

Workshop: Biosafety issues associated with RNAi

Meeting of WG 3: planning future activities

WG3 Deliverables:

- **Compilation of reviews** on the available knowledge on aspects of RNAi systems that are essential for an effective risk assessment (identification of known or new knowledge gaps in the area of food/feed and potential environmental hazards specific to RNAi)
- Development of a **data base on targets and off-targets** of characterised dsRNA and miRNA sequences (jointly with WG1 and WG2)
- Development of **specific biosafety protocols and post-market monitoring requirements and methods for RNAi plants** (current or new data requirements for risk assessment with regard to specific hazards)
- Production of risk assessment examples based on **case studies of current RNAi applications and developments**

Reviews on knowledge of RNAi systems essential for an effective risk assessment

EXTERNAL SCIENTIFIC REPORT



APPROVED: 16 may 2017

doi:10.2903/sp.efsa.2017.EN-1246

Literature review of baseline information to support the risk assessment of RNAi-based GM plants

Jan Paces¹, Miloslav Nic², Tomas Novotny², Petr Svoboda¹

¹ Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic (IMG)

² EcoMole Ltd.

Abstract

This report is the outcome of an EFSA procurement aiming at investigating and summarising the state of knowledge on (I) the mode-of-action of dsRNA and miRNA pathways, (II) the potential for non-target gene regulation by dsRNA-derived siRNAs or miRNAs, (III) the determination of siRNA pools in plant tissues and the importance of individual siRNAs for silencing. The report is based on a

Main tasks addressed as defined by EFSA:

1. Mode-of-action of dsRNA and miRNA pathways
2. Potential for non-target (off-target) gene regulation by dsRNA-derived siRNAs or miRNAs
3. Determination of siRNA pools in plant tissues and importance of individual siRNAs for silencing

WG3 Deliverable:

Compilation of reviews on the available knowledge on aspects of RNAi systems that are essential for an effective risk assessment

- Areas of risk assessment where the Literature review provides knowledge?
- Knowledge gaps important for food/feed and environmental risk assessment identified by the Literature review?
- Additional aspects relevant for biosafety to be addressed?

Specific risk assessment issues of RNAi-based GM plants

Molecular characterization of the RNAi GM plant as a basis for risk assessment

- Minimal target site requirements for siRNA and miRNA?
- Prediction of off-target effects in the plant → bioinformatics, transcriptomics
- Composition and silencing capacity of the siRNA pool derived from dsRNA breakdown
- Expression level of dsRNA; (co-occurrence of methylation of homologous DNA?)



Identification of potential hazards

Food/feed risk assessment

- Adverse non-target effects in mammals
- Safety relevant compositional changes in GM plant

Environmental risk assessment (ERA)

- Adverse off-target effects in non-target organisms
- Resistance development in target organism (HIGS)

Other types of adverse unintended effects, i.e. non-specific dsRNA responses, saturation of RNAi machinery

Exposure

- Expression level of dsRNA in GM plants
- Characteristics of GM plant (dsRNA vs. siRNA), e.g. insects do not seem to take up siRNAs
- dsRNA stability in the environment (e.g. soil)
- Bioavailability after oral exposure (e.g. enzymatic and non-enzymatic degradation after ingestion)
- Other exposure routes (respiratory, ocular and dermal)?

Knowledge on risk assessment issues provided by EFSA External Scientific Report

Minimal target site requirements for siRNA and miRNA

- Focus on mammalian systems which are distinct from plants and arthropods with respect to base pairing requirements and tolerated mismatches (concept of nt 2-7 as „seed sequence“)

→ **Generally: Sequence homology based target prediction is insufficient**

Prediction of off-target effects in the plant, in mammals and in non-target organisms

- Besides base pairing most critical parameters are

1. **siRNA/miRNA amount entering the RNAi pathway**, i.e. bound to AGO proteins – influenced by concentration, length, terminal nucleotides, structural features
2. **Target site accessibility** (RNA/RNA and RNA/protein binding affected by local secondary structures)

Composition and silencing capacity of the siRNA pool derived from dsRNA breakdown

- In contrast to well-defined miRNAs large numbers of different siRNAs are derived from a longer dsRNA

→ **Due to high sequence variability of siRNAs silencing capacity of long dsRNAs difficult to predict**

→ **Each individual siRNA is present at low concentration, thereby limiting off-target effects**

Food/feed

- miRNA pathway is dominant small RNA pathway in somatic mammalian cells; endo-siRNA pathway in germline (mouse oocytes)
- plant miRNAs have features distinct from mammalian miRNAs (3'-end methylation)

Knowledge gaps and additional aspects important for food/feed and environmental risk assessment

Food/feed

- Horizontal transmission of RNA interference into other species with different RNAi machinery → Controversial : Plant miRNAs transferred to and affecting mammalian genes (**exposure**)
- Despite long history of safe exposure of mammals to dsRNA: do mass amounts of dsRNA (e.g. produced in chloroplasts or externally applied) induce interferon response? (**hazard**)

ERA - Hazard

- Identifying biosensors (model organisms) for monitoring relevant non-target effects *in vivo*
- Interplay of specific RNAi pathway and non-specific dsRNA immune responses in nematodes, arthropods, plants

ERA – Exposure

- Safe threshold for environmental concentration of small RNAs? Minimum threshold for RNAi in different organisms?
- Effect of length and sequence on stability of dsRNA/siRNA in rotting plants and in soil?
- Barriers to dsRNA exposure after direct (GM plants, free dsRNA) or indirect (prey) uptake, e.g. limited half-life of dsRNA in digestive tract of arthropods, different dsRNA uptake systems

Exposure - general

- Transitivity (generation of secondary siRNAs) in organisms lacking RdRP (insects, mammals)? Are there alternative pathways for signal amplification?
- Use of tissue specific or inducible promoters for more specific expression and limited exposure of dsRNA

Biosafety aspects addressed at 1st WG3 meeting

- **N. Papadopoulou:**

Risk assessment of RNAi-based GM plants → EFSA commissioned 3 external scientific reports on issues relevant for food/feed and environmental risk assessment

- **S. Arpaia:**

Possible environmental effects of RNAi-based GM plants → arthropod toxicity tests on selected species through different exposure routes as most reliable data source for ERA

- **K.M Parker:**

Environmental fate of dsRNA → distribution in different environmental matrices

- **O. Christiaens:**

RNAi-based pest control: environmental safety and non-target effects → factors involved in RNAi efficiency

- **I. Menzler-Hokkanen:**

Minimizing non-target organism exposure in dsRNA applications by choice of trap plant, timing, method

- **B. Mateescu:**

Models for investigating the functional transfer of plant-derived dsRNA in mammalian cells

- **H. Kuiper:**

Food safety assessment strategies → case-specific approach, targeted compositional analysis based on molecular characterization and bioinformatics; further development of profiling methods needed

Biosafety aspects addressed at 2nd WG3 meeting

Day 1

Applications, mechanisms of action and biosafety considerations of RNAi targeting insect herbivores

→ **What are the main issues to be brought forward?**

- Expression levels in plants relevant for exposure
- Relevance of non-specific dsRNA effects (immune stimulation) in insects?
- Target gene selection
- Basis for differential responses to RNAi in different insects?
- Framework for *in silico* ERA based on 21mer matches and taxonomic relatedness
- Other soil organisms (e.g. nematodes) affected?

Day 2

General biosafety issues associated with RNAi:

- miRNAs in plant development and stress response – a contribution to ERA of RNAi plants
- Potential reduction of insecticide treatment by using RNAi technology (against PPV)
- RNAi to increase valuable metabolites or to reduce harmful components (e.g. allergens) in crops
- Safety of food (fruits) from transgenic RNAi fruit-trees as compared to fruits from a non-GM cultivar

Which topics should be raised during the Plenary Meeting in Poznan?

- Information to be obtained from WG1 and WG2 for **case study risk assessments and development of specific biosafety protocols**
- Development of a **data base on targets and off-targets** of characterised dsRNA and miRNA sequences – (jointly with WG1 and WG2)?

-