Risk Assessment Reference:
RNAi technology

Introduction

RNAi technology is a group of techniques used to turn off the expression of genes, which is also known as gene silencing. These techniques are based on natural processes that take place in eukaryotic cells to regulate gene expression or to protect the organism against invading microorganisms. These processes are called **RNA interference (RNAi)** or **RNA silencing pathways** because the silencing agents are short RNA molecules (Vaucheret, 2006). The RNA molecules are called small RNAs (sRNA). More information about these pathways can be found in scientific reviews (Ghildiyal and Zamore, 2009; Guo et al., 2016).
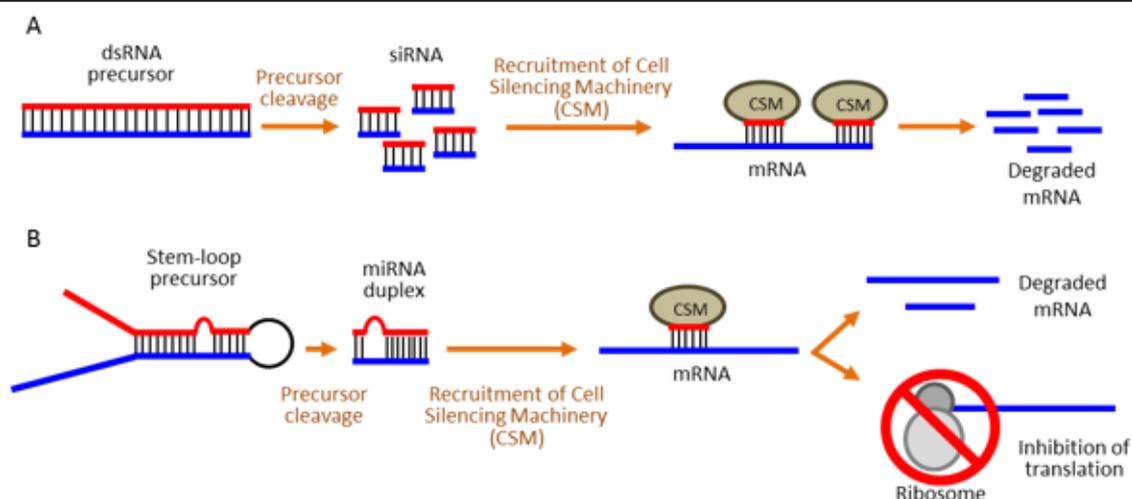
sRNA mode of action

The sequence of the sRNA is a full or partial match to the sequence of the genes that they silence. This allows the binding of the sRNA to the messenger RNA (mRNA) of their target genes. The binding of the sRNA to the mRNA triggers the degradation of the mRNA or the inhibition of its translation (Eamens et al., 2008). These processes, which are carried out by the gene silencing machinery of the cell, decrease the amount of protein produced from the target gene.

Since target gene selection is based on sequence similarity and the gene silencing machinery is present in most eukaryotic organisms, sRNA can be potentially customised to silence any gene in any organism.

Classes of sRNA

Different classes of sRNA are distinguished according to how they are produced and the type of silencing machinery they recruit (Eamens et al., 2008). The sRNA used most commonly to generate genetically modified organisms (GMOs) are short interfering RNA (siRNA) and microRNA (miRNA).

siRNA are made from long double stranded RNA (dsRNA) precursors originated either exogenously (e.g. derived from viruses or transposons) or endogenously (e.g. derived from the centromere and other repetitive sequences in the genome). These precursors are processed into multiple siRNA of approximately 21 nucleotides long that trigger the degradation of the target mRNA (Figure 1A; Carthew and Sontheimer, 2009).

Figure 1. Production and mode of action of siRNA (A) and miRNA (B).

miRNA are encoded by endogenous genes and silence other endogenous genes that encode proteins (Jones-Rhoades et al., 2006). miRNA genes are transcribed into a precursor that folds forming a

stem-loop structure in which the stem is dsRNA (Figure 1B). The precursor is processed to release the miRNA, which induces the degradation of the target mRNA or the inhibition of its translation or both (Jones-Rhoades et al., 2006).

RNAi techniques involving the generation of a GMO

Living organisms can be genetically modified[1] with RNAi constructs that produce novel sRNA. These novel sRNA can silence endogenous genes and change the characteristics of the living organism.

RNAi constructs that produce siRNA generally contain a fragment of the target gene repeated in both sense and antisense orientation separated by an intron (Figure 2A). After transcription, the RNA complementary regions anneal and the intron is eliminated, which results in a dsRNA precursor that is processed by the cell machinery into siRNA (Wesley et al., 2001).

RNAi constructs that produce miRNA use natural miRNA encoding genes in which the miRNA sequence has been changed to match the target gene (Figure 2B; Schwab et al., 2006).
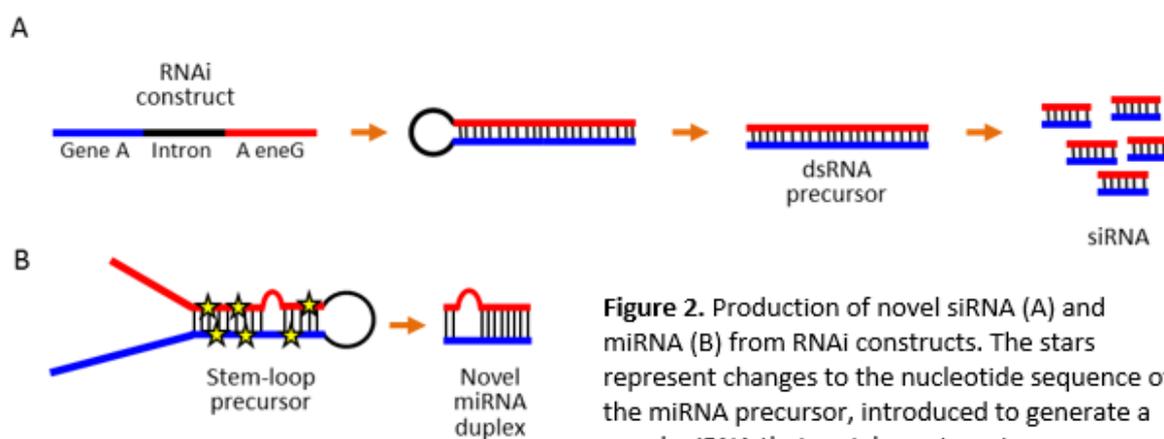
Figure 2. Production of novel siRNA (A) and miRNA (B) from RNAi constructs. The stars represent changes to the nucleotide sequence of the miRNA precursor, introduced to generate a novel miRNA that matches a target gene.

Possible unintended effects of RNAi genetically modified (GM) plants

RNAi GM plants produce novel sRNA that enter the RNAi pathway. These novel sRNA can silence endogenous plant genes to improve the characteristics of the crop. They can also be complementary to pathogen or pest genes, in which case they increase the resistance of the plant to diseases and pests.

There are two possible unintended effects associated with RNAi GM plants:

- Off-target gene silencing – the sRNA binds the mRNA of a gene that is not the intended target gene; and
- Silencing of genes in non-target organisms – the sRNA binds the mRNA of a gene in an organism that is not the intended target organism.

Off-target gene silencing

Efficient gene silencing in plants requires a high level of sequence similarity between the sRNA and its target (Schwab et al., 2006). In addition, RNAi constructs are designed to specifically target the intended gene and to avoid the silencing of similar genes. These both reduce the probability of the introduced sRNA silencing an off-target gene within the plant. In the unlikely event that the introduced sRNA silenced off-target genes, this could potentially lead to changes other than the intended effects, including changes to levels of endogenous toxins or allergens (Figure 3A).

Silencing of genes in non-target organisms

sRNA produced in GM plants could unintentionally change gene expression in animals (Figure 3B). For this to happen, animals must feed on large quantities of the RNAi plant material, the sRNA must resist degradation in the gastrointestinal tract and bloodstream, animal cells must be competent to take up

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[1] See risk assessment reference about plant genetic modification in the OGTR website.

the sRNA in sufficient quantities to activate the endogenous RNAi machinery, sRNA would need to match a gene being expressed in the animal cell and effectively reduce gene expression (Figure 3C; Roberts et al., 2015). This reduced gene expression may or may not be harmful to the animal.

Dietary RNAi in humans and other vertebrates

There are conflicting reports about the possibility of humans and other vertebrates being able to take up miRNA from plants consumed in the diet. Two main methods have been used to study this subject: feeding experiments and sequencing of miRNAs present in blood and other animal tissues.

Feeding studies. In these studies, animals are fed plant material or purified plant miRNA and after feeding, the presence of plant miRNA in blood and/or other tissues is investigated.

Several feeding studies support that humans and mice are able to absorb miRNA from the diet (Zhang et al., 2012a; Baier et al., 2014; Liang et al., 2014; Mlotshwa et al., 2015; Yang et al., 2015; Zhou et al., 2015; Chin et al., 2016). In one of these studies the absorbed miRNA was found to have an effect on gene expression in mice (Zhang et al., 2012a). This work tracked the metabolic fate of the plant miRNA miR-168a, that is produced abundantly in rice and other plants and happens to have a near perfect sequence match to a mammalian gene. miR-168a was detected in mouse livers after feeding mice a rice-only diet and was reported to reduce the expression of the matching mammalian gene in the liver by approximately 50%. The effect of miR-168a on the mouse gene ceased when rice was no longer included in the food. A criticism of these feeding studies is that huge amounts of miRNA or plant feed were administered to mice. For instance, a large quantity of rice was fed to the mice in the Zhang et al (2012a) study, equivalent to a human eating approximately 33 kg/day of cooked rice, an unrealistic quantity in any human diet.

Many other feeding studies have tried to replicate the dietary uptake of miRNA in mice, piglets, monkeys and humans, however, none of these detected in blood the plant miRNAs that were otherwise abundant in the supplied diet (Zhang et al., 2012a; Dickinson et al., 2013; Snow et al., 2013; Witwer et al., 2013; Petrick et al., 2015; Kang et al., 2017; Huang et al., 2018).

Sequencing of miRNA. Next-generation sequencing techniques have been used to sequence and measure the levels of all miRNA present in animal blood or other tissues. Some studies have reported the presence of plant miRNA and other exogenously derived miRNA (xenomiRs) in human plasma or breast milk and have concluded that miRNA can be absorbed from the diet (Wang et al., 2012; Zhang et al., 2012a; Beatty et al., 2014; Lukasik and Zielenkiewicz, 2014; Chin et al., 2016). Only in Zhang et al (2012a) were the levels of plant miRNA comparable with levels of endogenous animal miRNA. In the other studies the detected xenomiRs were at extremely low concentrations, in the order of $1-5$ miRNA copies per cell, in contrast to endogenous miRNA that can reach 50,000 copies per cell (Kang et al., 2017). It has been estimated that miRNA need to be present at a 1,000 to 10,000 copies per cell to silence genes (Denzler et al., 2014).

Other reports have detected the same low levels of xenomiRs in blood and other tissues and concluded that the level detected is biologically insignificant or a technical artefact (Zhang et al., 2012b; Tosar et al., 2014; Bağcı and Allmer, 2016; Kang et al., 2017). Supporting the notion that the detected low levels are technical artefacts is the detection of plant miRNAs and other xenomiRs in tissue samples derived from organisms that did not feed on the source of the xenomiR (Tosar et al., 2014; Kang et al., 2017). For instance, Tosar et al (2014) analysed a sRNA dataset of lancelets[2] obtained by the group of Zhang et al (2012a) (the authors of the first study claiming dietary absorption of plant miRNA in humans) and found that miR168a was also present in this sample even if lancelets did not feed on plants. Kang et al (2017) also observed that some xenomiRs found consistently in 824 datasets of sRNAs from human tissues and body fluids were specific to rodents, however rodents are not a common component of the human diet.

Although the debate is still ongoing (Nawaz et al., 2019), most scientific evidence in the published literature on RNAi does not support the view that dietary sRNA have an impact on human gene expression (FSANZ, 2013-response to Heinemann et al). First, there is a vast repertoire of RNA molecules in living organisms, the environment and our diet, which establishes a history of safe

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[2] A small elongated marine invertebrate that resembles a fish but lacks jaws and obvious sense organs.

consumption by humans (Petrick et al., 2013). Of the total RNA molecules consumed in food, only a small percentage is sRNA, even in the case of RNAi GM plants, which suggests that the amount of RNAi is not high enough to induce potent gene silencing (Petrick et al., 2013; Chan and Snow, 2017). In addition, major biological and physiological barriers prevent significant uptake of sRNA via the gastrointestinal tract and consequent systemic exposure to these molecules (Petrick et al., 2013). These barriers include degradation in the stomach, low absorption in the intestine and enzymatic degradation of absorbed sRNA in the blood stream (Figure 3C). Any surviving sRNA would have to enter into the cytoplasm of the cells to trigger RNAi, which is impeded as sRNAs do not diffuse through the cell membrane (Martineau and Pyrah, 2007).

Dietary RNAi in insects

Some insects can take up sRNA from their diet and if the sRNA match an insect gene, this can result in gene silencing. This susceptibility to dietary RNAi varies widely between insect classes (Scott et al., 2013). Lepidopteran insects (butterflies and moths) have low susceptibility to dietary RNAi, while Orthoptera (grasshoppers), Coleoptera (beetles) and Hemiptera (stink bugs, aphids, cicadas, etc) are more sensitive, although there is variability in these Orders as well (Guan et al., 2018). In bees, ingested sRNA from pollen are not efficiently taken up by the digestive tract or dispersed to other tissues under normal conditions (Masood et al., 2016). The underlying causes of this variability are unknown and may rely on species-specific barriers to exposure, such as a gastrointestinal tract that favours sRNA degradation, barriers to cellular uptake, and the capacity of the organism to amplify and spread the silencing signal from one part of the organism to another (Roberts et al., 2015).

Gene silencing can be achieved by feeding susceptible insects on RNAi GM plants expressing dsRNA that target insect genes (Scott et al., 2013). For this to happen, it is essential that the introduced dsRNA give rise to a high number of siRNA with perfect complementarity to the insect gene (Ivashuta et al., 2015). This notion is supported by the fact that plant-feeding insects that are susceptible to dietary RNAi have been shown to accumulate plant derived siRNA in their bodies without an apparent effect on global gene expression or body mass (Ivashuta et al., 2015).

Dietary RNAi in other organisms

Genes can be silenced by external application of sRNA in nematodes, *Botrytis cinerea* (fungus) and *Arabidopsis thaliana* (plant) (Wang et al., 2016; Mitter et al., 2017). This means that these organisms may be susceptible of becoming an unintended target of RNAi GM plants. How widespread this phenomenon is in fungi and plants is currently unknown.

Fungi and nematodes are able to take up sRNA when infecting the plant cell since RNAi GM plants expressing sRNA against fungi or nematode genes are protected against the diseases caused by these organisms (Huang et al., 2006; Li et al., 2010; Machado et al., 2018). However, fungi, nematodes or plants feeding on decaying RNAi GM plant debris in the soil may be unlikely to be unintended targets of RNAi mainly because RNAi reagents degrade quickly in the environment (Mitter et al., 2017).
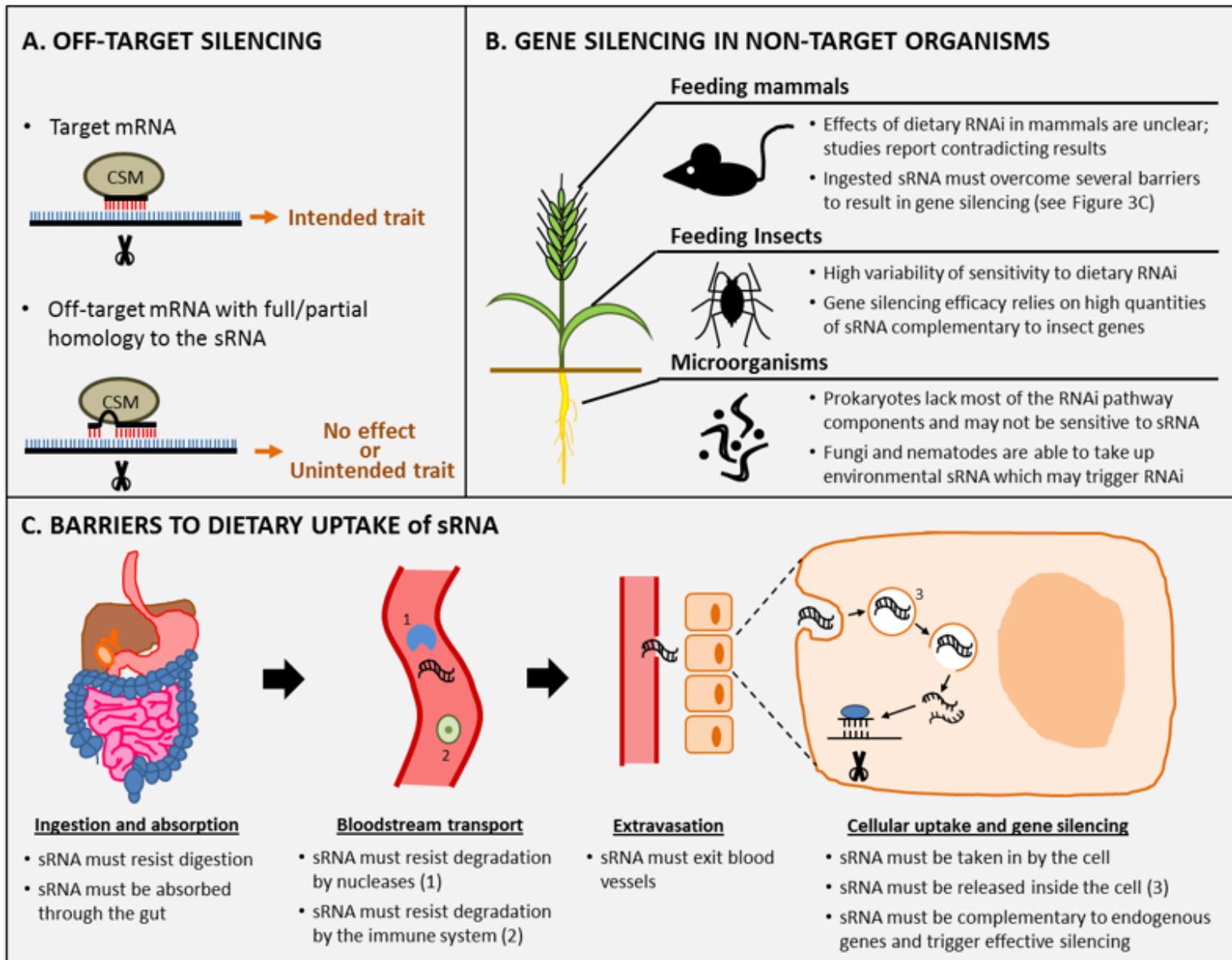
Approvals of RNAi GM plants

The Regulator has approved field trials of RNAi GM plants under licences DIR 93, 112, 117, 121, 131, 136 (OGTR website) and no adverse effects have been notified. In Australia, the only RNAi GM plants approved for commercial release are blue carnations (DIR 134 and OGTR GMO Register) and high oleic safflower (DIR 158). FSANZ has approved food for consumption from the RNAi GM plants high oleic acid soybean (A1018, A1049), insect resistant corn (A1097), reduced acrylamide and reduced browning potato (A1128, A1139), low lignin lucerne (A1085), and is assessing an application of high oleic safflower (A1156). Other RNAi crops approved overseas are common bean, papaya and pineapple (Biosafety Clearing-House).

Conclusion

There have been no reports of adverse effects on human health and safety or the environment as a result of the use of RNAi technology for crop improvement. Nevertheless, the potential for adverse

unintended effects as a result of RNAi technology is assessed on a case-by-case basis, in the context of the proposed dealings, in each risk assessment for release of a GM plant.

Figure 3. Unintended effects of RNAi GM plants. A. Off-target silencing: RNAi reagents expressed in GM plants could potentially silence off-target genes with partial or full complementarity which may result in unintended traits in the plant. (CSM stands for Cell Silencing Machinery) **B. Gene silencing in non-target organisms:** Gene silencing in non-target organisms can only occur in organisms that eat sufficient amounts of RNAi GM plant material; have few barriers to RNA uptake; and have genes with sufficient sequence homology to the RNAi reagents. **C. Barriers to sRNA dietary uptake:** sRNA ingested by mammals must resist digestion; degradation in the bloodstream; and subsequently be taken up by the cell to be able to silence genes. This pathway is unlikely to occur.

REFERENCES

Bağcı, C., and Allmer, J. (2016). One step forward, two steps back; xeno-microRNAs reported in breast milk are artifacts. PLoS One *11*, e0145065.

Baier, S.R., Nguyen, C., Xie, F., Wood, J.R., and Zempleni, J. (2014). MicroRNAs Are Absorbed in Biologically Meaningful Amounts from Nutritionally Relevant Doses of Cow Milk and Affect Gene Expression in Peripheral Blood Mononuclear Cells, HEK-293 Kidney Cell Cultures, and Mouse Livers–3. The Journal of Nutrition *144*, 1495-1500.

Beatty, M., Guduric-Fuchs, J., Brown, E., Bridgett, S., Chakravarthy, U., Hogg, R.E., and Simpson, D.A. (2014). Small RNAs from plants, bacteria and fungi within the order Hypocreales are ubiquitous in human plasma. BMC Genomics *15*, 933.

Carthew, R.W., and Sontheimer, E.J. (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell *136*, 642-655.

Chan, S.Y., and Snow, J.W. (2017). Formidable challenges to the notion of biologically important roles for dietary small RNAs in ingesting mammals. Genes & Nutrition *12*, 13.

Chin, A.R., Fong, M.Y., Somlo, G., Wu, J., Swiderski, P., Wu, X., and Wang, S.E. (2016). Cross-kingdom inhibition of breast cancer growth by plant miR159. Cell Research *26*, 217-228.

Denzler, R., Agarwal, V., Stefano, J., Bartel, D.P., and Stoffel, M. (2014). Assessing the ceRNA hypothesis with quantitative measurements of miRNA and target abundance. Molecular Cell *54*, 766-776.

Dickinson, B., Zhang, Y., Petrick, J.S., Heck, G., Ivashuta, S., and Marshall, W.S. (2013). Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. Nature Biotechnology *31*, 965-967.

Eamens, A., Wang, M.B., Smith, N.A., and Waterhouse, P.M. (2008). RNA Silencing in Plants: Yesterday, Today, and Tomorrow. Plant Physiology *147*, 456-468.

FSANZ (2013). Response to Heinemann et al on the regulation of GM crops and foods developed using gene silencing. Available online at this [link](#).

Ghildiyal, M., and Zamore, P.D. (2009). Small silencing RNAs: an expanding universe. Nature Reviews Genetics *10*, 94-108.

Guan, R.B., Li, H.C., Fan, Y.J., Hu, S.R., Christiaens, O., Smagghe, G., and Miao, X.X. (2018). A nuclease specific to lepidopteran insects suppresses RNAi. Journal of Biological Chemistry *293*, 6011-6021.

Guo, Q., Liu, Q., Smith, N.A., Liang, G., and Wang, M.-B. (2016). RNA silencing in plants: Mechanisms, technologies and applications in horticultural crops. Current Genomics *17*, 476-489.

Huang, G., Allen, R., Davis, E.L., Baum, T.J., and Hussey, R.S. (2006). Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. Proceedings of the National Academy of Sciences *103*, 14302-14306.

Huang, H., Davis, C.D., and Wang, T.T. (2018). Extensive Degradation and Low Bioavailability of Orally Consumed Corn miRNAs in Mice. Nutrients *10*, 215.

Ivashuta, S., Zhang, Y., Wiggins, B.E., Ramaseshadri, P., Segers, G.C., Johnson, S., Meyer, S.E.*, et al.* (2015). Environmental RNAi in herbivorous insects. RNA *21*, 840-850.

Jones-Rhoades, M.W., Bartel, D.P., and Bartel, B. (2006). MicroRNAs and their regulatory roles in plants. Annual Review of Plant Biology *57*, 19-53.

Kang, W., Bang-Bertelsen, C.H., Holm, A., Houben, A., Müller, A.H., Thymann, T., Pociot, F.*, et al.* (2017). Survey of 800+ datasets from human tissue and body fluid reveals XenomiRs are likely artifacts. RNA *23*, 433-445.

Li, J., Todd, T.C., Oakley, T.R., Lee, J., and Trick, H.N. (2010). Host-derived suppression of nematode reproductive and fitness genes decreases fecundity of *Heterodera glycines* Ichinohe. Planta *232*, 775-785.

Liang, G.F., Zhu, Y.L., Sun, B., Shao, Y.H., Jing, A.H., Wang, J.H., and Xiao, Z.D. (2014). Assessing the survival of exogenous plant microRNA in mice. Food Science & Nutrition *2*, 380-388.

Lukasik, A., and Zielenkiewicz, P. (2014). In silico identification of plant miRNAs in mammalian breast milk exosomes–a small step forward? PLoS One *9*, e99963.

Machado, A.K., Brown, N.A., Urban, M., Kanyuka, K., and Hammond-Kosack, K.E. (2018). RNAi as an emerging approach to control Fusarium head blight disease and mycotoxin contamination in cereals. Pest Management Science *74*, 790-799.

Martineau, H.M., and Pyrah, I.T. (2007). Review of the Application of RNA Interference Technology in the Pharmaceutical Industry. Toxicologic Pathology *35*, 327-336.

Masood, M., Everett, C.P., Chan, S.Y., and Snow, J.W. (2016). Negligible uptake and transfer of diet-derived pollen microRNAs in adult honey bees. RNA Biology *13*, 109-118.

Mitter, N., Worrall, E.A., Robinson, K.E., Li, P., Jain, R.G., Taochy, C., Fletcher, S.J.*, et al.* (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. Nature Plants *3*, 16207.

Mlotshwa, S., Pruss, G.J., MacArthur, J.L., Endres, M.W., Davis, C., Hofseth, L.J., Peña, M.M.*, et al.* (2015). A novel chemopreventive strategy based on therapeutic microRNAs produced in plants. Cell Research *25*, 521-524.

Nawaz, M.A., Mesnage, R., Tsatsakis, A.M., Golokhvast, K.S., Yang, S.H., Antoniou, M.N., and Chung, G. (2019). Addressing concerns over the fate of DNA derived from genetically modified food in the human body: A review. Food Chemical Toxicology *124*, 423-430.

Petrick, J.S., Brower-Toland, B., Jackson, A.L., and Kier, L.D. (2013). Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: A scientific review. Regulatory Toxicology and Pharmacology *66*, 167-176.

Petrick, J.S., Moore, W.M., Heydens, W.F., Koch, M.S., Sherman, J.H., and Lemke, S.L. (2015). A 28-day oral toxicity evaluation of small interfering RNAs and a long double-stranded RNA targeting vacuolar ATPase in mice. Regulatory Toxicology and Pharmacology *71*, 8-23.

Roberts, A.F., Devos, Y., Lemgo, G.N.Y., and Zhou, X. (2015). Biosafety research for non-target organism risk assessment of RNAi-based GE plants. Frontiers in Plant Science *6*, 958.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D. (2006). Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. Plant Cell *18*, 1121-1133.

Scott, J.G., Michel, K., Bartholomay, L.C., Siegfred, B.D., Hunter, W.B., Smagghe, G., Zhu, K.Y.*, et al.* (2013). Towards the elements of successful insect RNAi. Journal of Insect Physiology *59*, 1212-1221.

Snow, J.W., Hale, A.E., Isaacs, S.K., Baggish, A.L., and Chan, S.Y. (2013). Ineffective delivery of diet-derived microRNAs to recipient animal organisms. RNA Biology *10*, 1107-1116.

Tosar, J.P., Rovira, C., Naya, H., and Cayota, A. (2014). Mining of public sequencing databases supports a non-dietary origin for putative foreign miRNAs: underestimated effects of contamination in NGS. RNA *20*, 754-757.

Vaucheret, H. (2006). Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes and Development *20*, 759-771.

Wang, K., Li, H., Yuan, Y., Etheridge, A., Zhou, Y., Huang, D., Wilmes, P.*, et al.* (2012). The complex exogenous RNA spectra in human plasma: an interface with human gut biota? PloS One *7*, e51009.

Wang, M., Weiberg, A., Lin, F.-M., Thomma, B.P., Huang, H.-D., and Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. Nature Plants *2*, 16151.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M., Rouse, D.T., Liu, Q., Gooding, P.S.*, et al.* (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. The Plant Journal *27*, 581-590.

Witwer, K.W., McAlexander, M.A., Queen, S.E., and Adams, R.J. (2013). Real-time quantitative PCR and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of dietary miRNAs: limited evidence for general uptake of dietary plant xenomiRs. RNA Biology *10*, 1080-1086.

Yang, J., Farmer, L.M., Agyekum, A.A., Elbaz-Younes, I., and Hirschi, K.D. (2015). Detection of an abundant plant-based small RNA in healthy consumers. PloS One *10*, e0137516.

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

Zhang, Y., Wiggins, E., Lawrence, C., Petrick, J., Ivashuta, S., and Heck, G. (2012b). Analysis of plant-derived miRNAs in animal small RNA datasets. BMC Genomics *13*, 381.

Zhou, Z., Li, X., Liu, J., Dong, L., Chen, Q., Liu, J., Kong, H.*, et al.* (2015). Honeysuckle-encoded atypical microRNA2911 directly targets influenza A viruses. Cell Research *25*, 39-49.