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Commentary

RNAi-based GM plants: food for thought for risk assessors

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Summary

RNA interference (RNAi) is an emerging technology that offers new opportunities for the generation of new traits in genetically modified (GM) plants. Potential risks associated with RNAi-based GM plants and issues specific to their risk assessment were discussed during an international scientific workshop (June 2014) organized by the European Food Safety Authority (EFSA). Selected key outcomes of the workshop are reported here.

Introduction

In 2013, 175 million hectares of genetically modified (GM) plants were cultivated, mostly in the United States, Brazil, Argentina, India and Canada. The majority of these plants are engineered to express one or more novel proteins, providing specific agronomic traits such as insect resistance or herbicide tolerance. GM plants intended for market release can also be designed to induce silencing of target genes *in planta* or in insect pests through RNA interference (RNAi), which is an emerging technology that offers new opportunities for the generation of new traits in GM plants (referred to hereafter as RNAi-based GM plants) (as recently reviewed by Koch and Kogel, 2014). RNAi involves small RNAs (small interfering RNAs [siRNA] or microRNAs [miRNA]) that bind to messenger RNA (mRNA) with sequence homology and leads to the silencing of target gene expression by cleavage of the mRNA via an enzyme complex. RNAi-based GM plants express either a double-stranded RNA (dsRNA), which is subsequently cleaved into a pool of active siRNA molecules that can interact with different parts of the target mRNA, or an artificial miRNA precursor, which is cleaved to a mature and active miRNA.

As part of the premarket risk assessment in the European Union (EU), the European Food Safety Authority (EFSA) evaluates any risks that GM plants and derived food and feed products may pose to human and animal health and the environment. To discuss potential risks associated with RNAi-based GM plants and to identify issues specific to their risk assessment, EFSA organized an international scientific workshop (4–5 June 2014, Brussels, Belgium, for more details see http://www.efsa.europa.eu/en/events/event/140604.htm), bringing together experts from academia, risk assessment bodies, non-governmental organizations, the European Commission and the private sector.

During the workshop, plenary sessions, focusing on the molecular biology of RNAi, RNAi-based GM plant applications and general issues for their risk assessment, were followed by three breakout sessions, each of which considered one of the three main areas of GM plant risk assessment: molecular characterization; food/feed risk assessment; and environmental risk assessment. Discussions in the breakout sessions will inform the problem formulation, which is the first step in the risk assessment process, and which enables a structured, logical approach to identifying harmful effects requiring characterization, while excluding non-harmful effects as irrelevant. Selected key outcomes of the discussions between participants in the breakout groups are summarized below.

Considerations for the molecular characterization of RNAi-based GM plants

As RNAi activity is based on sequence homology between small RNAs and mRNAs, not only target genes but also off-target genes with sufficient sequence homology can be silenced, potentially leading to adverse effects on animal and human health or the environment. Off-target gene silencing could occur in the GM plant itself, or in other organisms that are exposed to the GM plant (e.g. Lundgren and Duan, 2013; Ramesh, 2013; US EPA, 2014).

Several small RNA-target match programs enable the identification of possible off-target genes. Experimental data in plants show that gene silencing can occur even in the presence of some mismatches between target genes and small RNAs, and therefore such programs typically allow for some sequence mismatches when generating a list of potential target genes. Depending on the stringency of the prediction criteria applied, the prediction will vary in sensitivity and specificity, with uncertain biological significance. As most prediction criteria are based on data derived from miRNA-target matches, the applicability of these data for the prediction of siRNA (derived from dsRNA)-target matches is still under debate.

A specific challenge for dsRNA-expressing GM plants is the uncertainty about the pool of generated siRNAs that could trigger off-target gene effects. The composition of the siRNA pool may not necessarily encompass all possible siRNAs that could be

theoretically derived from the respective dsRNA. Although the composition can be elucidated by next generation sequencing, this method might lead to inaccurate results due to a possible ligation bias in the construction of the siRNA library.

It was also noted that the identification of a potential off-target gene may not necessarily imply its responsiveness to the small RNA. The off-target mRNA could be protected (e.g. encapsidated mRNA of transposons) or the position of the sequence homology may not be adequate to achieve effective silencing. However, a match may point to the potential for unintended interactions requiring further characterization.

The detection of off-target genes is also hampered by the lack of knowledge on genomes and their expression, especially in non-model plant lines and other species. These limitations at the molecular level will have implications for the prediction of potential off-target gene effects, and the usability of bioinformatic data in support of the food/feed and environmental risk assessment.

Considerations for the food/feed risk assessment of RNAi-based GM plants

For the food/feed risk assessment of GM plants expressing novel proteins, a comparative approach is typically followed to identify potential (intended and unintended) changes in the GM plant. This approach also assesses: (i) the agronomic, phenotypic and compositional characteristics of the GM plant; (ii) the toxicity and allergenicity of the newly expressed protein(s) and their metabolites; and, if appropriate; (iii) the nutritional characteristics of the GM plant. Any identified biologically relevant differences are assessed further to determine whether safety issues or concerns exist.

Overall, the above described strategy was considered appropriate for identifying and evaluating potential adverse effects relevant to animal and human health arising from RNAi-based GM plants (see also FSANZ, 2013; US EPA, 2014), although the risk assessment would focus on the newly expressed RNAi molecules instead of the novel proteins. Consequently, potential adverse effects of RNAi molecules were discussed. Relevant considerations in this context were the history of safe consumption of RNAi molecules naturally occurring in plants, as well as their limited stability in the digestive tract and low uptake into the blood stream after ingestion. It was further noted that the bulk of information available from pharmaceutical studies does not indicate adverse effects due to the toxicological and pharmacokinetic profile of RNAi molecules (limited bioavailability, quick metabolism and rapid excretion). Therefore, testing of purified RNAi molecules in oral toxicity studies was not considered relevant and the usefulness of studies with whole food/feed in rodents was viewed controversially. A single study suggesting that endogenous miRNAs present in plant material could lead to a miRNA-induced off-target effect in mammals upon oral ingestion (Zhang *et al.*, 2012) was heavily debated; the results of this study were not confirmed by several other studies addressing this question (Dickinson *et al.*, 2013; Witwer *et al.*, 2013). In addition, adverse effects such as the saturation of the RNAi machinery and immunostimulatory effects were not considered relevant in humans under realistic exposure conditions.

Considerations for the environmental risk assessment of dsRNA-expressing GM plants

The breakout discussions focused on GM plants expressing dsRNA to control insect pests, as those are currently under development. A typical hypothesis addressed during the environmental risk assessment of such GM plants is that they do not adversely impact arthropod species other than the target species at field exposure levels (e.g. US EPA, 2014). Discussions therefore centred on the assessment of potential adverse effects on non-target arthropods (NTAs).

For most dsRNA-expressing GM plants, arthropod exposure is expected to occur primarily through oral ingestion of living or dead plant material. Various barriers, such as the rapid degradation of dsRNA in soil (Dubelman *et al.*, 2014), the lack of digestive stability of dsRNA and diverse cellular uptake mechanisms, can limit exposure. However, the current understanding on some of these barriers was considered insufficient to make generalizations across non-target taxa and to refine exposure estimates.

Potential adverse effects on NTAs are typically evaluated within different tiers that progress from laboratory studies representing worst-case exposure conditions to more realistic but less controlled field studies. Overall, participants considered the tiered approach appropriate for the non-target testing of dsRNA-expressing GM plants.

Laboratory studies aimed to analyse toxic effects on arthropods frequently contain dsRNA in the test diet. No consensus was found on whether siRNAs should be included as well, as it is not yet clear whether arthropods would be able to take them up at the cellular level. Ideally, test substance doses applied in these studies should be higher than the maximum amount of dsRNA to which NTAs are exposed to in the environment. Laboratory studies conducted at the so-called maximum hazard dose are feasible (Bachman *et al.*, 2013), and add certainty to the risk assessment. Owing to the latency of silencing in affected organisms, measurement endpoints should also target sublethal effects on growth, development and reproduction of the test organisms, so that they are likely to indicate possible off-target gene effects in NTAs.

No consensus was reached on the use of plant material as test substance. Some participants recommended tests with plant material, as they reflect more realistic exposure conditions, and might help to capture unknown complexities and variability in the GM plant that cannot easily be predicted (see also US EPA, 2014). Other participants argued that such tests will not add weight of evidence to the risk assessment, owing to confounding factors that may obscure the interpretation of the results. To avoid that other, unidentified plant characteristics are responsible for the observed effects, it was noted that plant material from a suitable line with a genetic background similar to that of the GM line should be used as comparator. Ideally, this comparator should be grown under same environmental/agricultural conditions as the GM plant.

Concluding remarks

EFSA will take the outcomes of the workshop into account to determine in which areas of the existing EU risk assessment frame for GM plants refinements might be needed for RNAi-based GM plants. However, an apparent conclusion was that bioinformatic analyses could play an important role in the risk assessment of RNAi-based GM plants. The unequivocal detection of off-target genes *in planta* could drive the food/feed risk assessment through the identification of potential changes that require further characterization. For the environmental risk assessment, bioinformatic analyses could guide the selection of

non-target species which harbour genes that share a certain level of homology with the gene targeted in the pest and which should be the focus of further assessment. However, bioinformatic data cannot be reliably used as a standalone to predict the presence of RNAi activity at present. More research is needed on the exact rules for small RNA-target matches, to design more efficient algorithms and make more reliable predictions. There is also a necessity to expand knowledge on genomes and their expression, especially in non-model lines and other species.

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