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Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: A scientific review

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ABSTRACT

Gene expression can be modulated in plants to produce desired traits through agricultural biotechnology. Currently, biotechnology-derived crops are compared to their conventional counterparts, with safety assessments conducted on the genetic modification and the intended and unintended differences. This review proposes that this comparative safety assessment paradigm is appropriate for plants modified to express mediators of RNA-mediated gene regulation, including RNA interference (RNAi), a gene suppression mechanism that naturally occurs in plants and animals. The molecular mediators of RNAi, including long double-stranded RNAs (dsRNA), small interfering RNAs (siRNA), and microRNAs (miRNA), occur naturally in foods; therefore, there is an extensive history of safe consumption. Systemic exposure following consumption of plants containing dsRNAs that mediate RNAi is limited in higher organisms by extensive degradation of ingested nucleic acids and by biological barriers to uptake and efficacy of exogenous nucleic acids. A number of mammalian RNAi studies support the concept that a large margin of safety will exist for any small fraction of RNAs that might be absorbed following consumption of foods from biotechnology-derived plants that employ RNA-mediated gene regulation. Food and feed derived from these crops utilizing RNA-based mechanisms is therefore expected to be as safe as food and feed derived through conventional plant breeding.

1. Introduction

RNA interference (RNAi) is an RNA-based mechanism that modulates endogenous gene expression in eukaryotes including plants, insects, fungi, nematodes, and mammals. Because RNAi is a widely occurring biological process, the RNA molecules that mediate this mechanism are a ubiquitous component of the diet for animals including humans. RNAi is mediated by small RNA molecules that bind to and suppress transcription and/or translation of specific messenger RNAs (mRNAs); specificity is driven by base pairing between target mRNAs and these small RNAs. Because of the specificity of RNAi, there is great interest in application of this mechanism for crop improvement and for development of human therapeutics. Based on evidence supporting continual exposure to dietary RNA (including siRNAs, miRNAs, and longer dsRNAs) and biological barriers that limit uptake and biological activity of ingested RNA, there is no reason to expect that consumption of foods or feeds from biotech crops employing traits produced through an RNAi-based mechanism or other RNA-mediated mechanism are any less safe than their conventional counterparts. However, to realize the potential for applications of these mechanisms in agricultural biotechnology, it is necessary to establish scientifically sound principles for evaluating their safety in crop plants. Herein we consider the weight-of-the-evidence supporting the safe use of RNAi in crop plants in the context of the current paradigm for evaluating the safety of biotechnology-derived crops (referred to throughout as biotech crops). This evidence is also considered in the context of one study that suggested oral activity of a plant miRNA after dietary consumption (Zhang et al., 2012a). Based on the weight-of-the-evidence for RNA dietary safety and the robust nature of the current internationally accepted principles for the safety

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeat; dsRNA, double-stranded RNA; FDA, food and drug administration; LDL, low density lipoprotein; miRNA, microRNA; mRNA, messenger RNA; OECD, organisation for economic cooperation and development; RdRP, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex; RNAi, RNA interference; rRNA, ribosomal RNA; shRNA, short hairpin RNA; siRNA, small interfering RNA; tRNA, transfer RNA; WHO, World Health Organization.

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evaluation of biotech crops, this review proposes that these principles are applicable to crops modified using RNA-based mechanisms such as RNAi.

2. RNAi: Background and plant applications

2.1. General features of RNAi

Gene suppression was first observed in plants as a cellular mechanism for the recognition and degradation of foreign RNA including viral RNA (Dougherty et al., 1994; Napoli et al., 1990). Fire, Mello, and colleagues defined the RNA-mediated mechanism of gene suppression (i.e., RNAi) in nematodes (Fire et al., 1998), and RNAi-mediated gene suppression has since been observed in fungi, worms, insects, and mammals (Brodersen and Voinnet, 2006; Dykxhoorn et al., 2003; Ghildiyal et al., 2008; Jones-Rhoades et al., 2006; Li and Liu, 2011; Mallory and Vaucheret, 2006; Sandy et al., 2005; Vazquez, 2006). Prokaryotes also utilize RNA-mediated gene silencing through the CRISPR system that is analogous to, but mechanistically distinct from, RNAi (Wiedenheft et al., 2012). Based on these observations, it is apparent that modulation of gene expression through RNA-mediated mechanisms is nearly ubiquitous.

The triggers for RNAi-mediated gene suppression are small double-stranded RNAs (dsRNAs) of 21–27 nucleotides; these small RNAs include small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Hammond, 2005; Zamore et al., 2000). siRNAs and miRNAs are derived from processing of longer dsRNA sequences that do not encode proteins. In plants, biogenesis of siRNAs and miRNAs from precursor dsRNAs involves multiple Dicer-like proteins (Liu et al., 2009), endonucleases that cleave longer dsRNAs. Mature siRNA duplexes contain an interfering antisense or guide strand complementary to a target mRNA sequence and a passenger strand (Caplen et al., 2001; Elbashir et al., 2001; Zamore et al., 2000). RNAi-mediated gene suppression involves incorporation of the guide strand into an RNA-induced silencing complex (RISC) with concomitant degradation of the passenger strand (Tomari and Zamore, 2005). RNAi-mediated gene suppression occurs through either mRNA degradation or translational inhibition (Bartel, 2009; Carthew and Sontheimer, 2009; Fabian et al., 2010; Guo et al., 2010; Huntzinger and Izaurralde, 2011).

2.2. Differences in RNAi mechanisms in different kingdoms

Although the general mechanism of RNAi is conserved across eukaryotes, there are some important phylogenetic differences. A general distinction is that plant miRNAs are usually perfectly or nearly perfectly complementary to their target genes and induce direct mRNA cleavage by RISC, whereas miRNAs in animals trigger either translational repression (Fabian et al., 2010) or target mRNA cleavage (Guo et al., 2010; Huntzinger and Izaurralde, 2011). There is some evidence, however, that miRNAs can also act in the translationally inhibitory mode in plants (Brodersen et al., 2008). Another aspect of RNAi observed in nematodes and plants is intercellular spreading of gene suppression. Classic examples of this phenomenon are systemic transport of viral resistance from a local site of infection to distant sites in plants and systemic spreading observed in *Caenorhabditis elegans* (Jose and Hunter, 2007). The phenomenon of intercellular spreading of RNAi appears to be restricted to plants, fungi, and a subset of invertebrate species (Jose and Hunter, 2007; Voinnet, 2005). Intercellular spreading may be attributed to the activity of RNA-dependent RNA polymerase (RdRP) that is present in plants, worms, and perhaps other invertebrates but does not appear to be present in *Drosophila* or vertebrates (Tomari and Zamore, 2005).

Nematodes also exhibit intercellular and systemic transport of RNA molecules, processes not observed to any significant extent in mammals. RNAi-mediated gene suppression is induced in *C. elegans* by soaking the worms in siRNA-containing solutions (Maeda et al., 2001; Tabara et al., 1998), by feeding bacteria expressing an siRNA to *C. elegans* (Newmark et al., 2003; Timmons et al., 2001), or by injecting RNA isolates from siRNA producing plants into worms (Boutla et al., 2002). There have also been demonstrations of RNAi-mediated gene suppression in larvae of certain species of insects and nematodes upon feeding of plant material engineered to produce dsRNAs targeting genes in these organisms (Baum et al., 2007; Fairbairn et al., 2007; Huang et al., 2006; Mao et al., 2007; Yadav et al., 2006). Although there has been speculation that amplification of the RNAi signal and systemic transport and spreading might be present in mammals under certain environmental conditions (Jose and Hunter, 2007), there is no *in vivo* evidence for these functions in mammals. Taken together with the mammalian barriers to uptake of dietary RNA (Depicted in Fig 1), this information strongly suggests that no adverse effects should be anticipated in mammals following consumption of dietary RNA. This assertion is also discussed below (Section 3.4.5.) in the context of a publication that indicates possible mammalian responses to ingested small RNAs.

2.3. Applications of RNAi in plants

Examples of naturally occurring RNA-mediated gene suppression traits that were selected through conventional breeding include soybean seed coat color (Tuteja et al., 2004) and maize stalk color (Della Vedova et al., 2005), both of which are mediated through suppression of chalcone synthase. Additionally, a conventional low glutelin rice variety resulted from a naturally occurring insertion of a region into the genome that expresses a long dsRNA that suppresses *glutelin* via an RNA-mediated mechanism (Kusaba et al., 2003). RNA-mediated gene suppression has also been leveraged in the production of biotechnology-derived food crops such as

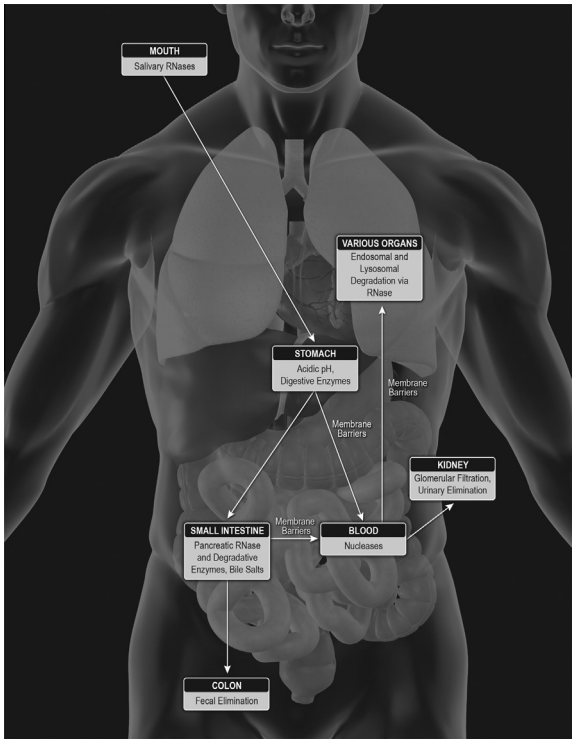

Fig. 1. Biological barriers to uptake and activity of ingested RNA.