Biosafety assessment of current RNAi application:

Case study of insect resistant MON 87411 maize

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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**

Current RNAi applications and developments I

Type	GMO	Target gene	Phenotype	Regulatory status (authorization)
Delayed fruit softening	Tomato FlavrSavr (Monsanto) and related events	Polygalacturonase (sense/antisense)	Reduced cell wall breakdown, delayed softening	**USA**: Food/feed (1994), cultivation (1992) **Canada**: Food (1995)
Reduced ripening/ senescence	Tomato 1345-4 (DNA Plant Technol. Corp.)	ACC synthase	Ethylene reduced Delayed fruit ripening	**USA**: Food, feed, cultivation (1995) **Canada**: Food (1995)
	Carnation 66 (Florigene)	ACC synthase	Ethylene reduced Longer vase life	**Australia** and **Norway**: Cultivation (1995/1998)
Fatty acid metabolism	Soybean DP 305423 (DuPont/Pioneer)	gmfad 2-1 (partial)	High oleic acid, low linoleic acid	**USA** and **Canada**: Food, feed, cultivation (2009) **EU**: Food and feed (2015)
	Soybean 260-05 (DuPont/Pioneer)	gmfad 2-1	High oleic acid, low linoleic acid	**USA/Canada**: Food, feed, cultivation (1997/2000)
	Soybean MON 87705 (Monsanto)	fad2-1A fatb1-A	High oleic acid, low linoleic acid	**USA** and **Canada**: Food, feed, cultivation (2011) **EU**: Food and feed (2015)

Current RNAi applications and developments II

Type	GMO	Target gene	Phenotype	Regulatory status (authorization)
Starch metabolism	Potato EH92-527 (Amflora, BASF)	gbss (antisense)	Amylose reduced, amylopectin increased	**EU**: Food, feed, cultivation (2010) – **cancelled in 2013**
	Potato AM04-1020 (BASF)	gbss (antisense)	Amylose reduced, amylopectin increased	**USA**: Food/feed (2014)
Product quality	Potato Innate (J.R. Simplot)	asn1 (dsRNA) ppo5 (dsRNA)	Asparagine/acrylamide reduced + reduced PPO/black spot	**USA/Canada**: Food, feed, cultivation (2014/2016) **Australia**: Food (2017)
	Arctic Apple GD743/ GS784 (Okanagan Specialty Fruits Inc.)	ppo (dsRNA)	Reduced PPO/non-browning phenotype	**USA/Canada**: Food, feed, cultivation (2015)
Virus resistance (Examples)	Papaya 55-1 (SunUp) (Cornell University/ University of Hawaii)	PRSV-cp	Resistance to PRSV	**USA**: Food/feed (1997), cultivation (1996) Canada: Food (2003)
	Plum C-5 (USDA/ARS)	PPV-cp	Resistance to PPV	**USA**: Food/feed (2009), cultivation (2007)

Coleopteran insect resistance: MON 87411 maize

Regulatory status (Authorization)

Country	Food	Feed	Cultivation
Australia	2015		
Brazil	2016	2016	2016
Canada	2015	2015	2015
Japan	2016	2014	2014 (expires 2018)
New Zealand	2015		
South Korea	2016	2016	
Taiwan	2015		
USA	2014	2014	2015
European Union	pending	pending	

Genetic elements introduced in MON 87411 maize

- **Suppression cassette containing IR sequence of a 240 bp fragment of western corn rootworm (WCR; *Diabrotica virgifera*) *Snf7** gene (DvSnf7)**

→ dsRNA transcript recognized by WCR RNAi machinery, resulting in siRNA-mediated down-regulation of targeted DvSnf7 gene

- *cry3Bb1* gene cassette

→ B.t. Cry3Bb1 protein binding to specific insect midgut receptors, eventually leading to disruption of cell integrity

Plant incorporated Protectants (PIPs)

- cp4 *epsps* gene cassette

→ 5-enolpyruvylshikimate-3-phosphate synthase tolerant to herbicide glyphosate

* Class E vacuolar sorting protein involved in sorting of transmembrane proteins on the way to lysosomal degradation. Suppression of sorting disturbs cell homeostasis and leads to cellular death and insect mortality.

Approaches for biosafety assessment of MON 87411

USA

USDA-APHIS: Biotechnology Regulations 7CFR part 340 → Determination of nonregulated status

Plant pest risk assessment

→ MON 87411 maize **unlikely to pose a plant pest risk**

e.g. based on „bioassays involving the insecticidal dsRNA to a range of insects varying in ecological function and to insects closely related taxonomically to corn rootworm“

U.S. EPA: Federal Food, Drug & Cosmetic Act and Federal Insecticide, Fungicide and Rodenticide Act

→ **Plant-incorporated protectant (PIP) registered as pesticide** (Active ingredients: *DvSnf7* dsRNA and Cry3Bb1 in MON 87411), but only for agronomic evaluation, seed increase and production in breeding nurseries (no commercial planting!)

U.S. FDA: Federal Food, Drug & Cosmetic Act

→ Safety assessment of PIPs under regulatory purview of EPA

→ Safety assessment with respect to its use in food or feed

- genomic stabilty over five breeding generations
- compositional equivalence to conventional maize varieties

Data delivered for safety assessment of DvSnf7 dsRNA

General assumptions:

- Nucleic acids including RNA have a history of safe use and are generally considered as safe (**GRAS**) by U.S. FDA
- U.S. EPA has established **tolerance exemption for nucleic acids** that are part of PIP products

- Expression levels in different tissue types of MON 87411
- Estimates for human and animal exposure from food and feed
- Insect diet bioassays to characterize the spectrum of activity
- Quantitative ecological risk assessment
  - Maximum expected environmental concentrations (MEECs)
  - No observed effect concentrations (NOECs)
  - Estimated margins of exposure (MOEs)

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Expression levels of DvSnf7 RNA and estimates for exposure from food and feed

- Mean expression levels in 19 tissue types across five replicated field sites (2011-2012), e.g.

Tissue type	µg/g fw (Mean ± SD)	µg/g dw (Mean ± SD)
- Whole plant developmental stage V3-V4	0.0105 ± 0.0042	**0.0848 ± 0.0438**
- Leaf developmental stage V14/R1	0.0144 ± 0.0067	0.0569 ± 0.0285
- Forage	0.0013 ± 0.0004	0.0043 ± 0.0013
- Grain	**0.00009** ± 0.00003	0.0001 ± 0.00003
- Pollen	0.0001 ± 0.00007	0.0001 ± 0.00009

- Human exposure to DvSnf7 RNA from dietary intake
  - Maize consumption (DEEM-FCID exposure estimates) x Level of DvSnf7 RNA in MON 87411 (µg/g fw)

    → **0.2 ng/kg bw/day** for U.S. population; **0.4 ng/kg bw/day** for non-nursing infants

    (500 million fold lower than estimated daily consumption of total RNA!)

- Animal exposure to DvSnf7 RNA from consumption in feeds

  → **81 ng/kg bw/day** in lactating cow; **10.5 ng/kg bw/day** in broiler chicken

Insect diet bioassays

- **Bioassays to characterize activity spectrum of 240 bp dsRNA „active“ region**
  - DvSnf7_240 dsRNA synthesized *in vitro*, applied at different test concentrations (ng/ml) in diet
  - 14 insect species selected based on taxonomic relatedness to WCR:
    7 x coleoptera (4 families), 1 x Hemiptera, 2 x Hymenoptera, 4 x Lepidoptera

→ **12 days-LC50 (WCR): 1.2 ng/ml**; 12 days-LC50 (SCR): 4.4 ng/ml
(for comparison: highest DvSnf7 RNA expression in whole plant is ≈ 85 ng/g dry weight)

→ Even in the closest related Chrysomelidae species Leptinotarsa decemlineata no negative impact, i.e. **no observed effect concentration (NOEC) ≥ 500 ng/ml**)

Quantitative ecological risk assessment

- **Evaluation of potential risks to non-target organisms (NTOs)**

  Taking into account most likely **hazard** and **exposure** scenarios

  → Test organisms: 1 x soil decomposer, 5 x beneficial insects (e.g. *Apis mellifera*)

  → Test concentrations of DvSnf7_968 RNA (*in vitro* produced) based on expression in tissues most relevant for exposure in the environment

  1. Calculation of maximum expected environmental concentration (**MEEC**) to NTOs
  2. Margin of exposure (MOE) > 10 used to set test concentrations

  → Determination of **NOEC** for NTOs

  → **Margins of exposure (= NOEC/MEEC) of ≥ 30** (indicative of minimal risk in worst-case laboratory assays, U.S. EPA 2010)

- **Environmental fate of DvSnf7_968 RNA**
  - No specific data provided
  - Only reference made to general considerations (large amounts of nucleic acids release into the environment from conventional plants and other organisms) and to *nptII* gene degradation study

EFSA guidance for risk assessment of GMO authorization according to Regulation (EC) 1829/2003

Hazard identification and characterization

Information requested/provided		Possible hazards/adverse effects
- Information on expression of insert - Agronomic and phenotypic data - Comparative compositional analysis - Nutritional assessment → **Compositional and nutritional equivalence to conventional maize** - Toxicology (90-days feeding study in rodents with whole GM feed; no 28-days toxicity study with dsRNA) → **No toxicologically relevant effects**	? ? ?	- Safety relevant compositional changes in GM plant caused by non-specific silencing in plant (driven by specific risk hypothesis?) - Adverse non-target effects in humans/animals - Other types of adverse unintended effects in humans/ animals, i.e. non-specific dsRNA responses like immune stimulation, saturation of RNAi machinery

Exposure assessment – anticipated intake or extent of use

- Expression level of dsRNA in GM plants and daily consumption→ Exposure estimates from food and feed

Regulation (EC) 1829/2003 - Environmental risk assessment (ERA) for MON 87411 in accordance with Annex II of Directive 2001/18/EC

- Potential hazards from plant characterization and comparative assessment?
- Hazard characterization
- Exposure characterization:
  - **no cultivation** in the EU
  - accidental spillage of MON 87411 grain
  - faeces of animals fed MON 87411
  - imported plant material

  → **<u>Negligible exposure</u>**
- Risk characterization

Specific areas of risk adressed which are most relevant for RNAi insect resistant maize:

- Interactions with target organism (→ development of resistance in target organism?)
- Interactions with non-target organisms (→ adverse effects on NTOs with important ecosystem functions?)
- Effects on biogeochemical processes (→ impact on relevant soil microorganisms?)

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Do we need more data and/or more knowledge for risk assessment of RNAi plants?

Food/feed safety

- Evaluation of possible off-target effects in the plant

→ Omics data (Transcriptomics, metabolomics) in addition to compositional analysis?

- Predicting possible non-target effects in humans and (farm) animals

→ Bioinformatic search for potential targets of DvSnf7 dsRNA in human and animal genes?

however: - Limited predictability; additional factors like target site accessibility important
- Plant miRNA uptake by humans and effect on human gene expression is controversial issue
- Long history of safe exposure of mammals to dsRNA

- Exposure assessment

→ Data on actual uptake and fate of DvSnf7 dsRNA in humans and animals?

Additional data needed for ERA in case of cultivation!