Global Risk Assessment Considerations for *in planta* RNA-mediated Control of Insect Pests

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How Can RNA-Based Technologies Help Agriculture?

- An **important tool** derived from a **naturally occurring process** that supports **sustainable agriculture**.

- Offer growers **new modes of action** in managing hard to control pests and viruses, thereby enabling them to i**ncrease yields and maintain productivity and durability**.

Existing Applications of RNA-Based Technologies in Agriculture

Delayed ripening

Altered flower color

Enhanced pathogen resistance

Altered nutritional composition

Specialty crop plant oils

⟵ Altered seed coat color

Buff seed coat in soybean

Low glutelin rice

Control of Coleopteran Pests Through RNA interference

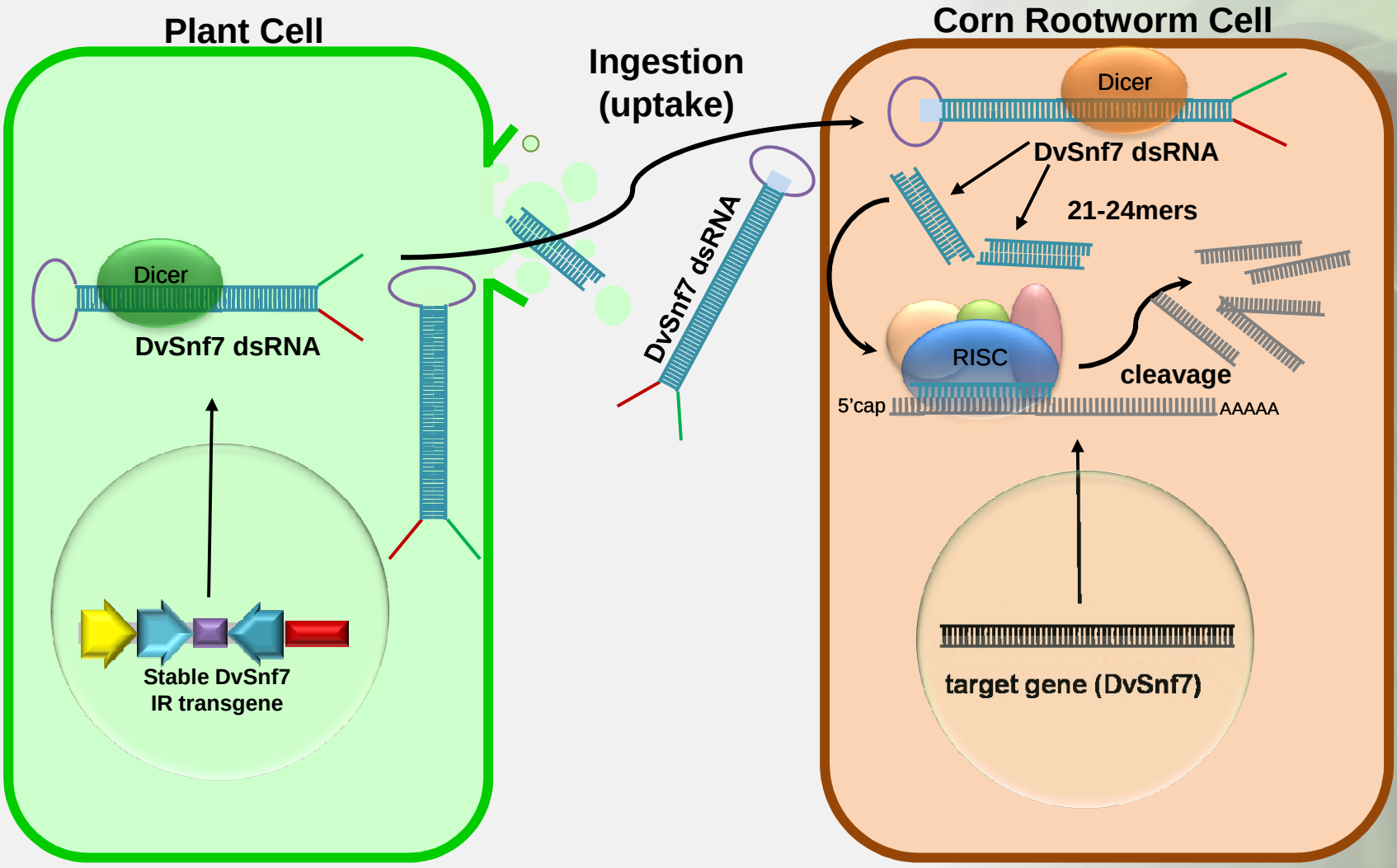
James A Baum, Thierry Bogaert, William Clinton, Gregory R Heck, Pascale Feldmann, Oliver Ilagan, Scott Johnson, Geert Plaetinck, Tichafa Munyikwa, Michael Pleau, Ty Vaughn & James Roberts

November 2007, Volume 25 No 11 pp1187-1328

Western Corn Rootworm
Coleoptera: Chrysomelidae:
Diabrotica virgifera virgifera

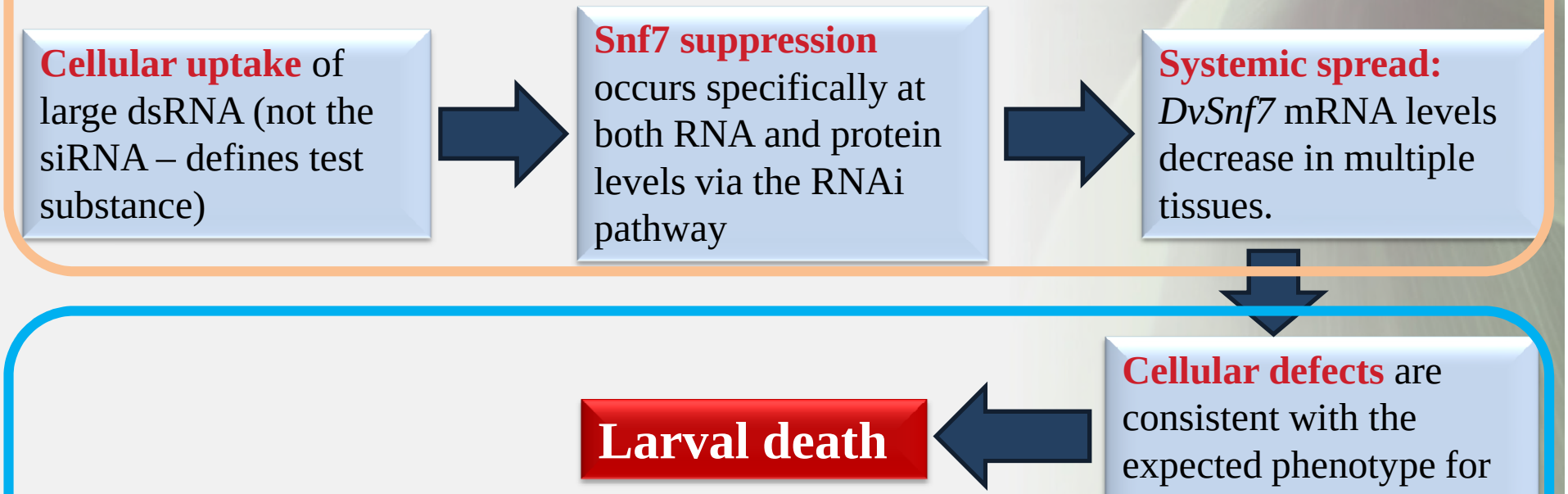
Non-transgenic corn Transgenic corn

dsRNA is Orally Delivered to Suppress Gene Expression in Corn Rootworm



Key DvSnf7 Mode of Action Publications

Bolognesi R, et al. (2012) Characterizing the Mechanism of Action of Double-Stranded RNA Activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). PLoS ONE 7(10): e47534.



Ramaseshadri P, et al. (2013) Physiological and Cellular Responses Caused by RNAi- Mediated Suppression of Snf7 Orthologue in Western Corn Rootworm (*Diabrotica virgifera virgifera*) Larvae. PLoS ONE 8(1): e54270.

Robust Comparative Safety Assessment Approach for Biotech Crops…

- **Gene(s)**
  - Source(s)
  - Molecular characterization
  - Insert/copy number/gene integrity
- **Protein(s)**
  - History of safe use and consumption
  - Function/specificity/mode-of-action
  - Expression Characterization
  - Toxicology/Allergenicity
    - In silico and In vivo assessments
- **Food/Feed Safety**
  - Proximate analysis
  - Key nutrients and anti nutrients
  - Animal performance assessment
- **Environmental Studies**
  - Safety to non-target organisms
  - Soil degradation
  - Out-crossing
  - Susceptibility to disease

Existing Approach is Appropriate for Crops Utilizing RNA-Mediated Gene Suppression…

- **Gene(s)**
  - Source(s)
  - Molecular characterization
  - Insert/copy number/gene integrity
- **dsRNA**
  - History of safe use and consumption
  - Function/specificity/mode-of-action
  - Expression Characterization
  - Ingested RNA is not toxic in higher organisms, nor allergenic

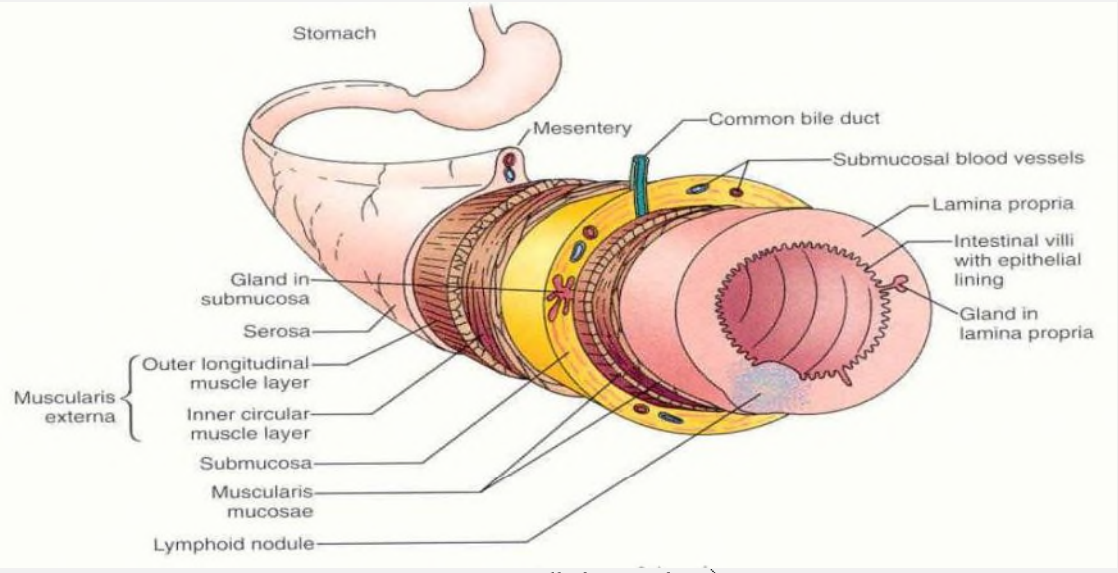
- **Food/Feed Safety**
  - Proximate analysis
  - Key nutrients and anti nutrients
  - Animal performance assessment
- **Environmental Studies**
  - Safety to non-target organisms
  - Soil degradation
  - Out-crossing
  - Susceptibility to disease

RNA-based Products Approved by Regulatory Agencies

A number of products utilizing RNA-based technology have been reviewed received approval by international regulatory agencies.

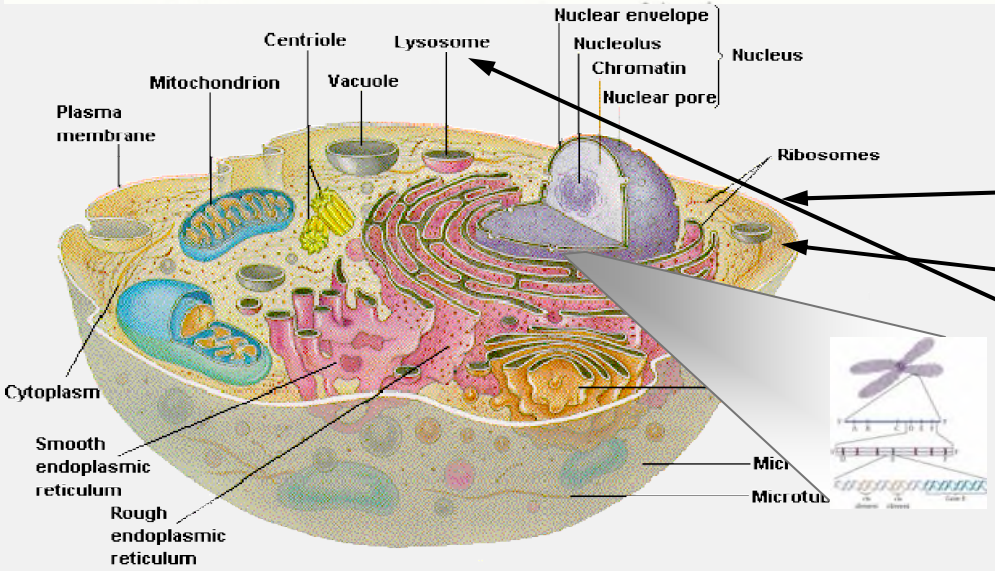
Compositionally modified soybean	Flavr Savr™ Tomato	Amylopectin potato	Virus resistant:
• Dupont (U.S., CAN, MEX, AUS/NZ, KOR, CHN, TPE)-1997/2011 • Monsanto (U.S., CAN, MEX, AUS/NZ, JPN, KOR, COL)-2011/2012	• Calgene (U.S., CAN, MEX)-1994/1995	• BASF (EU)-2010	• Papaya (CAN, U.S.)-1996 • Squash (CAN, U.S.)-1994 • Potato (U.S., CAN, MEX, AUS/NZ, JPN, KOR, PHI)-1998 • Beans (Brazil)-2011 • 'HoneySweet' plum trees resistant to Plum Pox Virus (U.S. EPA)-2007

Biological Barriers Protect Against Ingested RNA



Systemic Barriers

- Salivary nucleases
- Stomach acid
- Pancreatic nucleases
- Intestinal epithelium
- Vascular endothelium
- Blood/systemic nucleases



Cellular Barriers

- Plasma membrane barrier
- Endosomal sequestration
- Lysosomal degradation

Safety of Exogenous dsRNA in Higher Organisms

dsRNA has been consumed safely over millennia

- Small RNAs and long dsRNAs with identity to human (and animal) transcripts are safely consumed in staple crops[1,2]
- RNAi is not new to agriculture: underlies domesticated crop phenotypes and traits in approved biotech crops

After decades of research, oral RNA/DNA therapeutics remain an elusive goal

- Very low oral bioavailability for oligo therapeutics (<1%)[3]
- Direct injection, formulation, and stabilizing modifications needed for systemic activity[3]
- siRNA drugs are extensively metabolized; half-life of ~5 mins; cleared by kidney within mins of i.v. dose[4,5].
- RNA drugs have been safely administered at doses of up to 200 mg/kg i.v. in rats[5]

Dietary RNAs: Not active and negligible uptake

- Zhang et al (2012): novel report of activity and significant uptake of ingested miRNAs in mammals[6]
- Dickinson et al (2013): Zhang's findings resulted from nutritional differences, not ingested miRNAs[7]
- Witwer et al (2013), Snow et al (2013) and Dickinson et al (2013) together show negligible small RNA uptake from the diet in rodents, bees, primates, and humans[7,8,9]

RNA-based Technology Has Potential for High Taxonomic Specificity

The sequence specificity of RNAi allows:

- Targeted suppression of essential gene(s) in pests
- Development of highly efficacious and highly selective products with a low likelihood to adversely impact NTOs

Baum et al. (2007) showed

- Using the V-ATPase A target with WCR and CPB, that single species specificity is not achieved when multiple 21 nt sequence lengths are common among the species target sequences

Whyard et al. (2009) showed

- Using the tubulin target and four Drosophila species, that single species specificity of transfected dsRNA can be achieved when targeting the 3' UTR where no 19 to 21 nt sequence length was shared among the species

Baum et al., (2007) Nature Biotechnology 25:1322-1326
Whyard et al., (2009) Insect Biochemistry and Molecular Biology 39:824-832

Taxonomic Specificity Informs Non-target Species Selection

Work done at Monsanto confirms the conclusion of Whyard et al. (2009) that insecticidal dsRNAs can achieve high taxonomic specificity

We used a hypothesis-based taxonomic approach to establish the relationship between biological activity and taxonomic relatedness with a CRW active RNA in diet feeding assays (Bachman et al. 2013)

Results from testing 18 species from 10 families and 4 orders in sub-chronic or chronic bioassays shows that activity with a CRW active RNA is only evident at the subfamily level (next slide)

Characterization of the spectrum of activity informs the assessment plan by aiding non-target species selection and can narrow the scope of hazard testing

Bachman et al., (2013) Transgenic Research 22:1207-22

A CRW Active dsRNA is Highly Specific

Order	Common Name	Species	Activity	Duration	Endpoint
Coleoptera	Western Corn Rootworm	*Diabrotica virgifera virgifera*	✚	12 d	S, G
	Southern Corn Rootworm	*Diabrotica undecimpunctata howardi*	✚	12 d	S, G
	Colorado Potato Beetle	*Leptinotarsa decemlineata*	▬	12 d	S, G
	Red Flour Beetle	*Tribolium castaneum*	▬	12 d	S, G
	Pink-spotted Lady Beetle	*Coleomegilla maculata*	▬	21 d	S,G, E
	Mexican Bean Beetle	*Epilachna varivestis*	▬	28 d	S,G,E
	Ground Beetle	*Poecilus chalcites*	▬	35 d	S,G,E
Lepidoptera	European Corn Borer	*Ostrinia nubilalis*	▬	12 d	S,G
	Fall armyworm	*Spodoptera frugiperda*	▬	8 d	S,G
	Corn earworm	*Helicoverpa zea*	▬	12 d	S,G
	Silkworm	*Bombyx mori*	▬	14 d	S,G
Hymenoptera	Jewel Wasp	*Nasonia vitripennis*	▬	20 d	S
	Eulophid Wasp	*Pediobius foveolatus*	▬	21 d	S
	Honey Bee adult	*Apis mellifera*	▬	14 d	S, B
	Honey Bee larvae	*Apis mellifera*	▬	17 d	S, G, E
Hemiptera	Insidious Flower Bug	*Orius insidiosus*	▬	20 d	S, G
Collembola	Springtail	*Folsomia candida*	▬	28 d	S, R
Haplotaxida	Earthworm	*Eisenia andrei*	▬	14 d	S, G

S= Survival; G= Growth; E = Emergence; B= Behavior; R = Reproduction

Factors to Consider when Assessing Risk to NTOs from dsRNA during Problem Formulation

Exposure

- Barriers to exposure such as endonucleases in saliva (Lygus), midgut fluids, and the hemolymph
- Magnitude, duration and temporal nature of exposure after application
- For Plant Incorporated Protectants, tissue-specific expression that eliminates routes of exposure

Relative Sensitivity

- Not all taxa demonstrate quantifiable responses to environmental exposure to dsRNA and there is a large range of sensitivities across taxa

Sequence Match

- Sequence information can ONLY indicate whether an insect is potentially sensitive but could be used to exclude organisms from testing
- Bioinformatics can be used to address specific questions that reduce uncertainty in the hazard assessment, but its application is limited due to barriers, sensitivity and exposure; **therefore sequence cannot be used as a standalone to predict hazard to NTOs**
- Our research has demonstrated that ≥21 nt contiguous sequence embedded in a ≥60 nt dsRNA is required for oral activity in a highly sensitive insect species (CRW)

Summary

- RNA-based technologies offer **new modes of action** in managing hard to control pests and viruses
- RNA-based suppression is based on a naturally occurring process, and has been used as a longstanding approach to achieve desired phenotypes
- RNA has a history of safe consumption
- RNA offers specific gene suppression that greatly reduces potential impact on non-target organisms
- The current safety assessment strategy for RNA-based products is based on the same safety assessment paradigm as other biotechnology-derived crops that have been reviewed and approved globally

References

1. Ivashuta, et al., (2009) Food Chem. Tox. 47, 353-360.
2. Jensen, et al., (2013) GM Crops and Food, 4, 1-8.
3. Reviewed by Petrick et al., (2013) Reg. Tox. Pharm., 66, 167-176.
4. Christensen, et al.,(2013) Drug Metab. Distr. 412, 1211-1219.
5. Thompson et al., (2012) Nucleic Acid Ther. 22, 255-264.
6. Zhang, et al., (2012) Cell Research, 22: 107-126.
7. Dickinson, et al., (2013) Nature Biotech 31, 965-967.
8. Witwer, et al., (2013) RNA Biology 10: 7, 1-7.
9. Snow, et al., (2013) RNA Biology 10: 6, 1-10.