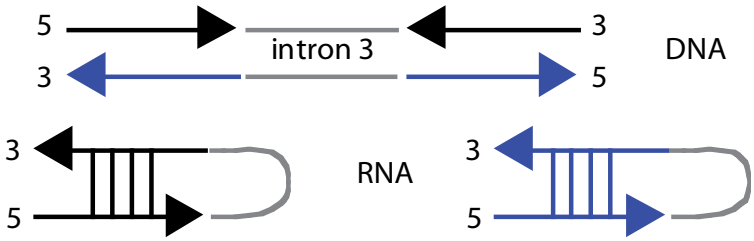

Figure 3. Hypothetical structures of regulatory RNA molecules in GM wheat.
Arrows indicate repeated sequences and their orientation. The numbers 5 and 3 indicate strand polarity. The grey line labeled intron 3 is the third intron of *SEI*.

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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.

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

Nucleotide range		Perfect match length	Overall identity (%)
GBE	*SEI*		
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

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 siRNA effects kill cells (Fedorov et al., 2006), but lesser and still distressing 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′ untranslated region (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.

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?

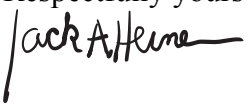
I. Bioinformatic studies to identify any likely unintended targets of the intended siRNAs in humans and species used as indicators of key ecological functions or which are protected. These studies would have looked for perfect matches or similar sequences in the coding region and introns (Seinen et al., 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.
IV. Any potential off-target effect identified through the bioinformatic 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), 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 bioinformatic 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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18 July 2012

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