Workshop Report

WG 3. Biosafety of RNAi applications for plant protection.

Tuesday 2 April : 2pm : Tarragona, Spain.

This workshop was held in conjunction with the 15th ISBR symposium and was open to delegates from the symposium. Antje Dietz-Pfeilstetter (Vice leader WG3), and Jeremy Sweet (vice Chair iPlanta) opened the workshop by informing attendees about COST and iPlanta and introducing the workshop programme. This was followed by eight presentations, as shown in the programme, with questions permitted after each presentation. There followed a general discussion on specific data requirements for the risk assessment of RNAi GM plants and topical applications of RNAi. While it was recognised that data requirements related very much to a case-by-case problem formulation and assessment, many also considered that additional (nice to know) information should be supplied to satisfy regulators and decision makers. The use of bioinformatics to identify which exposed non-target organisms (NTOs) were at risk was also discussed. It was considered too premature to utilise only bioinformatics information, as not enough was known about the specificity of small RNAs and as certain types of mismatches are tolerated in RNAi-mediated gene silencing. Non-specific effects could also occur, and thus exposed NTOs should be tested where a potential harm could result. Delegates agreed that mammalian uptake of small RNAs from GM RNAi plants was very unlikely and that environmental persistence of dsRNA was low. However formulations of topically applied dsRNA could significantly change exposure pathways and environmental persistence. The absence of specific regulations for topical applications of dsRNA was discussed and it was considered that the scientific community should be encouraging the development of appropriate regulations and registration procedures. It was noted that several workshop attendees and iPlanta partners will participate in the forthcoming OECD Conference on of RNAi based pesticides in Paris where some of these issues will be discussed.
The workshop was attended by approximately 30 people in addition to the presenters and chairs and closed at 7pm.

Agenda of invited presentations:

- **Review of baseline information on RNAi to support the environmental risk assessment of RNAi-based GM plants** : Olivier Christiaens, Teodora Dzhambazova, Kaloyan Kostov, Salvatore Arpaia , Mallikarjuna Reddy Joga, Isabella Urru**, Jeremy Sweet**, Guy Smagghe.

- **Phage-based bacterial production and exogenous application of dsRNAs for plant protection.** **Annette Niehl***,* Marjukka Soininen, Minna Poranen, Manfred Heinlein

- **Viral-based dsRNA delivery systems for use in pest and disease control.** **Olivier Christiaens**, Clauvis N.T. Taning, Luc Swevers, Guy Smagghe.

- **Problem formulation for environmental risk assessment of spray applications of insecticides containing double-stranded RNA.** **Alan Raybould,** Helen Thompson & Andrea Burns

- **Authorisation of sprayable RNAi based plant protection products: Challenges for environmental risk assessment and risk management.** **Achim Gathmann,**

- **Silencing an essential gene in grain aphid through plant-mediated RNA interference generates aphid-resistant wheat plants.**
  Yongwei Sun, Caroline Sparks, **Huw Jones**, Mandy Riley, Frédéric Francis, Wenming Du and Lanqin Xia.

- **Problem Formulation in the ERA of RNAi-based GM Wheat with Resistance to *Fusarium* Pathogens. Felix Moronta-Barrios**, Antje Dietz-Pfeilstetter, and Wendy Craig .

- **Data requirements for the environmental assessment of RNAi plants**: **introduction to a discussion. Antje Dietz-Pfeilstetter**, Salvatore Arpaia, Jeremy Sweet, Olivier Christiaens.

iPlanta funded : 7 people . Antje Dietz-Pfeilstetter, Jeremy Sweet, Olivier Christiaens, Felix Moronta-Barrio, Huw Jones, Annette Niehl, Achim Gathmann.

Abstracts

A review of baseline information on RNAi that supports the environmental risk assessment of some RNAi-based GM plants

Olivier Christiaens[1], Teodora Dzhambazova[2], Kaloyan Kostov[2], Salvatore Arpaia[3], Mallikarjuna Reddy Joga[1], Isabella Urru[3], Jeremy Sweet[4], Guy Smagghe[1]

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Abstract

A systematic literature search was performed to collect all available peer-reviewed studies on RNAi in invertebrate species (Nematoda, Arthropoda, Mollusca and Annelida) to provide a baseline information on this technology for EFSA that could support the environmental risk assessment of RNAi-based GM plants. In this literature search, we retrieved a total of 5,076 publications. Based on this database, an overview was compiled of all studies using oral delivery of small RNAs (sRNAs) to these invertebrates. This overview includes information on tested species, life stage, sRNA molecule type, target gene, concentrations used, outcomes, etc. A second part of our assignment was to provide several narrative reviews on different topics such as environmental and cellular uptake of sRNAs, RNAi efficiency and factors involved in sensitivity, possible exposure routes of small RNAs to (non-)target organisms, potential unintended effects by sRNAs on invertebrate species in the agroecosystem and also on the availability and use of genomic data in risk assessment of RNAi-based GM crops. The analysis of the studies shows that in most cases, information on dsRNA expression in GM plants is not adequate for an exposure analysis and in some studies, no detection of dsRNA in plants was carried out. We conclude that it is necessary to characterize 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. We conclude that fate of dsRNA originating from GM plants in different trophic levels needs to be determined in order to assess effects at higher trophic levels. The 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 and there is limited sequence information in both targets and off targets. There is no certainty on the number of nucleotides that must match the target sequence and on the allowed

number or types of mismatches. In addition there is no clear evidence on the number of siRNAs processed from dsRNA that are necessary for gene silencing. Thus we conclude that genomic data alone is unlikely to be sufficient to predict silencing effects.

Phage-based bacterial production and exogenous application of dsRNAs for plant protection

Annette Niehl[1,2], Marjukka Soininen[3], Minna Poranen[3], Manfred Heinlein[1]

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Abstract

Virus infection causes severe damage on cultivated plants and therefore represents a serious threat to global food production. Recent experiments demonstrate the ability of exogenously applied double-stranded (ds)RNAs to protect plants against virus infection by triggering RNA interference. We engineered bacterial cells containing a phi6 phage-derived RNA replication system for the efficient in vivo production of large amounts of high-quality dsRNA sequences homologous to *Tobacco mosaic virus* (TMV). The produced TMV-derived dsRNAs inhibited infection by TMV when applied to *Nicotiana benthamiana* plants. The established dsRNA production system enables the cost efficient production of dsRNAs and application of dsRNA molecules as a highly flexible and nontransgenic approach for protecting crops against viruses and other pathogens.

Reference: Niehl, A., M. Soininen, M. M. Poranen and M. Heinlein. Synthetic biology approach for plant protection using dsRNA. Plant Biotechnology Journal. 2018. DOI: 10.1111/pbi.12904

Viral-based dsRNA delivery systems for use in pest and disease control

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Abstract

RNA interference (RNAi), a gene silencing mechanism triggered by double-stranded RNA (dsRNA), holds great promise as a novel and environmentally friendly pest and disease control strategy. The first insect control strategies which are expected to be commercialized are specifically targeting certain beetle species. While most beetles appear to be very sensitive to orally delivered dsRNA, this is not the case for many other insects. A great amount of research is being conducted to develop efficacious dsRNA delivery strategies in an attempt to overcome some of the barriers which have been identified as impeding efficient RNAi in these insects. One of these delivery approaches is viral delivery of dsRNA. Viruses can be modified to express insect-specific dsRNA, either in plants or in the insect itself. This approach has several benefits which allow a more efficient control of the target pest or disease. Alternatives, such as the use of viral-like particles to enhance the delivery of dsRNA are now also investigated and might provide a less controversial alternative to the use of engineered viruses as well. Here, an overview will be given on the state of the art and the possibilities of these virus-based delivery systems and a proof-of-concept of an engineered insect virus expressing a fruitfly-specific dsRNA will be presented.

Problem formulation for environmental risk assessment of spray applications of insecticides containing double-stranded RNA

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Abstract

Risk comprises the probability and severity of harm that may result from taking a course of action, such as applying a pesticide. Risk is high when severe effects are likely and low when harmful effects are predicted to be rare or trivial, or both. Estimates of risk contribute to decisions about whether to take certain actions; other relevant factors may be estimates of the likely benefits of those actions and the risks from not taking action. Efficient and effective risk assessment relies on problem formulation, which includes several vital steps: 1. agreement on what effects should be regarded as harmful; 2. formulation of hypotheses about how the proposed activity may lead to such harmful effects; 3. tests of those hypotheses with existing data; and 4. a plan to acquire new data for hypothesis testing should tests with existing data be insufficient for decision-making. By concentrating on predicting harm, problem formulation guides risk assessment away from haphazard collecting of data of unknown relevance for decision-making. Problem formulation is particularly valuable when considering products of new technology, when there is a temptation for risk assessment to become basic research into the properties of the technology, rather than a method for evaluating whether particular uses of specific products of the technology are likely to be harmful. We illustrate how problem formulation can guide the ecological risk assessments for spray applications of insecticides containing dsRNA active ingredients that induce RNAi in target insects.

Authorisation of sprayable RNAi based plant protection products: Challenges for environmental risk assessment and risk management

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Abstract

RNA interference (RNAi) is a means of reducing or switching-off the expression of individual genes, often described as 'gene silencing'. RNAi is a natural process with important defence and regulatory functions in animals, plants and fungi. RNA technology is widely used in GM plants. Prominent examples are virus resistances, e.g. in squash, papaya or plum, quality traits, e.g. in potato and apple, oil composition of soybeans, and pest regulation of the western corn root worm.

Additionally, sprayable RNAi based plant protection products are in the pipeline aiming at different targets such as flea beetles in oil seed rape, fusarium diseases in barley or weed control to overcome resistant weeds.

RNAi is a new mode of action in "conventional plant protection products". This might challenge the risk assessment and risk management. For some aspects/areas, the characteristics of RNAi as active ingredient, needs adaptions of existing or the development of new risk assessment tools. For other parts, it might on the other hand ease the risk assessment. Additionally, new formulations to resist degradation of sRNA such as liquid encapsulation, conjunction with polymers or nanoparticles might challenge risk assessments.

The presentation will introduce the new challenges, identify similarities and differences in risk assessment of biotechnical and classical plant protection products, and discuss how these challenges might be considered in the authorisation process of sprayable RNAi plant protection products.

Silencing an essential gene involved in infestation and digestion in grain aphid through plant-mediated RNA interference generates aphid-resistant wheat plants

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Abstract

Grain aphid (*Sitobion avenae* F.) is the most dominant and destructive pest of wheat causing significant yield loss of cereal plants each year. Expression of double strand RNA (dsRNA) in transgenic plants designed against insect target genes has been shown to give protection against pests through RNA interference (RNAi). In this study, we identified a novel potential RNAi target gene (*SaZFP*) which was involved in ingestion and digestion in grain aphid based on transcriptomic profiling of the alimentary canal of grain aphid upon feeding on wheat plants and *in vitro* dsRNA artificial diet assay. We generated stable transgenic wheat lines expressing dsRNA for targeted silencing of *SaZFP* in grain aphid. After feeding on transgenic wheat plants expressing *SaZFP*-dsRNA, attenuated expression levels of *SaZFP* mRNA in aphids were observed compared with those of aphids feeding on wild-type plants. The decreased *SaZFP* expression levels were correlated with significantly prolonged development, reduced fecundity and survival, and dramatically decreased reproduction of aphids. We also observed altered aphid feeding behaviour such as delayed delivery of saliva into sieve element and shorter ingestion phase. Furthermore, we found the silencing effect was persistent and transgenerational as decreased survival and fecundity were observed in both surviving aphids and their offspring. Taken together, we not only identified a novel effective RNAi target in grain aphid, but also demonstrated that plant-mediated RNAi of an essential gene involved in infestation and digestion can be exploited as an efficient strategy for aphid control in wheat.

Problem Formulation in the ERA of RNAi-based GM Wheat with Resistance to *Fusarium* Pathogens

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Abstract

Genetically modified (GM) organisms are becoming ever more complex through the application of new molecular techniques. One of these techniques uses RNA interference (RNAi) for pathogen control. RNAi-based GM plants can produce species-specific double-stranded RNA (dsRNA) to disrupt essential physiological functions of plant pathogens via host-induced silencing of gene expression (HIGS). This innovative, highly specific technology combines pronounced selectivity for the target organism with minimal side effects as compared with chemical treatments, and has the potential to introduce novel pest and disease resistance, thereby increasing crop productivity and reducing post-harvest losses. Evidently, it is critical to ensure that the dsRNA and corresponding small-interfering RNA species do not result in any unintended gene silencing (often referred to as off-target gene silencing) that can negatively impact the physiology of the host plant, any plant-associated beneficial fungi, potential non-target herbivores, and/or their predators. However, current understanding of the susceptibility of organisms to environmental exposure to dsRNA, as well as the likelihood of off-target gene effects, is incomplete. In this study, European environmental protection policy objectives formed the starting point of an assessment of potential impacts from the cultivation of RNAi-based GM wheat designed to resist *Fusarium* pathogens via HIGS. We have applied Problem Formulation, consisting of a catalogue of risk hypothesis and their causal pathways to potential environmental harms, as the first step towards their science-based environmental risk assessment (ERA). The results support: (i) the identification of knowledge gaps arising in the area of potential environmental harms specific to RNAi-based fungus resistance, and; (ii) the development of risk assessment guidelines which relate to specific

effects of RNAi crops on the environment. Certainly, these outcomes will prove valuable for informing future ERAs of RNAi-based pathogen-resistant GM plants.

Data requirements for the environmental assessment of RNAi plants: introduction to a discussion

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Abstract

RNAi-based genetically modified (GM) plants have been in use for a long time, starting with the development of the FLAVR SAVR tomato and of several virus resistant crops, e.g. papaya resistant to papaya ring spot virus. More recently control of plant pests and fungal pathogens has been achieved in host-induced gene silencing (HIGS) approaches. Especially these new types of applications raise specific environmental biosafety issues like potential unintended effects on non-target organisms caused by pest- or pathogen-specific dsRNA produced in RNAi plants. Current approaches to environmental risk assessment of RNAi plants within the EU legal framework will be presented in comparison with a case study from the US. Relevant questions will be addressed as an introduction to a discussion on data requirements to determine possible harm, exposure and eventually risk of RNAi-based GM plants.