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Regulation and biosafety assessment of RNAi based pesticidal approaches

Antje Dietz-Pfeilstetter

GfPb-Symposium, Neustadt 3-4 September 2018

Routes for exposing pests/pathogens to dsRNA as trigger for gene silencing

Introduction of a **transgene** construct containing an inverted repeat (IR) / hairpin (hp) of an essential pest/pathogen gene as a plant incorporated protectant (PIP)

→ **Host-induced gene silencing (HIGS)**

Direct **pesticidal application** of dsRNA targeting essential pest/pathogen genes, e.g. by spraying on plant leaves

→ **Spray-induced gene silencing (SIGS)**

RNAi based transgenic plants with resistance to pests or pathogens

Type	GMO	Target gene	Phenotype	Regulatory status
Virus resistance	Plum C-5 „Honeysweet“ (USDA/ARS)	PPV-*cp*	Resistance to PPV (plum pox virus)	**USA**: Food/feed (2009), cultivation (2007) **Europe**: Field tests
	Papaya 55-1 „SunUp“ (Cornell University/ University of Hawaii)	PRSV-*cp*	Resistance to PRSV (papaya ring spot v.)	**USA**: Food/feed (1997), cultivation (1996) **Canada**: Food (2003)
Insect resistance	MON 87411 maize (Monsanto Co.)	*Diabrotica virgifera DvSnf7*	Resistance to corn rootworm	**USA**: Food/feed (2014), cultivation (2015); commercial planting 2017 **Canada**: Food/feed and cultivation (2015) **Europe**: pending

Genetic elements introduced into 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

Biosafety assessment of MON 87411 - US approach

USDA-APHIS: Biotechnology Regulations 7CFR part 340

→ **Determination of non-regulated status**

Basis: Plant pest risk assessment for MON 87411

→ No plant pest risk identified from transformation and/or expression of new genetic material

→ Disease and pest incidence and/or damage not increased

→ Unlikely to adversely impact beneficial non-target organisms (based on bioassays with dsRNA)

→ Not likely to have impact on weediness or invasiveness

→ No significant changes to agricultural/cultivation practices expected

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)

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 stability 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 (considering most likely **hazard exposure** scenarios)
  - Maximum expected environmental concentrations (MEECs)
  - No observed effect concentrations (NOECs)
  - Estimated margins of exposure (MOEs)

EFSA guidance for risk assessment of food and feed from GM plants according to Regulation (EC) 1829/2003

Hazard identification and characterisation

Information requested/provided

- Molecular characterisation
- Information on expression of insert
- *in planta* RNAi off-target screen
- Agronomic and phenotypic data
- Comparative compositional analysis
- Nutritional assessment
- Toxicology (90-days feeding study in rodents with whole GM feed; 28-days toxicity study with dsRNA inconclusive; not considered necessary by GMO panel)

Assessment of possible hazards/adverse effects of dsRNA

? - Safety relevant compositional changes in GM plant caused by non-specific silencing in plant (driven by specific risk hypothesis?) → **No**

? - Adverse non-target effects in humans/animals?

- Other types of adverse unintended effects in humans/ animals (non-specific dsRNA responses like immune stimulation, saturation of RNAi machinery → **No** (dietary non-coding RNA ubiquitous, rapid degradation in gastrointestinal tract, limited cellular uptake)

Exposure assessment – anticipated intake or extent of use

Expression level of dsRNA in GM plants and daily consumption → Estimates for exposure from food/feed

Environmental risk assessment (ERA) for MON 87411 in accordance with Dir 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

→ **Risk** characterization

Negligible exposure

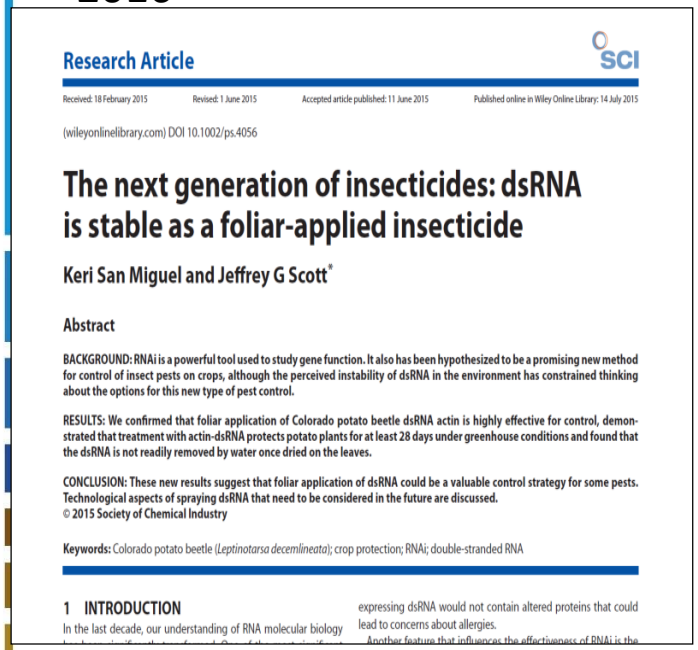
Specific areas of risk adressed which are most relevant for RNAi maize **in case of cultivation**:

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

Examples of recent developments in the field of spray-induced gene silencing (SIGS)

I. RNAi based exogenous pesticides

2016



Research Article

SCI

Received: 18 February 2015 Revised: 1 June 2015 Accepted article published: 11 June 2015 Published online in Wiley Online Library: 14 July 2015

(wileyonlinelibrary.com) DOI 10.1002/ps.4056

The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide

Keri San Miguel and Jeffrey G Scott*

Abstract

BACKGROUND: RNAi is a powerful tool used to study gene function. It also has been hypothesized to be a promising new method for control of insect pests on crops, although the perceived instability of dsRNA in the environment has constrained thinking about the options for this new type of pest control.

RESULTS: We confirmed that foliar application of Colorado potato beetle dsRNA actin is highly effective for control, demonstrated that treatment with actin-dsRNA protects potato plants for at least 28 days under greenhouse conditions and found that the dsRNA is not readily removed by water once dried on the leaves.

CONCLUSION: These new results suggest that foliar application of dsRNA could be a valuable control strategy for some pests. Technological aspects of spraying dsRNA that need to be considered in the future are discussed.

Keywords: Colorado potato beetle (*Leptinotarsa decemlineata*); crop protection; RNAi; double-stranded RNA

1 INTRODUCTION

In the last decade, our understanding of RNA molecular biology

expressing dsRNA would not contain altered proteins that could lead to concerns about allergies.

2017

PLOS | PATHOGENS

RESEARCH ARTICLE

An RNAi-Based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery

Aline Koch[1], Dagmar Biedenkopf[1], Alexandra Furch[2], Lennart Weber[3], Oliver Rossbach[4], Eltayb Abdellatef[1], Lukas Linicus[1], Jan Johannsmeier[1], Lukas Jelonek[5], Alexander Goesmann[5], Vinitha Cardoza[6], John McMillan[6], Tobias Mentzel[7], Karl-Heinz Kogel[1]*

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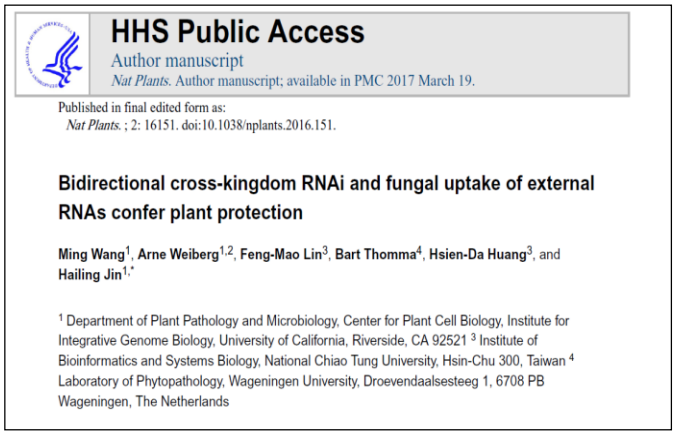
Citation: Koch A, Biedenkopf D, Furch A, Weber L, Rossbach O, Abdellatef E, et al. (2016) An RNAi-Based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs Involves a Plant Passage and Is Controlled by the Fungal

Abstract

2016



HHS Public Access

Author manuscript

Nat Plants. Author manuscript; available in PMC 2017 March 19.

Published in final edited form as:

Nat Plants. ; 2: 16151. doi:10.1038/nplants.2016.151.

Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection

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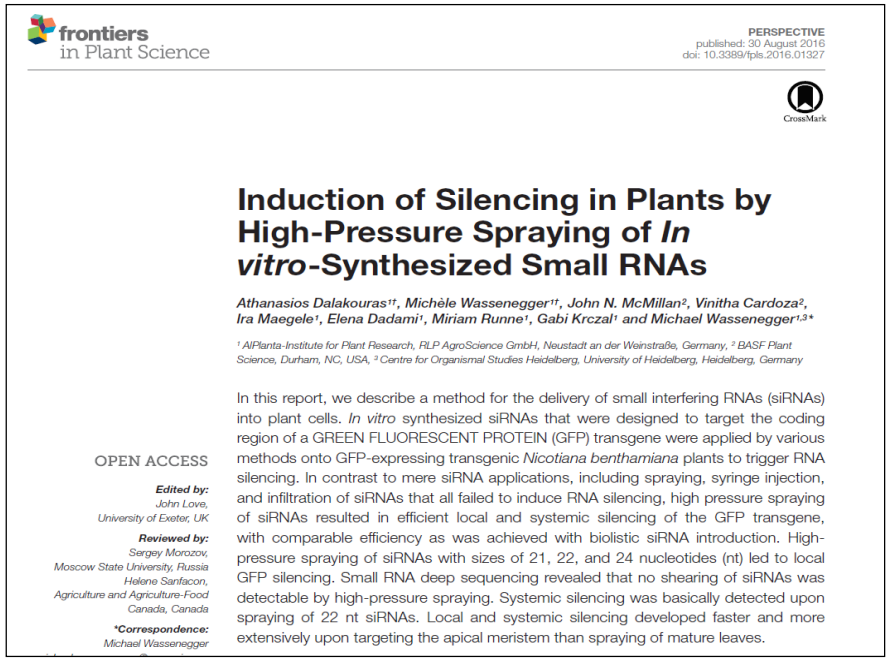
Examples of recent developments in the field of spray-induced gene silencing (SIGS)

II. Techniques for dsRNA synthesis and application

2016



frontiers in Plant Science

PERSPECTIVE
published: 30 August 2016
doi: 10.3389/fpls.2016.01327

Induction of Silencing in Plants by High-Pressure Spraying of *In vitro*-Synthesized Small RNAs

*Athanasios Dalakouras[1†], Michèle Wassenegger[1†], John N. McMillan[2], Vinitha Cardoza[2], Ira Maegele[1], Elena Dadami[1], Miriam Runne[1], Gabi Krczal[1] and Michael Wassenegger[1,3]**

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In this report, we describe a method for the delivery of small interfering RNAs (siRNAs) into plant cells. *In vitro* synthesized siRNAs that were designed to target the coding region of a GREEN FLUORESCENT PROTEIN (GFP) transgene were applied by various methods onto GFP-expressing transgenic *Nicotiana benthamiana* plants to trigger RNA silencing. In contrast to mere siRNA applications, including spraying, syringe injection, and infiltration of siRNAs that all failed to induce RNA silencing, high pressure spraying of siRNAs resulted in efficient local and systemic silencing of the GFP transgene, with comparable efficiency as was achieved with biolistic siRNA introduction. High-pressure spraying of siRNAs with sizes of 21, 22, and 24 nucleotides (nt) led to local GFP silencing. Small RNA deep sequencing revealed that no shearing of siRNAs was detectable by high-pressure spraying. Systemic silencing was basically detected upon spraying of 22 nt siRNAs. Local and systemic silencing developed faster and more extensively upon targeting the apical meristem than spraying of mature leaves.

OPEN ACCESS

Edited by:
John Love, University of Exeter, UK

Reviewed by:
Sergey Morozov, Moscow State University, Russia
Helene Sanfacon, Agriculture and Agriculture-Food Canada, Canada

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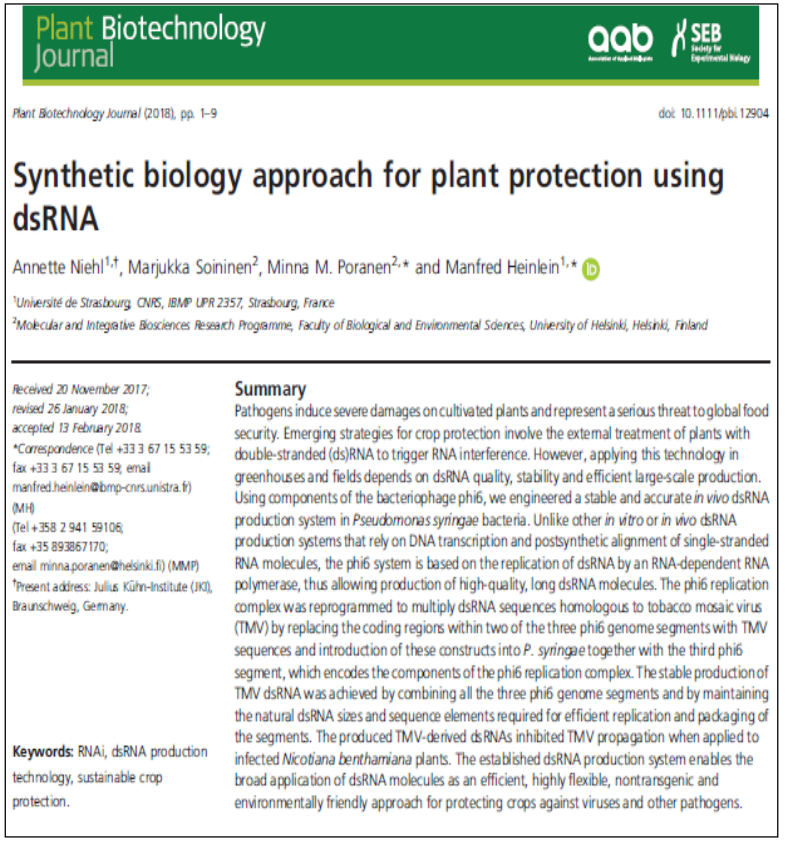
2017

Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses
Neena Mitter et al., Nature Plants (2017)

2018



Plant Biotechnology Journal

Plant Biotechnology Journal (2018), pp. 1–9 doi: 10.1111/pbi.12904

Synthetic biology approach for plant protection using dsRNA

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Received 20 November 2017;
revised 26 January 2018;
accepted 13 February 2018.

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Summary
Pathogens induce severe damages on cultivated plants and represent a serious threat to global food security. Emerging strategies for crop protection involve the external treatment of plants with double-stranded (ds)RNA to trigger RNA interference. However, applying this technology in greenhouses and fields depends on dsRNA quality, stability and efficient large-scale production. Using components of the bacteriophage phi6, we engineered a stable and accurate *in vivo* dsRNA production system in *Pseudomonas syringae* bacteria. Unlike other *in vitro* or *in vivo* dsRNA production systems that rely on DNA transcription and postsynthetic alignment of single-stranded RNA molecules, the phi6 system is based on the replication of dsRNA by an RNA-dependent RNA polymerase, thus allowing production of high-quality, long dsRNA molecules. The phi6 replication complex was reprogrammed to multiply dsRNA sequences homologous to tobacco mosaic virus (TMV) by replacing the coding regions within two of the three phi6 genome segments with TMV sequences and introduction of these constructs into *P. syringae* together with the third phi6 segment, which encodes the components of the phi6 replication complex. The stable production of TMV dsRNA was achieved by combining all the three phi6 genome segments and by maintaining the natural dsRNA sizes and sequence elements required for efficient replication and packaging of the segments. The produced TMV-derived dsRNAs inhibited TMV propagation when applied to infected *Nicotiana benthamiana* plants. The established dsRNA production system enables the broad application of dsRNA molecules as an efficient, highly flexible, nontransgenic and environmentally friendly approach for protecting crops against viruses and other pathogens.

Keywords: RNAi, dsRNA production technology, sustainable crop protection.

- RNAi-based pesticides have new mode of action
→ Inadequacies of current testing frameworks

- In most countries (e.g. EU) no regulation in place

↓

Initiative of the OECD Working Group on Pesticides (WGP)

→ In **May/July 2015**: Establishment and Call for nominations for the *Ad hoc* Expert Group on Novel Technologies and Their Use as Pesticides

Initial focus on RNA interference (RNAi) based pesticides

→ **Members** of the expert group (nominated by OECD governments, invited experts):

1. experienced in pesticide risk assessment

2. experts on RNAi technology

Objectives of the OECD Expert Group on RNAi-based pesticides

Provide a **forum for countries and developers of technology** in order to:

- Consider the **applicability of current approaches to environmental and human health risk assessment** of conventional and biological pesticides
- Investigate if additional **data requirements** or new types of data requirements need to be developed
- Work towards **harmonization** of data requirements, of methodologies for hazard and risk assessment and of methods for collecting relevant information
- Establish **linkages**, share information and ideas about appropriate regulation of RNAi-based pesticides and identify areas of common interest **with other groups**
- Promote **communication** between regulatory authorities and with the general public on RNAi-based pesticides

Survey among participants to define priority issues for the Expert Group

- 1. Off-target effects (i.e. effects on non-target organisms) from exposure to RNAi-based pesticides
- 2a. Environmental fate of RNAi-based pesticide
- 2b. Definition of the active ingredient/active constituent (AI/AC) for regulatory purposes
- 2c. Human routes of exposure and ADME (absorption, distribution, metabolism, excretion)

↓

Actions:

- Development of a working paper on off-target effects and environmental fate of RNAi-based pesticides
- Work on the definition of AI/AC

Definition of AI/AC

Exogenous dsRNA for pesticidal use: Externally applied dsRNA that triggers RNA interference in a target organism

- General and broad definition
- Does not include the manufacturing process
- Does not point to possible modifications

OECD working paper
RNAi-based Pesticides: Assessment of Environmental fate and the risks of off-target (non-target) effects

Objective(s):

- Provide scientific information related to RNAi
  - → RNA interference mechanisms
  - → Current and future applications of RNAi-based pesticides
- Address regulatory and risk assessment issues

Current state (August 2018) of the working paper: 2nd draft commented by experts

Seminar proposal:

Regulation of Externally Applied dsRNA-based Products for Management of Pests

→ **Application** to receive TAD (OECD´s trade and agriculture directorate) funding for a conference within the Co-Operative Research Programme: Biological Resource Management for Sustainable Agricultural Systems

- Conference programme and proposed speakers submitted in the application
- Information about recent workshops/conferences on similar topics included
- **Conference** envisaged for **10-12 April, 2019**

Thank you