Response to Heinemann et al on the regulation of GM crops and foods developed using gene silencing

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Key points:

- A recent scientific article (Heinemann et al, 2013) claims that small double-stranded RNAs (dsRNAs) generated in GM plants as a result of using gene silencing techniques can create biosafety risks that are not being adequately assessed by regulators such as Food Standards Australia New Zealand (FSANZ). They suggest changes to the safety assessment process to address their concerns.

- FSANZ has carefully examined the arguments put forward in the article, and has thoroughly researched the scientific literature on gene silencing. The weight of scientific evidence published to date does not support the view that small dsRNAs in foods are likely to have adverse consequences for humans.

- In formulating their hypothesis, the authors have not taken into account the fact that small dsRNAs are ubiquitous in the environment and in the diverse range of organisms we consume as food, including plants and animals. This establishes a long history of safe human consumption which pre-dates the use of such techniques in GM plants.

- The authors failed to adequately acknowledge that developing oral therapies based on small dsRNAs targeted against human viruses and other diseases such as cancer has so far been unsuccessful because of the barriers that exist to their uptake, distribution and targeting within the body.

- The authors have also underestimated the strengths of the GM food safety assessment to detect possible unintended effects, including those that could arise from the use of gene silencing.

- There is no scientific basis for suggesting that small dsRNAs present in some GM foods have different properties or pose a greater risk than those already naturally abundant in conventional foods.

- The current case-by-case approach to GM food safety assessment is sufficiently broad and flexible to address the safety of GM foods developed using gene silencing techniques. This approach enables additional studies to be requested should that be necessary to further inform the safety assessment of a particular GM food.

- FSANZ will continue to monitor the scientific literature for any new developments which may be relevant to GM food safety assessment.

Response to Heinemann et al on the regulation of GM crops and foods developed using gene silencing

Summary

In March 2013, Professor Jack Heinemann and co-authors (Heinemann *et al*., 2013) published an article in the scientific journal *Environment International* in which they claim genetic modification of plants using gene silencing mechanisms can create biosafety risks that are not adequately considered in either environmental or food safety/risk assessment. The major concern of the authors is that genetically modified (GM) foods represent a potential source of exposure to new ribonucleic acid (RNA) molecules in the diet that could affect the expression of human genes with possible adverse consequences for human health. They propose a number of changes to the current safety assessment approach to address their concerns.

The article focuses on some of the properties of double-stranded or duplex RNA (dsRNA), the formation of which is now known to be a trigger for gene silencing (RNA interference, RNAi) in eukaryotic organisms (Carthew and Sontheimer, 2009; Fire, 2007). Several GM crops have been developed using gene silencing approaches to confer the new trait, and many more are under development. In certain circumstances, gene silencing has been observed in the laboratory stages of GM plant development as an unintended consequence of genetic modification.

Food Standards Australia New Zealand (FSANZ) has carefully examined the arguments advanced in this paper as well as consulted the scientific literature more broadly to examine the scope of research into RNAi mechanisms across different species and in different biological contexts. Based on this appraisal, FSANZ considers the current GM food safety assessment process adequately addresses the safety of GM foods developed using gene silencing approaches.

In formulating their hypothesis, the authors have not taken into account the presence of a vast repertoire of RNA molecules in living organisms, the environment and our diet, which establishes a history of safe consumption by humans. It is also clear from recent work on the development of small RNA molecules as possible therapeutic agents that a number of major biological and physiological barriers prevent significant uptake via the gastrointestinal tract and consequent systemic exposure to these molecules, which the authors have failed to adequately acknowledge. Moreover, the authors appear to have underestimated the strengths of the GM food safety assessment to detect possible unintended effects in a GM plant, including those that could arise from the use of gene silencing.

Overall, the weight of evidence in the published literature on gene silencing and the role of dsRNA does not support the view that small RNAs ingested as part of the human diet have an impact on human gene expression. There is also no scientific basis for suggesting that dsRNAs present in GM food have different properties or pose a greater risk than those already naturally abundant in foods from conventional plants, animals and microorganisms such as yeasts.

FSANZ is already aware that the role of RNA in gene regulation and cellular processes is an active area of research and will therefore continue to monitor the scientific literature for any new developments which may be relevant to the safety of GM food. However, in terms of assessing the potential risks associated with the use of gene silencing and the presence of dsRNAs in particular, current safety data requirements already address a number of unique characteristics of the techniques and are sufficiently broad and flexible to accommodate the provision of additional data and information on a case by case basis.

Detailed Reasoning for FSANZ Response

1. **Gene silencing or RNAi is a universal mechanism that is naturally present in eukaryotic organisms.**

Gene silencing or RNAi are terms that refer to a highly conserved set of natural processes in which dsRNA induces the inactivation of related nucleic acid sequences via an RNA induced silencing complex (RISC) . This typically results in the "silencing" of certain target genes or invading nucleic acid, although in some circumstances genes may also be activated (Li *et al*., 2006). Versions of these processes have been found in virtually all eukaryotic organisms, including humans, and they all appear to share similar features (CERA, 2011), although particular fundamental differences between organisms (e.g. plants and animals, multicellular versus unicellular organisms) have been identified (Cerutti and Casas-Mollano, 2006).

The gene silencing machinery variously consists of ~20-30 nucleotide (nt) duplex-derived RNAs and, depending on the organism, RNA binding proteins from two distinct families: Dicer enzymes and effector proteins from the Argonaute superfamily. The small RNA components, short interfering RNAs (siRNAs) and microRNAs (miRNAs) are broadly distributed both phylogenetically and physiologically. The biochemistry, structure and function of the RNA and protein components of silencing complexes have been the subject of intense study over the past decade in organisms as diverse as yeast (*Saccharomyces* sp.), simple worms (*C. elegans*), insects (*Drosophila* sp.), plants (*Arabidopsis*), vertebrates and mammals including humans, and the underlying molecular processes are now being revealed.

Although the gene silencing or gene modulatory effects are similar across living organisms, there are fundamental differences in the biochemistry of RISC components and how they interact. Studies have revealed that siRNAs can be generated endogenously from a range of duplexes including natural sense-antisense pairs, in addition to long, fully complementary double-stranded RNA precursors introduced directly into the cytoplasm or taken up from the environment (Mello and Conte Jr, 2004). On the other hand, miRNAs are encoded by an array of endogenous genes in plants and animals and are generated intracellularly from processing of stem-loop regions of single-stranded mRNA by Dicer enzymes.

There are also differences across organisms in the nature of core proteins involved in the gene silencing pathways. For example, nematodes (worms) and mammals have only a single Dicer enzyme, whereas *Drosophila melanogaster* (fruit fly) produces two distinct Dicers, and *Arabidopsis thaliana* plants express four Dicer enzymes (Carthew and Sontheimer, 2009). In addition, the Argonaute proteins manifest great variation in functional domains according to the nature of the gene silencing pathway and the organism. For example, in plants, flies and worms, proteins in the Argonaute family clearly distinguish between siRNAs and miRNAs. In humans, four of the eight Argonaute proteins associate with both miRNAs and siRNAs. Further study is required to fully characterize the functional domains of Argonaute proteins in various species.

In constructing their argument, Heinemann *et al*. (2013), have brought together disparate areas of research on RNAi mechanisms. Much of the evidence presented on the potential of exogenous dsRNAs to modulate gene expression relates to experiments in insects and simple worms, however a review of more than 150 experiments in Lepidoptera (moths and butterflies) where applications of RNAi have largely failed (Terenius *et al*., 2011) shows that attempts to generate these effects are highly unreliable and depend on precise experimental conditions for eliciting a gene silencing effect.

The core of the argument presented in the Heinemann *et al*. (2013) paper is based on the research findings published by L. Zhang and others (Zhang *et al*., 2012a) in which certain

plant miRNAs derived from common food crops were reportedly found in the bloodstream of humans. Further, one miRNA, which is highly enriched in rice, was reported to inhibit the expression of a protein in human liver, leading the authors to suggest that miRNAs can influence gene expression across phylogenetic kingdoms. This paper lead to speculation (e.g. Jiang *et al*., 2012) that small duplex RNAs (eg siRNAs and miRNAs) present in foods could be taken up by epithelial cells lining the human gastrointestinal tract, be packaged into microvesicles, secreted into the bloodstream and subsequently make their way to target organs where they would enter cells and exert some effect on the expression of endogenous genes. No other evidence for this as a biological phenomenon in humans currently exists however.

While there have been several commentaries on the implications of these findings (Hirschi, 2012; Vaucheret and Chupeau, 2011; Zhang *et al*., 2012b), it is notable that there have been no other publications which corroborate the transmissibility of gene silencing effects from foods to humans.

Heinemann *et al.* (2013) refer, in their paper, to a number of submissions made in the past to FSANZ by the Centre for Integrated Research in Biosafety (INBI)/New Zealand Institute of Gene Ecology (NZIGE) and use these to reiterate arguments. For example, in 2005, before public release of the Zhang *et al* (2012a) paper, in a submission on the assessment of FSANZ Application A549 High Lysine Corn, INBI [then NZIGE] stated:

> *"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."*

FSANZ has not been able to identify any evidence in the scientific literature which supports this statement, and nor were any references substantiating this statement provided by the author of the submission, Prof. J. Heinemann. Instead, the author extrapolated from experiments with invertebrate animals such as fruit flies and simple worms (Cogoni and Macino, 2000; Meister and Tuschl, 2004). It is clear however that such extrapolations are not scientifically appropriate, given the diversity of RNAi mechanisms and machinery that exist between organisms.

It is known that some small RNAs are mobile within certain types of organisms, particularly plants (Dunoyer *et al*., 2010; Molnar *et al*., 2010; Vaucheret and Chupeau, 2011) and that sometimes amplification of these duplexes in the cell allows the silencing effects to spread from the cell where it was activated to the rest of the organism. It has also been reported that RNAi can be induced experimentally in worms and paramecia by feeding with cells expressing long dsRNA targeting one of the host genes. In terms of small RNAs, there is some evidence that they can be transferred from plant to insect pests and nematode worms via expression in leaves and plant roots respectively (Vaucheret and Chupeau, 2011). In these cases, gene silencing was achieved by engineering the plant to express dsRNA that was processed into siRNA homologous to an essential gene (the target) in the insect pest or parasite. However, as explained above, this is not a generally successful strategy and has failed to work in other insect orders.

Although several examples of exogenous gene silencing have been reported in the literature as outlined above, it remains to be determined whether the enormous repertoire of small RNAs present in plant- and animal-derived foods that make up the human diet could play an active physiological role in humans by influencing the expression of endogenous genes. Plants are reported to encode hundreds of thousands of different small RNAs (Rajagopalan *et al*., 2011) which, in theory, would have the potential to match short homologous sequences in mammalian genes. Similar reasoning applies to the abundant miRNAs present in animal tissues consumed as meat. Humans have evolved in conjunction with the

organisms used as food and even the unconfirmed findings reported in the Zhang *et al*. (2012a) paper do not raise safety concerns. Moreover, until replicated and shown to be a more general biological phenomenon, the results obtained by Zhang *et al*. (2012a) could be limited to the experimental conditions used by those researchers.

Regarding the links established between gene silencing mechanisms involving siRNAs and epigenetic effects (so-called transcriptional gene silencing), these have been reported in plants and simple worms (Molnar *et al*., 2010), but have been best characterized to date in yeast (Carthew and Sontheimer, 2009). There are no reports in the literature of epigenetic effects occurring in humans as a result of dietary dsRNAs, therefore the speculation in the INBI submission and the Heinemann *et al*. (2013) paper about long lasting, heritable effects from ingested small RNAs has no scientific basis.

The scientific literature is also populated with publications outlining attempts to develop RNAi therapeutics for a range of biomedical applications. If miRNAs and siRNAs survive passage through the gastrointestinal tract and elicit an effect on gene expression as described in the Zhang *et al*. (2012a) paper, they present possibilities for targeting and silencing genes associated with certain diseases. In the early 2000s, when knowledge of RNAi mechanisms was expanding rapidly, research efforts were enthusiastically applied to viral diseases such as hepatitis and AIDS, and cancer (Wasi 2003). However, the success of emerging therapeutics based on RNAi has been tempered by the need to develop specialized carriers to deliver siRNA drugs to a target organ or cell type. This has resulted in a focus on delivery vehicles such as biodegradable nanoparticles, lipids, bacteria and attenuated human viruses (Burnett *et al*., 2011). In 2012, it was demonstrated for the first time that RNAi-based therapeutics could be effective against a respiratory virus (RSV) in humans via topical administration of siRNA. The literature therefore does not provide compelling evidence that oral delivery of short RNA duplexes can lead to physiologically significant effects, even when dosage can be controlled and the target is a single known gene.

2. dsRNAs are a normal constituent of the human diet.

Nucleic acids, including RNA, are a natural albeit minor component of food. Animal tissues generally have a higher RNA content than plant tissues (Jonas *et al*., 2001). While dietary intake of RNA will be at low levels compared with other constituents, overall amounts will be influenced by the nature of the diet of individuals and vary widely.

Small RNA duplexes, such as siRNAs and miRNAs, are found in both plant and animal tissues (Carthew and Sontheimer, 2009; Ivashuta *et al*., 2009).These small RNAs make up less than 5% of the total RNA in plants (Petrick *et al*., 2013). For soybean, it has been calculated that small RNAs in the range of 21-24 nucleotides are present at levels of up to 1.61 µg/g seed, with comparable amounts present in corn and rice grain (Ivashuta *et al*., 2009). It is apparent therefore that in the case of GM plants using RNAi to silence or down-regulate an endogenous gene, some siRNAs may already be in the human diet.

A subset of small RNAs naturally occurring in rice have been found to have perfect homology to sequences in human and other animal RNA transcripts (Heisel *et al*., 2008; Ivashuta *et al*., 2009). Such homology is considered even more likely in the case of small RNAs present in animal tissues consumed as food (Petrick *et al*., 2013). Therefore, humans are already naturally exposed to RNA, including small RNAs, through food from a diversity of plant and animal sources. A subset of these RNA molecules will almost certainly have homology, including perfect homology, to human genes (Lewis *et al*., 2005).

3. A number of barriers exist to the systemic and cellular uptake of exogenous nucleic acids, including small RNAs, by humans

There are a number of barriers that must be overcome before an ingested small RNA could potentially exert a harmful effect by inducing silencing of an endogenous human gene. These barriers were considered recently by Petrick *et al*. (2013) in their review of the safety assessment of food and feed from crops where RNA-based approaches to gene regulation had been used.

In the case of ingested small RNAs, the first barrier is digestion. It is well known that the highly acidic conditions of the human stomach combined with the action of various digestive enzymes significantly degrades, denatures and depurinates ingested nucleic acids (Loretz *et al*., 2006; O'Neill *et al*., 2011). Digestibility experiments in rodents may overestimate the gastric stability of small RNAs in humans, as the pH of the stomach in rats and mice is typically higher and therefore not as chemically hostile to nucleic acid (O'Neill *et al*., 2011).

The second barrier is absorption across the intestinal epithelium. In humans, absorption of RNA, including siRNA, across the GI tract is said to be negligible (Akhtar, 2009; Jain, 2008) due to rapid degradation and poor transcytosis.

The next barrier is the systemic circulation. Naked, unmodified siRNA duplexes have been shown to undergo rapid degradation by ribonucleases in serum and this degradation is much faster in human serum compared to mouse serum (Haupenthal *et al*., 2006). Furthermore, such molecules have also been shown to undergo rapid clearance from the blood through liver excretion (where they are secreted into the gallbladder then emptied into the intestine)(Huang *et al*., 2011) and renal filtration (Gao *et al*., 2009; Kawakami and Hashida, 2007). Distribution of naked siRNA to other tissues is very limited and in many cases cannot be detected, suggesting that if such molecules make it into the blood compartment, they are excreted almost instantly (Kawakami and Hashida, 2007).

The next barrier is cellular uptake. Nucleic acids, including small RNA molecules such as siRNAs, do not readily diffuse across the cell membrane. This is primarily due to their negative charge and size (Meade and Dowdy, 2008). Should naked RNA manage to penetrate the cell membrane, it invariably remains within endosomal/lysosomal vesicles and is degraded (Gilmore *et al*., 2004).This inability to cross membranes is regarded as one of the major bottlenecks in the development of siRNA-based therapies (Aagaard and Rossi, 2007).

The combination of these barriers means it is highly unlikely that small RNAs will be able to exert any biological effects once ingested (Petrick *et al*., 2013). This is supported by an extensive database of studies on the development and potential use of these molecules as therapeutics, which shows they are only effective at overcoming these barriers and exerting a biological effect if they are modified (through chemical modification and/or the use of carriers) to enhance their *in vivo* stability and cellular uptake (Bramsen and Kjems, 2012; Burnett *et al*., 2011; Kawakami and Hashida, 2007). In the case of small RNAs present in food, these will be unmodified and therefore subject to the full array of physiological and cellular barriers.

Heinemann *et al*. (2013) however dismiss these facts, stating this “does not imply that all dsRNAs are safe because not all dsRNAs are equally efficiently taken up or stable and the effects of some may be enough to cause harm at concentrations lower than needed to cause the intended trait”. The reference used in this context is again the paper by Zhang *et al*. (2012a) described above, where naturally occurring plant miRNAs (from non-GM rice) were reported to be detected in human serum. Some of the findings in this study, and the interpretation of results, have been disputed (Petrick *et al*., 2013; Zhang *et al*., 2012b). There

is even a suggestion that the detection of plant miRNAs in animals is an artifact of the sequencing methodology used and that the accumulation of plant miRNAs via the diet is actually not common in animals (Zhang *et al*., 2012b). Therefore, as noted above, further confirmation and evaluation is required to determine whether uptake of miRNAs from the diet is a real and common phenomenon. In the meantime, the weight of evidence published to date does not support the view that small RNAs ingested as part of the human diet have an impact on human gene expression.

4. There is no scientific basis for presuming that dsRNAs produced by GM plants would pose a greater risk than dsRNAs naturally present in food

The main objective of a GM food safety assessment is to identify new or altered hazards in the food relative to a conventional counterpart, and if present to determine what risk, if any, they may pose to human health (Codex, 2004; FSANZ, 2007). Therefore, the key issue for FSANZ is whether dsRNA molecules present as a result of the genetic modification of crop plants represent a new or altered hazard, compared to dsRNA molecules already naturally present in foods.

Heinemann *et al*. (2013) state that the dsRNA molecules produced in GM plants as a direct (or indirect) consequence of the genetic modification are potentially more harmful than those naturally present, however they fail to provide a scientifically plausible hypothesis for why that should be so. Instead, they speculate on what might happen by extrapolating upon disparate areas of research across a range of species and biological processes. Such speculation, while of possible academic interest, is not supported by the current weight of evidence. Such speculation could equally be applied to the dsRNAs naturally present in foods with a long history of use, or from new conventionally-produced plant varieties, or from entirely new plant and animal sources of food such as new horticultural hybrids, marine algae or insects.

Heinemann *et al*. (2013) also claim that "unintended gene silencing is a common outcome of the genetic engineering process", however this is potentially misleading when considering the genetic stability of GM crops submitted for regulatory assessment. In the 1990s, post transcriptional gene silencing (PTGS) in plants was known to occur in the research environment. Matzke & Matzke (1995) reported that *cis*-inactivation could occur in plants where multiple linked copies of transgenes had been inserted, usually as a consequence of the transformation method. In more recent times however, molecular characterisation of GM crops selected for commercial applications typically shows the insertion of a single intact expression cassette at a single locus in the plant genome. Stability of the newly introduced trait over multiple generations is a prerequisite for regulatory approval and for commercial viability. Unintended gene silencing effects are therefore not commonly associated with GM crops and foods brought forward for regulatory approval.

5. The current safety assessment framework is adequate to address any potential risks posed by the production of dsRNA in GM plants.

Heinemann *et al*. (2013) suggest that small dsRNAs present in GM food as a result of the use of gene silencing techniques can pose a risk to human health. They present a case for changing the safety assessment approach for GM foods, where gene silencing techniques have been used, to routinely include a number of additional studies which in their opinion would address the potential for adverse effects. These additional studies include: bioinformatic analyses to search for sequence identity between the small dsRNAs present in GM food and human genes; profiling techniques to identify new dsRNAs molecules present in the GM food; testing of the dsRNAs in animal and human cells to monitor for changes in gene expression; and long-term toxicity testing in at least two animal species over two

generations. In certain cases, they suggest that human clinical trials might also be considered.

As stated above, the objective of the GM food safety assessment is to identify new or altered hazards relative to a conventional counterpart. If new or altered hazards are identified, an assessment is made to determine what risk, if any, they may pose to human health. That assessment is case-by-case , considers both the intended and unintended effects of the genetic modification, and compares the GM food with conventional foods having an acceptable standard of safety. International consensus has been reached on the types of studies necessary to inform this assessment, and this forms the basis for the data requirements specified by FSANZ. If questions remain following consideration of this core set of information, then FSANZ has the ability to request additional data or studies. Before requesting such studies however FSANZ must be satisfied that additional information will satisfy a legitimate risk assessment question, and be interpretable against the background of natural variation.

On the latter point, the inherent genetic variability in many common foods such as maize, where up to 85% of the genome is non-coding and rich in transposable elements, means that natural variability at the molecular level is vast. In addition to this, humans consume a diversity of plants, including an array of commercial varieties generated by conventional breeding and carrying (beneficial) mutations induced by chemicals or ionising radiation. Against this naturally high background of genetic diversity, the bioinformatics data suggested by Heinemann *et al*. (2013) would largely be uninterpretable. FSANZ also questions how useful bioinformatics analyses could be, given that RNA silencing is promoted by a perfect match between six nucleotides in a small RNA (known as the “seed” region) and its nucleic acid target. Notwithstanding the possibility of false positive associations, it has been estimated that over one third of human genes are identifiable potential miRNA targets (Lewis *et al*., 2005).

In addition to the inability to interpret bioinformatics data, it remains to be confirmed whether humans would be exposed systemically to particular small dsRNAs present in foods. The failure of efforts to develop oral siRNA-based therapeutics appears to indicate that small dsRNAs are not readily absorbed across the GI tract. This does not mean of course that small RNA molecules derived from foods will not occasionally be found in the bloodstream or tissues using highly sensitive methods of detection, as have been reported for other nucleic acids ingested as part of the diet. This is merely a consequence of normal physiological processes for digesting, degrading and excreting exogenous nucleic acid.

Heinemann *et al*. (2013) have also ignored the strengths of the current framework of assessment to determine unintended effects. They do not adequately acknowledge the phenotypic analysis of GM plants which establishes their similarity with non-GM plants in appearance and agronomic performance. Nor do they acknowledge the molecular characterisation which describes the nature of the genetic modification in detail, probes for possible localised effects at the site of integration, and establishes the stability of the genetic change over multiple generations. They do not give adequate consideration to the ability to monitor the expression of the new trait, and the compositional analyses which give a clear indication of any differences, both intended and unintended, between the GM and non-GM lines. FSANZ therefore considers the current assessment framework which is widely implemented around the world, adequately addresses the potential safety issues associated with foods derived from crops developed using gene silencing approaches. This conclusion is supported by others (CERA, 2011; Ivashuta *et al*., 2009; Parrott *et al*., 2010).

For these reasons, FSANZ does not consider that additional studies, along the lines of those proposed by Heinemann *et al*. (2013) are routinely necessary for the assessment of GM food. It is worth emphasising that GM food safety assessments are always undertaken on a

case-by-case basis, which means the approach used and the studies required depends on the type of food being evaluated and the specific nature of the genetic modification. FSANZ's safety data requirements are sufficiently broad and flexible to enable additional studies to be requested, whenever necessary to further inform the safety assessment.

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