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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

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

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

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

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

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

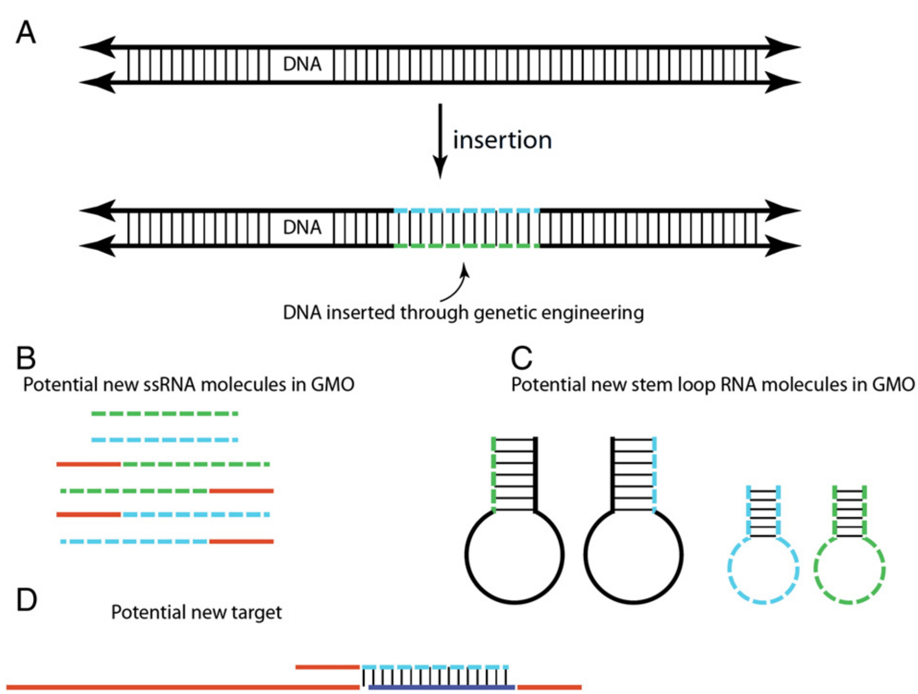

Fig. 1. Source of new dsRNA molecules from genetic engineering. (A) DNA inserted (dashed blue and green lines in the black double stranded DNA molecule) into a genome by genetic engineering creates new sequences regardless of the source of the DNA. The DNA used will create new sequences because it will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