

COST ACTION CA 15223 - MODIFYING PLANTS TO PRODUCE INTERFERING RNA

Working Group 3: Specific biosafety issues associated with RNAi

Minutes of the Workshop on “Biosafety issues associated with RNAi” – Naples (Italy) 3 July 2018

The workshop organized by the WG3 was structured in three different sections and consisted of 1) a scientific session based on the contributions by WG members, 2) a general discussion regarding the future activities of the working group and 3) a scientific session in the framework of the XI European Congress of Entomology, organized by the Italian Entomological Society and the Italian Academy of Entomology (see www.ece2018.com).

The first scientific session was attended by 36 people, with about 50% constituted by “external” attendees present at the Congress. The latter had then the possibility of hearing about the iPlanta COST action and exchange views on the issues discussed in the WG.

Scientific presentations were given by:

Hanspeter Naegeli (Institute of Veterinary Pharmacology and Toxicology, University of Zurich, CH and Chair of the GMO Panel, European Food Safety Authority, Parma, IT)

Elena Sturchio (Dipartimento Innovazioni Tecnologiche, INAIL, Rome, IT)

Antje Dietz-Pfeilstetter (Julius Kuehn-Institut, Institute for Biosafety in Plant Biotechnology, Braunschweig, Germany)

Roberta De Pinto and *Paul Henning Krogh* (University of Bari Aldo Moro, Italy; Department of Bioscience, Aarhus University, Denmark)

Salvatore Arpaia (Italian National Agency For New Technologies, Energy and Sustainable Economic Development - ENEA)

Jozsef Kiss (Plant Protection Institute, Szent Istvan University, Gödöllő, Hungary)

M. Karbarz (Department of Botany, Faculty of Biotechnology, University of Rzeszow, PL)

The abstracts of the presentations are included in the following pages

FOOD AND FEED SAFETY ASSESSMENT OF GENETICALLY MODIFIED CROP INVOLVING RNA INTERFERENCE

HANSPETER NAEGELI

Institute of Veterinary Pharmacology and Toxicology, University of Zurich, Switzerland

Small double-stranded RNAs for gene silencing are generated in genetically modified plants for multiple purposes. RNA interference (RNAi) refers to a process in which small RNAs induce the inactivation and/or degradation of target transcripts via an RNA-induced silencing complex. Several genetically modified crops have been developed using this gene-silencing approach to confer new traits, and many more are under development. Such intended traits include for example a tomato with delayed ripening, an apple with slow browning and an altered oil composition in soybean. RNAi-based mechanisms have also been used to improve crop nutritional values, confer virus resistances, reduce allergen levels, improve agronomic characteristics and provide insect protection. The applicability of RNAi as a potential tool of insect control has been described in several insect species. The example of root protection against western corn rootworm in transgenic RNAi maize demonstrates the effectiveness of this plant protection strategy and new RNAi-based events will presumably become available to complement existing pest management strategies.

With regard to food and feed safety assessment, the Panel on genetically modified organisms (GMO Panel) at the European food safety authority (EFSA) is of the opinion that currently recognized principles and guidance for the safety evaluation of genetically modified plants are also applicable to RNAi-based traits. Briefly, the first objective of the safety assessment of genetically modified food and feed is to identify new hazards in genetically modified products relative to those derived from conventional counterparts. If new hazards are identified, a follow-up assessment is made to determine what risk, if any, they may pose to human or animal health. In particular, double-stranded RNAs are ubiquitous in the diverse range of organisms consumed as food or feed, including plants and animals. This establishes a long history of safe consumption, which pre-dates the use of RNAi-based traits in genetically modified plants. Being a normal component of the human and animal diet, double-stranded RNA is regarded as safe and there is no scientific basis for suggesting that new small double-stranded RNAs present in genetically modified plants have different properties than those already existing naturally at abundant levels in conventional food and feed. An important strength of the existing safety assessment of genetically modified plants is the use of comparative analyses to detect possible unintended effects beyond the intended traits including those that could arise from off-target effects in the gene silencing process.

BIOTECH PROCESS OPTIMIZATION OF MICROALGAE-BASED PRODUCTS

E. STURCHIO¹, P. BOCCIA¹, M. ZANELLATO¹, M.G. BERARDINELLI¹, M. DE GIACOMO², R. ONORI²

¹Dipartimento Innovazioni Tecnologiche, INAIL, Rome, IT

²Dipartimento di Sicurezza Alimentare Nutrizione e Sanità Pubblica Veterinaria, Istituto Superiore di Sanità, Rome, IT

Risk assessment, food, algae, RNAi, NBT

The aims of the project are: 1. optimize, in terms of productivity and reliable process control, the production of phenols and carotenoids from microalgal species using the Experimental Design technique applicable at experimental and industrial production level; 2. identify the specific biosafety data requirements for the risk assessment and management of GM microalgae and their products. 3. develop a common platform to evaluate biosafety implications of new technologies in the EU, including technical and legal aspects of biosafety assessment.

Recently, algae have been used for the production of ethanol or biodiesel and research is on-going on genetic engineering of micro-algae, especially for the production of pharmaceuticals and cosmetics. However, no genetically modified micro-algae are currently on the market. Europe can become market leader in micro-algae based products for the food and feed markets in the next decade. However, two major factors can limit the European potential: the insufficient domestic demand for these products and the difficulties in achieving commercial authorization in the EU markets due to the complexity of the regulation of novel foods in Europe.

Several methods for DNA delivery have been applied successfully to micro-algae including RNA silencing by either antisense or RNAi technology. From a practical perspective, RNAi is becoming a customary method for directed gene silencing in algae. As the necessary molecular tools are developed, RNAi approaches are expected to contribute to the functional characterization of novel genes, as well as to the genetic engineering of algae. However, potential drawbacks to this promising technology are a possible lack of specificity resulting in off-target effects and variations in the degree of induced silencing for particular genes. This emphasizes the need for careful design of RNAi experiments, including appropriate negative controls and the use of multiple RNAi reagents for each target gene. The verification of RNAi results and the validation of their biological relevance, whenever possible by using alternative methods, are also important steps in any experimental design. Ultimately, RNAi technology, in conjunction with system level “omics” approaches, may provide much-needed insights into gene function, metabolic pathways, and regulatory networks to allow us to comprehend the role of algal species in nature, as well as to engineer these organisms for the synthesis of valuable bioproducts. So the development of gene editing technologies represents a potentially powerful force for good with regard to human and animal health addressing the challenges we continue to face in a growing global population. This now includes the development of approaches to modify microalgal strains for potential improvements in productivity, robustness, harvestability, processability, nutritional composition, and application. The rapid developments in this area demand a timely review and revision of the current definitions and regulations around genetically modified organisms (GMOs), particularly within Europe.

ACTIVITES OF THE OECD EXPERT GROUP ON RNAi BASED PESTICIDES

A. DIETZ-PFEILSTETTER

Julius Kuehn-Institut, Institute for Biosafety in Plant Biotechnology, Braunschweig, Germany

Risk assessment, pesticides, RNAi, regulation, dsRNA products

An important field of application of RNA interference (RNAi) is the development of genetically modified (GM) plants which are resistant to pathogens or pests by the expression of double stranded (ds)RNAs targeting essential pathogen/pest genes. This approach called “host induced gene silencing” (HIGS) is the basis of one of the insect resistance genes in the coleopteran resistant GM maize event MON 87411 currently under regulatory review within the EU. New developments using RNAi based pesticides instead of HIGS, however, aim to avoid the utilization of GM plants.

In order to discuss risk assessment and regulatory issues of these new pesticidal agents, the OECD working group on pesticides (WGP) initiated the establishment of an *ad hoc* expert group on RNAi based pesticides in 2015. Among the tasks of the group are recommendations of possible approaches for the registration of such pesticides as well as the organization of seminars and workshops on topics related to RNAi based pesticides. Corresponding to the main priority identified by the group, a working paper on environmental fate and off-target effects from exposure to RNAi based pesticides is currently drafted. In addition, a conference on the regulation of externally applied dsRNA products for pest management is envisaged for June 2019.

A PROTOCOL FOR ASSESSMENT OF DIRECT EFFECTS OF RNAi TO EARTHWORMS

ROBERTA DE PINTO, M.T. STRANDBERG¹, K. KOSTOV², P.H. KROGH¹

¹University of Bari Aldo Moro, Italy

²Department of Bioscience, Aarhus University, Denmark

³ABI, Sofia, Bulgaria

Risk assessment, food, RNAi, NBT, soil ecology, NTO.

Studies of non-target effects of RNAi require adjustment and modification of existing procedures such as the ISO and OECD standards as these procedures are mainly adopted to the soil exposure route. Earthworms, Lumbricidae, are an important part of the soil ecosystems and thus commonly included in the risk assessment of the new plant breeding techniques (NBT). However, the mechanisms and fate of ingested small interfering RNA molecules in this phylum are not well studied. We present a protocol for artificial exposure of RNAi molecules to earthworms aiming to study if dsRNA can cross the gut barrier, enter into the internal organs, and exert its silencing effect on the COI, cytochrome oxidase 1, mRNA, a crucial part of the respiratory energy producing system. The elements of the protocol include 1) reverse transcription of mRNA to cDNA, 2) dsRNA synthesis by PCR, 3) preparing chitosan/dsRNA nanoparticles acting as a carrier for food exposure of dsRNA (Zhang et al. 2015. *J. Vis. Exp.* (2015) DOI 10.3791/52523) 4) introduce dsRNA into the internal organs of earthworms and induce silencing and finally, 5) perform qPCR of the targeted mRNA to confirm the silencing effect. Thus, the protocol describes both how to study fate and effects of the RNAi molecules. If COI or another gene apt for silencing will be successful in the earthworm *L. terrestris*, this could become a suggested positive control agent for future non-target studies of RNAi. The testing of environmental effects of NBT require such positive control RNAi's being comparable to the mode-of-action of new untested RNAi compounds. If the mode of action is of another nature than the RNAi being tested, the positive control ultimately cannot validate the test conditions because they are not addressed by the positive control agent.

KNOWLEDGE GAPS IN THE EVALUATION OF THE BIOSAFETY OF RNAi-BASED GM PLANTS

S. ARPAIA¹, O. CHRISTIAENS³, T. DZHAMBAZOVA², K. KOSTOV², M. R. JOGA³, I. URRU¹, J. SWEET⁴, G. SMAGGHE³

¹ Italian National Agency For New Technologies, Energy and Sustainable Economic Development (ENEA), Italy

² Agrobioinstitute (ABI), Sofia, Bulgaria

³ Ghent University, Belgium

⁴ JT Environmental Consultants Ltd, Cambridge, UK

Risk assessment, non-target effects, bioinformatics

A systematic literature search was used for a review of the scientific information on RNA interference that supports the environmental risk assessment of RNAi-based genetically modified (GM) plants. The analysis of the studies shows that in most cases, information on dsRNA expression in GM plants is insufficient to run a robust exposure analysis and in several of these studies, detection of dsRNA in plants was not even conducted. We reckon that it will be necessary to characterize the expression levels in each GM event in order to determine exposure levels to both target and non-target organisms. Movement of dsRNA along trophic chains and the persistence of its biological activity have been shown only in a few multi-trophic systems. The fate of dsRNA originated in GM plants need to be determined in order to estimate possible non-target effects at higher trophic levels.

The potential use of bioinformatics to predict off-targets and non-target effects is problematic because there is no real consensus yet on the ‘rules’ for siRNA/RISC binding to the homologous mRNA. There is no certainty on the number of nucleotides that must match the target sequence identically and on the allowed number or type of mismatches. Furthermore, there is no clear evidence for the number of siRNAs, processed from long dsRNA for example, necessary to incite gene silencing. When considering the paucity of genomic data available for many relevant non-target species active in agro-ecosystems, it is clear that genomic data alone can not guarantee to predict absence of silencing effects.

COULD A COMPLEX FOOD WEBS ANALYSIS CONTRIBUTE TO THE ENVIRONMENTAL RISK ASSESSMENT OF RNAi –BASED GM PLANTS?

J. KISS¹, A. BALOG², Á. SZENASI¹, Z. PÁLINKÁS¹

¹Plant Protection Institute, Szent Istvan University, Gödöllő, HU

²Department of Horticulture, Faculty of Technical and Human Science, Sapientia Hungarian University of Transylvania, Sighisoara Str. 1C. Tirgu-Mures, RO

Environmental risk assessment, RNAi, food webs

“Integrated pest management (IPM) emphasises the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms”. Thus IPM is based on a system approach both in spatial and temporal context together with the functional groups of various living organisms in crops.

In our former field study (for details see 1 and 2), genetically modified (GM) and its non-GM comparator maize hybrid stands were compared for the functional diversity of plants and arthropods aiming to determine whether the structures of arthropods food webs were different among various treatments. A total number of 399,239 arthropod individuals sampled in the above maize stands were used for analyses. Food web parameters analyses as trophic groups’ number and the links between them indicated that neither the *Bt* proteins (providing coleopteran and/or lepidopteran resistance) nor the glyphosate tolerance and treatment changed the structure of food webs. Differences in the average trophic links/trophic groups were detected between GM and non-GM food webs for herbivore groups and plants but the food web structure remained relatively unchanged for all stands. The results suggest that at least in short-term period these particular GM maize hybrids do not have adverse effects on arthropod biota of maize crop eco-system. We suggest that a complex food web analysis in RNAi-based crops may provide additional important tool to support ERA.

References

1. Pálinkás, Z., Kiss, J., Zalai, M., Szénási, Á., Dorner, Z., North, S., Woodward, G., Balog, A. (2017) Effects of genetically modified maize events expressing Cry34Ab1, Cry35Ab1, Cry1F and CP4 EPSPS proteins on arthropod complex food webs. *Ecology and Evolution*, 7(7): 2286–2293.
2. Szénási, Á., Pálinkás, Z., Zalai, M., Schmitz, O.J. & Balog, A. (2014). Short-term effects of different genetically modified maize varieties on arthropod food web properties: an experimental field assessment. *Scientific Reports* 4, 5315; DOI:10.1038/srep05315.

MICRO RNA IN CROP IMPROVEMENT: FUTURE APPLICATION AND RISC

M. KARBARZ

Department of Botany, Faculty of Biotechnology, University of Rzeszow, Pigonia 35-310 Rzeszów, Poland

Crop, miRNA,

The main goal of current plant breeding programs is yield increasing to meet rapid population growth and global climate change. Molecular breeding is a toolbox where plant physiology, genetics and biotechnology tools and are put together to obtain better qualitative and quantitative traits of cultivated plants. One group of this tool are miRNA-derived approaches. Micro RNAs play fundamental role in the suppression of target gene expression. They are part of complicated regulatory network in crop plants development, metabolism and stress response. The information about miRNA biological function can be exploited for crop plants improvement strategies. One simple and straightforward approach is to modulate agronomic traits by constitutively overexpressing a specific miRNA. Three aspects of this approach should be considered: searching for natural variation in miRNA and their target genes, utilization of miRNAs in hybrid crop breeding, modification of miRNAs or their targets via CRISPR–Cas9 [1]. For these methods, not only technical aspects are important. The controversial cross – kingdom miRNA traffic should be taken into consideration[2]

References:

1. Tang, J. & Chu, C. (2017) MicroRNAs in crop improvement: fine-tuners for complex traits. *Nat. Plants* 3, 17077
2. Zhao, Y., Cong, L. & Lukiw, W.J. (2018) Plant and Animal microRNAs (miRNAs) and Their Potential for Inter-kingdom Communication *Cell Mol Neurobiol* 38: 133.

In the afternoon, an internal WG3 meeting took place for planning future activities in order to finalize the preparation of the deliverables foreseen for this working group. The discussion was introduced by S. Arpaia and A. Dietz-Pfeilstetter who summarized the expected deliverables of WG3. The subsequent discussion resulted in a number of decisions and plans for future activities:

- Plan for publication of reviews.

In May the EFSA external report on "Literature review of scientific information on RNAi that could support the environmental risk assessment of RNAi-based GM plants" was published. Therefore, trying to write a generic review merely updating the one sponsored by EFSA was not deemed relevant. Two proposals were voiced during the meeting: the first deliverable could be represented by the following chapters of the book on RNAi foreseen by WG5:

1. Regulatory landscape for RNAi plants
2. Food safety assessment of RNAi plants and products
3. Environmental safety assessment of RNAi plants
4. Regulation of topically applied RNAi-based products

A second proposal was made by a group of WG members (coauthors of the presentation by S. Arpaia) who would like to write a review on existing knowledge gaps based on the content of the presentation.

- Plan for preparing a selection of protocols for risk assessment of RNAi plants.

Regarding the collection of protocols for environmental risk assessment, some of the STSM (at University of Ghent, ENEA and University of Aarhus) have already produced some experimental protocols which could constitute the backbone of the specific collection to be developed, since the WG decided in the 2017 meeting that "case studies should be preferably based on data produced by iPLANTA participants". The WG is aware that similar efforts are ongoing by other scientific bodies (e.g. ILSI) and are involving some members of the WG, however it was decided that the two activities should be clearly separated even if some of the outcomes might be similar. A sub-working group meeting might be organized to initialize this collection

- Plan for exemplary case study risk assessments

It was also decided to solicit information from WG1 and WG2 for preparing case studies including molecular mechanisms, specific applications and risk assessments of specific RNAi plants. Such case studies may be selected by the integrated action of the core group and can be part of the book on RNAi.

- Plan for compiling a review of targets and off-targets of known dsRNA and miRNA sequences

Such data base does not exist at the moment and the workload foreseen could be sensibly more relevant than expected from a COST action. It was decided to think of a simpler list of such targets, based on the excel files elaborated by the authors of the EFSA report.

- Plan the next WG3 meeting.

Next WG meeting will be entirely dedicated to the preparation of the deliverables, therefore only one presentation will be given for each topic and the rest of the meeting will be dedicated to parallel working sessions. S. Arpaia and A. Dietz-Pfeilstetter, in agreement with the COST action coordinators,

will propose key persons to coordinate the preparation of the subgroups and further deliverables. Candidatures for hosting next working group meetings are Budapest, Cambridge and Berlin. The final decision will be taken after the general assembly of the action in February.

The final discussion highlighted the availability of additional experts that are interested in cooperating with the working group and are specialized in other possible non-target organisms (e.g. aquatic species), so far not explicitly considered by the working group. Finally, there was consensus in the working group that next Plenary meeting of the iPlanta COST consortium next February should give more room to discussion between different working groups in order to identify the case studies indicated above. Also the final layout of the book could contain information based on the selected case studies.

The second scientific session “Use of RNA Interference for Insect Control” within the framework of the European Congress of Entomology was based on presentations mostly from speakers that were not current members of the working group and was centered on the following program:

Literature review of baseline information on RNAi that could support the environmental risk assessment of RNAi-based GM plants (*Jeremy Sweet, JT Environmental Consultants Ltd, UK; Olivier Christiaens, Ghent University, Belgium; Teodora Dzhambazova, Agrobiointitute, Bulgaria; Kaloyan Kostov, Agrobiointitute, Bulgaria; Salvatore Arpaia, ENEA, Italy; Mallikarjuna Reddy Joga, Ghent University, Belgium; Isabella Urru, ENEA, Italy; Guy Smagghe, Ghent University, Belgium*)

Identification of lethal rna targets and their application in relevant pest species (*Julia Ulrich, Sonja Gabriele Mehlhorn, Benjamin Buer Bayer AG, Crop Science Division, Germany; Gregor Bucher, Department of Evolutionary Developmental Biology, Georg-August-University, Germany; Sven Geibel, Ralf Nauen, Bayer AG, Crop Science Division, Germany*)

RNAi-based biopesticides (*Guy Smagghe, Ghent University, Belgium*)

Gene silencing in *Tribolium castaneum* as a tool for the targeted identification of candidate RNAi targets in crop pests (*Eileen Knorr, Fraunhofer IME, Germany; Elane Fishilevich, Dow AgroSciences, Italy; Linda Tenbusch, Fraunhofer IME, Germany; Meghan L. F. Frey, Murugesan Rangasamy, Dow AgroSciences, Italy; Andre Billion, Fraunhofer IME, Germany; Sarah E. Worden, Premchand Gandra, Kanika Arora, Wendy Lo, Greg Schulenberg, Pablo Valverde-Garcia, Dow AgroSciences, Italy; Andreas Vilcinskis, Fraunhofer IME & Institute for Insect Biotechnology, JLU, Germany; Kenneth E. Narva, Dow AgroSciences, Italy*)

Encapsulation of dsRNA with guanlylated polymers increases RNAi efficiency in *Spodoptera exigua* (*Olivier Christiaens, Myriam G. Tardajos, Zarel M. Reyna, Mamoni Dash, Peter Dubruel, Guy Smagghe, Ghent University, Belgium*)

dsRNA complexation with block copolymers enables pest control by oral application (*Calum Ferguson, Nicholas Warren, Elwyn Isaac, Olivier Cayre, University of Leeds, UK*)

Technologies to control, surveil, and counter gene drives (*Roberto Galizi, Imperial College London, UK*)

Genomic and transcriptomic analysis of the olive fly reproductive system (*Maria-Eleni Gregoriou, Maria Adamopoulou, Stella Galatidou, Anna Angelopoulou, Kostas Mathiopoulos, University of Thessaly, Larisa, Greece*)

Chlorophyll degradation in a lepidopteran pest *Spodoptera littoralis* is mediated by a gut-specific chlorophyllide binding protein (*Vincensius S.P. Oetama, Stefan Pentzold, Yannick Pauchet, Wilhem Boland, Max Planck Institute for Chemical Ecology, Germany*)

Salvatore Arpaia

17 July 2018

Antje Dietz-Pfeilstetter



The Working Group meeting was organized in collaboration with:



UNIVERSITÀ
POLITECNICA
DELLE MARCHE



JT ENVIRONMENTAL CONSULTANTS



CEREBRO FAUCIBVS VTERO
AB ORBIS ORIGINE
TENENT

Accademia Italiana di Entomologia

<http://iplanta.univpm.it/>