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HORIZONTAL GENE TRANSFER IN GENETICALLY MODIFIED PLANTS – THE EU REQUIREMENTS

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GMO, Genetic engineering, HGT, Reg (EU) 503/2013, Dir (EU) 2018/350

Genetically modified (GM) plants and derived food and feed products are subject to a risk assessment and regulatory approval before entering the market in the EU. In this process, the European Food Safety Authority (EFSA) evaluates any risks that GM plants may pose to human and animal health and the environment. The safety assessment is performed based on risk assessment guidelines developed by the EFSA GMO Panel [1, 2] and following the requirements for the risk assessment of GMOs laid down in the Regulations (EU) No 503/2013 [3] and Directive (EU) No 2018/350 [4].

The plant-to-micro-organism gene transfer is one of the area of concerns to be addressed in the environmental risk assessment of GM plants.

The main EU requirements for the preparation of dossier for EFSA assessment will be introduced and used to trigger a discussion on how to implement them for the assessment of the potential plant-to-micro-organism gene transfer of dsRNA expressing plants.

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IMPLEMENTATION OF RNAI-BASED PEST CONTROL: BIOSAFETY AND REGULATORY CONSIDERATIONS

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RNAi, Environmental Risk Assessment, resistance management

With RNAi-based insect pest control products coming closer to commercialization in many countries worldwide, regulators are gathering information and forming their opinions on data requirements for the environmental risk assessment (ERA) and registration of these products. Recently, several extensive systematic literature searches and baseline information reviews were produced by the European Food Safety Authority (EFSA) and are now publicly available. Furthermore, the Organisation for Economic Cooperation and Development (OECD) pesticide committee has recently also organized a conference on RNAi-based pesticides and is expected to release a white paper soon, containing its conclusions and recommendations for risk assessment of these products. In this presentation, we give an overview of a number of important biosafety and ERA-related aspects of this new pest control strategy which could be the basis for a further discussion on the requirements for ERA of these products. These include the persistence of the dsRNA in the environment, the use of bioinformatics in ERA and we also discuss a number of knowledge gaps that impact ERA considerations. Finally, we also discuss the potential for resistance against these pesticides.

REGULATORY CONSIDERATIONS FOR dsRNA-BASED PRODUCTS: AN EU VIEW ON BIOSAFETY

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RNAi, dsRNA, environmental safety, mammalian safety

Recently, the first biotechnology-derived crop utilizing dsRNA designed to deliver highly specific insect control, was registered for cultivation in the U.S. and Canada, and also received import approvals globally. As part of this regulatory process, EFSA has published its positive opinion on this insect control trait and the European Commission has granted import authorization. Additionally, the EFSA review of a dsRNA-based bee health product also provides background on the safety profile of dsRNA-based products. While the use of RNA-based mechanisms to control insects and other pests has been perceived by some regulatory agencies as novel compared to the more familiar Bt biopesticides and Bt crops employed in agriculture, existing regulatory reviews demonstrate that the current regulatory framework is sufficient to also assess the durability and safety of dsRNA-based products. A strong weight of scientific evidence from the published literature and publicly available regulatory decisions address both mammalian and ecological risk assessment considerations, and therefore are able to provide insight into the biosafety assessment of dsRNA-based products.

FOOD & FEED SAFETY OF "GENE-SILENCED" CROPS: CONSEQUENCES OF RECENT TECHNOLOGICAL ADVANCES FOR RISK ASSESSMENT APPROACHES

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Crop biotechnology, RNA interference, food safety, animal feed safety, gene editing

An array of "gene silenced" transgenic crops have been created through application of RNA interference (RNAi) technology. These include crops with both improved agronomic traits, such as resistance towards plant pests such as papaya ringspot virus in papaya or corn rootworm in maize, and compositional characteristics, such as modified fatty acid profiles of oilseeds or the amylose/amylopectin ratio in starch crops. The technology exploits the machinery naturally present in eukaryotic cells for modulation of endogenous gene expression by RNA molecules, which grossly fall into either of two categories, namely small interfering RNAs (siRNA) or microRNAs(miRNAs), which suppress transcription and/or translation of specific messenger RNAs with sequences complementary to these smaller ones. For the creation of these "gene-silenced" crops, recombinant DNA techniques were commonly applied involving the insertion of transgenes coding for expression of RNA molecules with fragments of the targeted mRNA in sense or antisense direction. As for other types of transgenic crops, the assessment of their safety could be carried out according to the internationally harmonized principles of the assessment of genetically modified crops as laid down in Codex Alimentarius guidelines and further detailed in the annex to EU Implementing Regulation (EU) No. 503/2013. In brief, this entails a detailed characterization of the genetically modified crop at the molecular level and a comparison with a conventional non-GM counterpart with a history of safe use on compositional, agronomic, and phenotypic characteristics.

Recently, the advent of technologically advanced and precise methods of genome editing offer alternative technical options to alter the expression of plant genes. Examples include the creation of indels through of use of class 1 site-directed nucleases involving non-homologous end-joining of induced double-strand DNA breaks, such as with CRISPR Cas9. Another example is the use of RNA-dependent DNA methylation, to cause epigenetic changes to the sequence of interest, altering its expression over multiple generations. To which extent will the traditional risk assessment paradigm still apply to these crops at the borderline between mutagenesis and transgenesis. In this presentation, we will explore how the risk assessment for "gene silenced" crops might benefit from a focused analysis of

the impact of the modifications on cellular RNA expression. Transcriptomics as a holistic method to capture changes in gene expression can be combined with advanced statistical tools to tackle the complexity of the outcomes, particularly the “one-class” model.

CONSIDERATION OF SPECIFIC ISSUES FOR THE ENVIRONMENTAL RISK ASSESSMENT OF EXTERNALLY APPLIED DSRNA-BASED PESTICIDES

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SIGS, dsRNA delivery, plant uptake, amplification

RNAi technology can be used for plant protection either by the generation of genetically modified (GM) plants with stably integrated RNAi constructs targeting pest or pathogen genes (HIGS approach) or via the exogenous application of dsRNA-based pesticide products. While GM plants are regulated in Europe according to EU GMO regulations, dsRNA-based pesticides are subject to pesticide legislation. Apart from the specific testing and data requirements for pesticides there are some specific risk assessment issues which have to be considered for external applications of dsRNA pesticides in addition to the data required for GM RNAi plants. These issues which encompass different dsRNA stabilizing and delivery methods as well as uptake, translocation and amplification in plants will be discussed.

CURRENT STATUS OF RNA-BASED BIOCONTROL COMPOUNDS AND PERSPECTIVES TO REACH THE MARKET

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RNA interference, biocontrol, sprayable, dsRNA, regulatory

Facing current climate challenges and drastically reduced chemical options for plant protection, RNAi technology has been presented as one of the needed tools to support agriculture in a sustainable way. Applications of RNAi as an agricultural biotechnology tool has unveiled possible new solutions to the global problems of agricultural losses caused by pests, pathogens and other biotic and abiotic stresses. While the use of RNAi as a tool in agriculture is still limited to a few transgenic crops, and only adopted to restricted parts of the world due to expensive capital requirements and political/public concerns surrounding the cultivation and use of GM crops, scientists and industry are already seeking innovations in leveraging and exploiting the potential of RNAi in the form of sprayable RNA-based biocontrol products. This study highlights the expanding research and development pipeline, commercial landscape and regulatory environment surrounding the pursuit of sprayable RNA-based biocontrol products with improved environmental profiles.

PROBLEM FORMULATION IN THE ERA OF RNAI-BASED GM WHEAT WITH RESISTANCE TO FUSARIUM PATHOGENS

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Problem Fromulation, Environmental Risk Assessment, HIGS

Environmental Risk Assessment (ERA) is a fundamental element of risk analyses performed to facilitate regulatory decisions concerning market introductions of new agricultural products. Robust ERAs begin with an explicit problem formulation to set the context, and involves: formulating relevant risk hypotheses, devising plausible pathways to harm, and identifying information and data requirements essential to test the risk hypotheses. This procedure is widely used as a key first step and requirement for the ERA of genetically modified organisms. Here we apply problem formulation for assessing possible adverse effects on the environment of the hypothetical cultivation of an RNAi-based genetically modified wheat resistant to Fusarium pathogens. We present a catalogue of risk hypotheses and their causal pathways to harm, thereby supporting the identification of knowledge gaps pertaining to those potential environmental harms specific to the deployment of fungal pest control based on RNAi-plants.

SIRNA SPECIFICITY: MECHANISMS AND STRATEGIES TO REDUCE OFF-TARGET EFFECTS

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RNAi, miRNA-like off target effects, siRNA pooling, siRNA mobility

Short interfering RNAs (siRNAs) are processed from long double stranded RNA (dsRNA) and the guide strand is incorporated into the RNA-induced silencing complex (RISC). Within RISC, a member of the Argonaute protein family directly binds the guide strand and the siRNA guides RISC to fully complementary sites on target RNAs, which are then sequence-specifically cleaved by the Argonaute protein - a process commonly referred to as RNA interference or RNAi. Endogenous microRNAs (miRNAs) function similarly but do not lead to direct cleavage of the target RNA but to translational inhibition followed by exonucleolytic decay. This is due to only partial complementarity between the miRNA and the target RNA. SiRNAs, however, can function as miRNAs and partial complementarity can lead to miRNA-like off target effects in RNAi experiments. Since siRNAs are widely used for screening but also therapeutics and also crop protection purposes, such miRNA-like off target effects need to be eliminated. Strategies such as RNA modifications or pooling of siRNAs have been developed and are used to reduce off target effects.

RNAi AS USEFUL TOOLS TO TACKLE VIRUS INFECTION VECTORED BY APHIDS AND SAFE SUBSTITUTE FOOD FOR MACROLOPHUS INSECTS

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Aphids, sharka virus, RNAi, insect bugs, silencing

Aphid-vectored plum pox virus is the natural manner to spread sharka disease. After spending years of field natural testing, the high resistance character deployed by HoneySweet plum was confirmed. Through observations done in different countries (Skierniewice, Poland, Liria, Spain, Bistrita, Romania and Praha, Czech Republic) coherent information about the reduction of aphid impact versus siRNA accumulated in HoneySweet plants was confirmed. When viruliferous aphid vectors affected fruit-trees, a fight to PPV inoculated was engaged. Conventional or transformed trees that were infected and showing symptoms did not accumulate siRNA. Laboratory analysis showed that these clones, identified as susceptible, were remarkably diseased. The results for resistant clones that were significantly symptomless indicated the involvement of siRNA accumulated in cells. PPV threat via aphid inoculation was tackled. The role played by the viral siRNA in plants is specific and causes PPV RNA degradation. When other insects like Macrolophus bug were spread in plants to control any undesired pests. The development of molecular technology to specifically detect RNAi, permitted to detect these molecules in survival conditions. Interestingly Macrolophus bug used young leaves as food source. Molecular analysis showed that siRNA was sucked by these insects. Was there some threat? Under greenhouse conditions, we observed the lack of any biorisk, insects were alive. Over both cases (aphid and Macrolophus), the suitability of siRNA designated and engineered to tackle the undesired virus was confirmed. With the unintended sucking of siRNA by Macrolophus , these siRNA spread in whole plants do not pose any biorisk to carnivore insects used as biocontrol agent. With the development of biocontrol agents in agriculture to control pests, siRNA appears as any efficient biological weapon, and safe molecules for the environment.

COMPARATIVE ASSESSMENT OF GENETICALLY MODIFIED PLANTS – THE EU REQUIREMENTS

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GMO, Genetic engineering, Comparative analysis, Reg (EU) 503/2013

Genetically modified (GM) plants and derived food and feed products are subject to a risk assessment and regulatory approval before entering the market in the EU. In this process, the European Food Safety Authority (EFSA) evaluates any risks that GM plants may pose to human and animal health and the environment. The safety assessment is performed based on risk assessment guidelines developed by the EFSA GMO Panel [1, 2, 3] and following the requirements for the risk assessment of GMOs laid down in the Regulation (EU) No 503/2013 [4].

The comparative assessment represents one of the pillars for the risk assessment of GM plants.

The main EU requirements for the preparation of dossier for EFSA assessment will be introduced and used to trigger a discussion on how to implement them for the comparative assessment of dsRNA expressing plants.

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FIELD TRIALS FOR RISK ASSESSMENT OF GM PLANTS: ECOLOGICAL AND COMPOSITIONAL STUDIES - WHY DO THEY DIFFER, AND WHAT IS THE RELEVANCE FOR RNAI?

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Risk assessment for European GM food-feed (FF) applications are assessed using a set of field trials that usually cover both agronomic and phenotypic characterization and compositional analysis of the GM plants. Guidance for both types of assessment has been extensively revised in recent years, but both rely on the concept of a 'history of safe use'. This implies that a food derived from a common crop (such as maize), which has been consumed for thousands of years, can be considered as having a history of safe use. Hence, if the GM maize under assessment can be shown to have essentially the same composition and characteristics as the same variety of unmodified maize, it too should be safe to consume. For any particular endpoint, assessment proceeds with a statistical test of the difference between these two varieties: the GM and the unmodified (often the latter is termed a 'conventional comparator').

However, if a difference is found, then lack of safety is not immediately demonstrated, because the degree of difference needs to be evaluated for its biological relevance. Evaluation of relevance must reflect the fact that other commercially-grown varieties of maize, each with its own history of safe use, will likely yield different results for that particular endpoint, which will generate a statistical distribution of values which may or may not encompass that of the GM variety. If it does, then the implication is that the GM is not too dissimilar to the commercial varieties, and vice-versa. Field trials have therefore tended, for many years, to include some commercial varieties, in addition to the GM and its conventional comparator. Such considerations lead formally to a second statistical test, between the GM and the commercial varieties, called a test of equivalence. Effectively, if a difference may be found between the GM and its conventional comparator, and if the GM is found to be equivalent to the commercial varieties, the difference may not be deemed biologically relevant.

The guidance for European environmental risk assessment of GM plants has also been updated, although lack of applications has not enabled evaluation of how the guidance performs in practice. Since all agriculture has some effect on the environment, there is no analogous management, cultivation or crop that can be said to have, environmentally, a 'history of safe use'. Therefore, commercial varieties cannot be relied upon to give any background or contextual information concerning environmental safety and are not included in field trials. Instead, trials usually feature just two treatments, the GM and its

conventional comparator. However, it is just as important in ERA, as in FF studies, to place any difference found into context. Once again, the mere existence of a difference does not necessarily have environmental safety implications, because it is the biological relevance of the difference that is crucial. Here, however, rather than objectively using data from commercial varieties to place differences into context, the equivalence test that examines relevance, requires 'limits of concern' which perforce must be set subjectively. These limits of concern are intended to represent the minimum relevant ecological effect that is deemed biologically relevant.

The question raised by this paper is: for a genetic modification arising from a different method than that traditionally used since the 1990s, do these field trials, as described above, still have applicability? And specifically, if RNAi is the methodology pursued, is the field trial approach still valid? Is less data required? Or more? I have no firm answers but hope that these questions will form a good basis for discussion.