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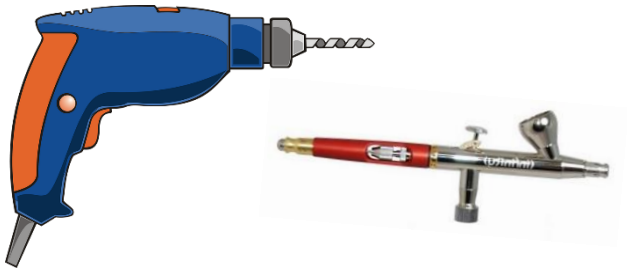
Gesellschaft für Pflanzenbiotechnologie e.V.

Modern Agriculture without chemical Pesticides?

Neustadt a.d. Weinstr., September 03-04, 2018

RNA interference and Delivery of exogenous RNA molecules

Michael Wassenegger



RLP AgroScience GmbH

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- Introduction of RNA interference (RNAi) in plants

- Patents on RNA delivery into plant cells

- Own work on delivery of RNA molecules into plant cells

- Benefits and problems of RNAi exploitation

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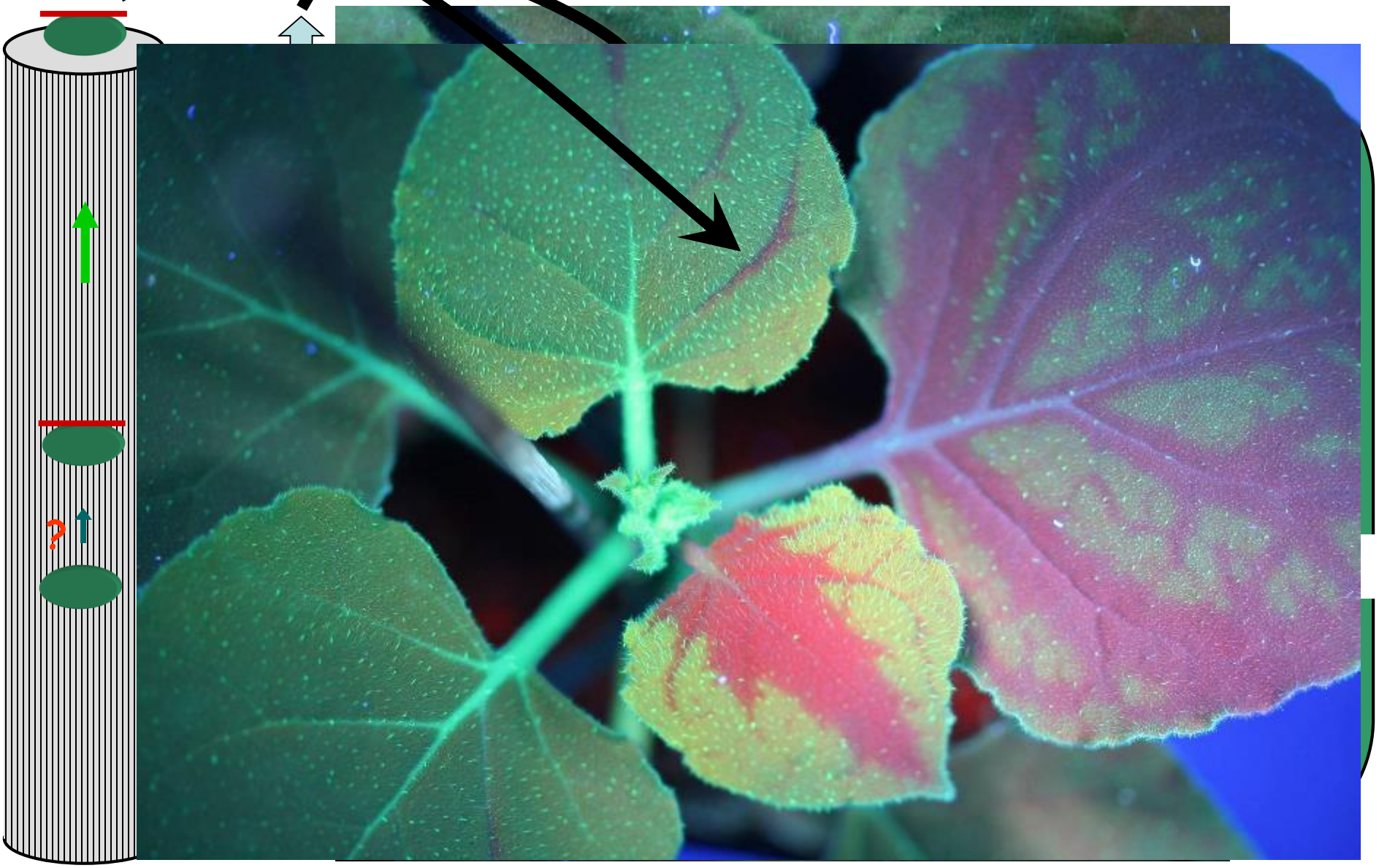
Current model of RNAi in plants



Patents on RNA delivery into plant cells

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

61)Such agents for conditioning of a plant to permeation by polynucleotides are applied to the plant by any convenient method, *e.g.*, spraying or coating with a powder, emulsion, suspension, or solution; similarly, the polynucleotide molecules are applied to the plant by any convenient method, *e. g.*, spraying or wiping a solution, emulsion, or suspension.

(19) **United States**
(12) **Patent Application Publication**
Tang

(10) **Pub. No.: US 2013/0047298 A1**
(43) **Pub. Date: Feb. 21, 2013**

(54) **METHODS AND COMPOSITIONS FOR INTRODUCTION OF EXOGENOUS DSRNA INTO PLANT CELLS**

(76) Inventor: **Guo-Qing Tang**, Durham, NC (US)

(21) Appl. No.: **13/585,947**

(22) Filed: **Aug. 15, 2012**

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(60) Provisional application No. 61/523,877, filed on Aug. 16, 2011.

Publication Classification

(51) **Int. Cl.**
A01N 57/16 (2006.01)
A01P 21/00 (2006.01)

(52) **U.S. Cl.** **800/278**; 504/128

(57) **ABSTRACT**

This invention provides a method to silence an endogenous target gene expression in plants by applying a specific dsRNA onto the exterior surface of a plant. Application, such as by spraying or brushing a plant with dsRNA is done without wounding the plant tissue and cells such as by mechanical-type wounding, particle bombardment or mechanical infection with viral vectors. The present invention enables the regulation of gene expression in plants. In some embodiments of the invention, the dsRNA is directed to an essential gene of a plant pathogen or pest, whereby the pathogen and/or pest damage is controlled, resulting in desired agronomic performance.

[0012] This invention disclosure is a novel approach of plant hormone-mediated penetration of dsRNA into plant cells and the subsequent induction of plant endogenous gene silencing by application of the dsRNA to a surface of a plant structure, e.g. a leaf surface. Gene silencing was successful in a crop species (maize) rather than model plants (Arabidopsis etc). Thus, the present invention establishes that external application of dsRNA can be used to silence or otherwise modulate endogenous plant gene expression.

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Own work on delivery of RNA molecules into plant I

Dalakouras *et al*. (2016) Induction of silencing in plants by high-pressure spraying of *in vitro*-synthesized small RNAs.
Front Plant Sci 7, 1327.

In order to develop RNA-based herbicides, uptake of RNA molecules by the plant cell is required.

Spraying experiments according to the Monsanto patent using various synthetic siRNAs
(*Nicotiana benthamiana* 16C line stably expressing GFP)

Before spraying

Day light **UV-hand lamp**

10 days post spraying (dps)

Day light **UV-hand lamp**

No GFP silencing was detectable during the whole experimental period!

(Plants were monitored every 2 days for 3 weeks)!

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Summary of all RNA applications that failed

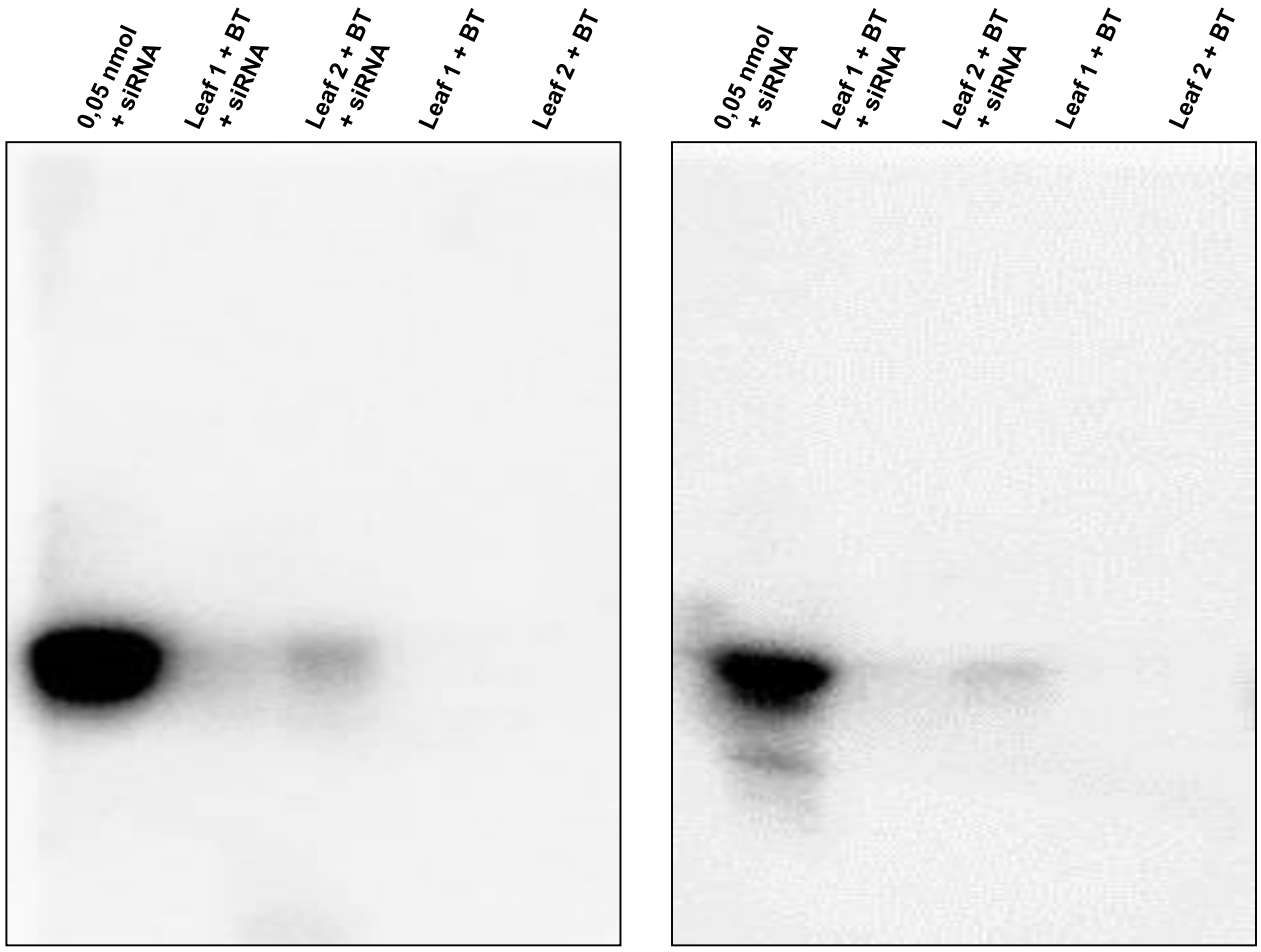
- Spraying experiments according to the Monsanto patent
- Spraying experiments according to the Syngenta patent
- RNA infiltration experiments, including vacuum infiltration
- Germination assay

Germination assay

3 seeds per well in MS medium
+ synthetic siRNAs

Analysis of siRNA stability 3 dps

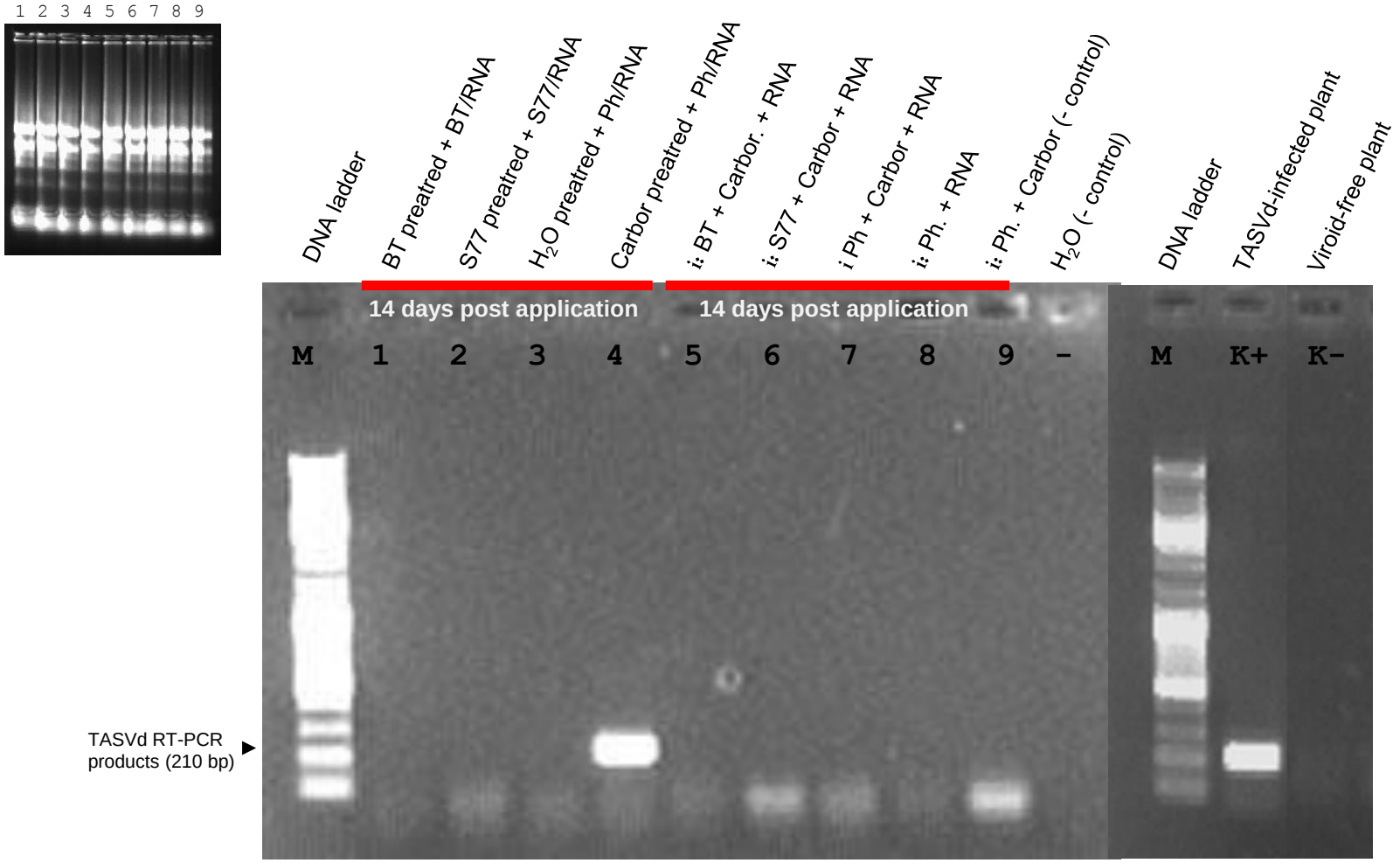
Synthetic siRNA #5 sprayed (low pressure) onto *N. benthamiana* wild type leaves



Sense probe **Anti-sense probe**

BT: Break-Thru

Tomato apical stunt viroid (TASVd) infection analysis of systemic leaves



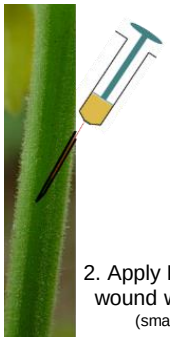
RT-PCR analysis on 1.2% agarose gel

RNA: total RNA from a TASVd-infected plant; **BT**: Break Thru; **S77**: Silwet 77; **Ph**: Phoshate buffer
i: infiltration; **Carbor**: Carborundum

RNA applications that did NOT fail

Application by injection

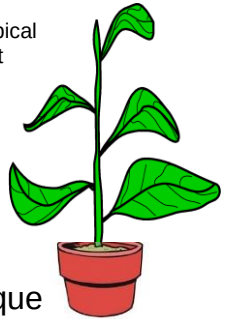
1. Wounding with a thick needle



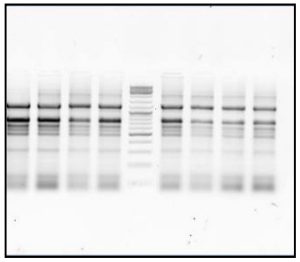
2. Apply RNA into the wound with syringe (small needle)

Cut and apply technique

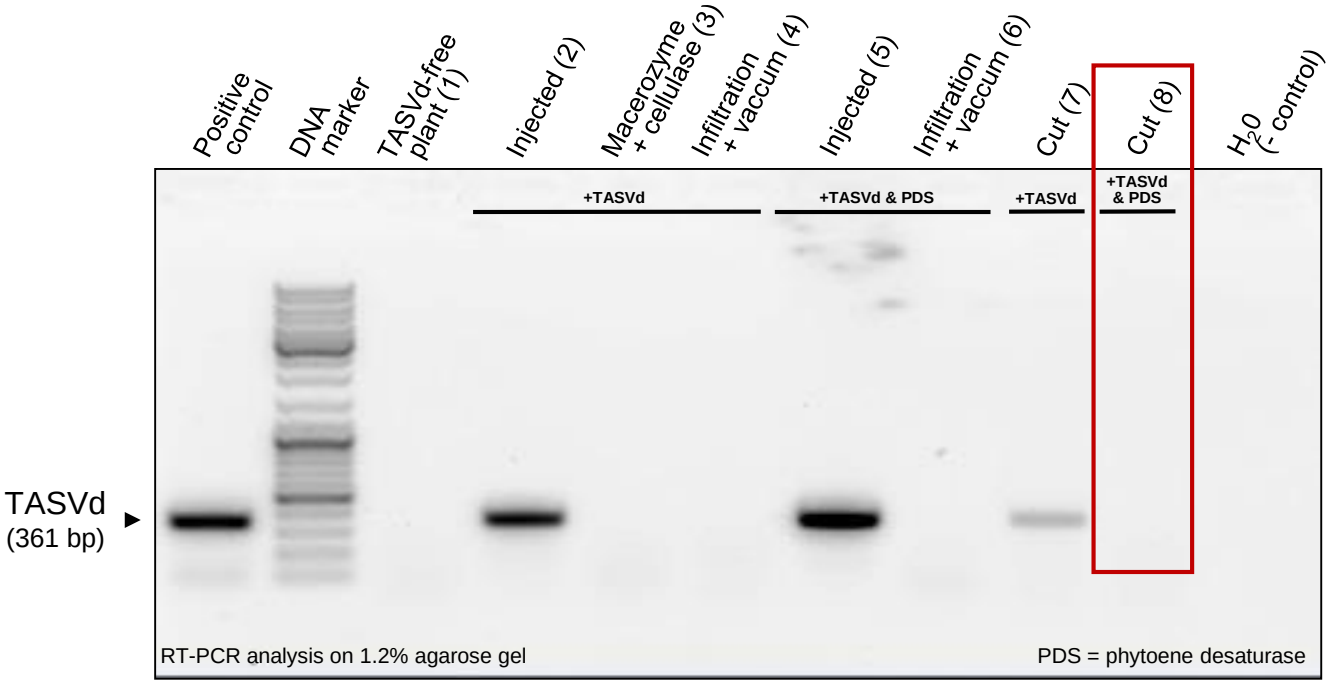
1. Cut apical shoot



2. Apply RNA with a pipette



RNA quality and quantity control (pooled tissue) of apical leaves.



Conclusion:

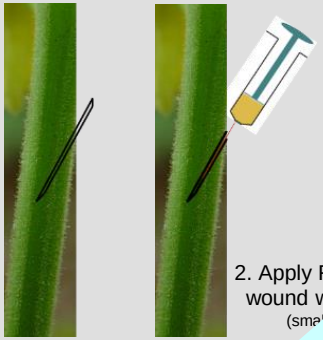
RNA uptake appeared to be dependent on cell wounding.

Note: PDS silencing was not detectable!

Summary of all RNA applications that did not fail

Application by injection

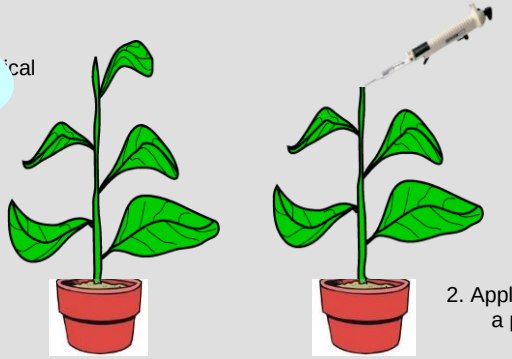
1. Wounding with a thick needle



2. Apply RNA i… wound with … (sma…

Cut and apply technique

…ical



2. Apply RNA with a pipette

Not suitable for field applications

(High costs and/or technical problems)

Biolistic intr…

High pressure spraying

Compressor > 3 bar

Airbrush > 3 bar

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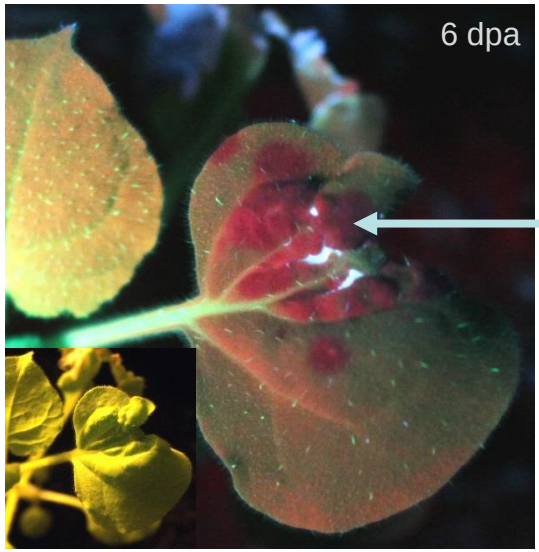
High pressure spraying of siRNA #15



Spraying excluding the leave bud

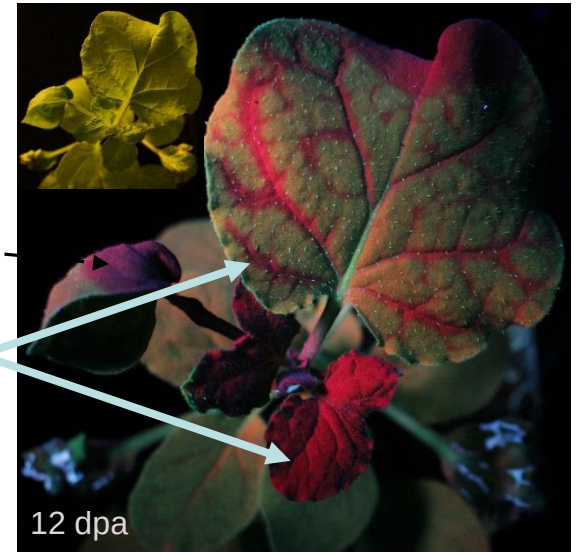
Local silencing

Systemic silencing

Spraying including the leave bud

Local silencing

Systemic silencing

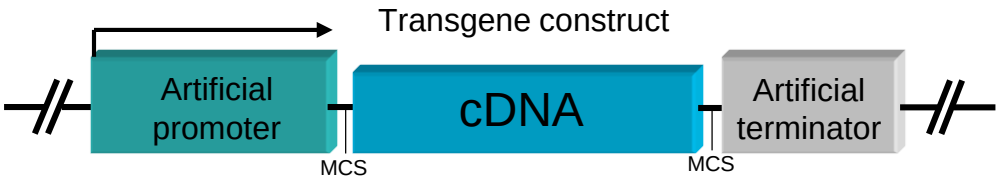


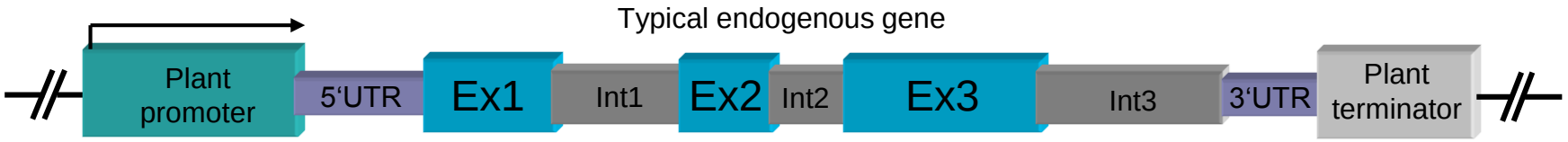
Intrinsic problems hampering RNA exploitation:

Endogenous *versus* exogenous nucleic acids

In general, endogenous genes are less prone to silencing than exogenous sequences (transgenes, viruses) are.

What is/are the difference/s between transgenes and 'typical' endogenes?





UTR = Untranslated region; Ex = exon; Int = intron

MCS = Multiple cloning site

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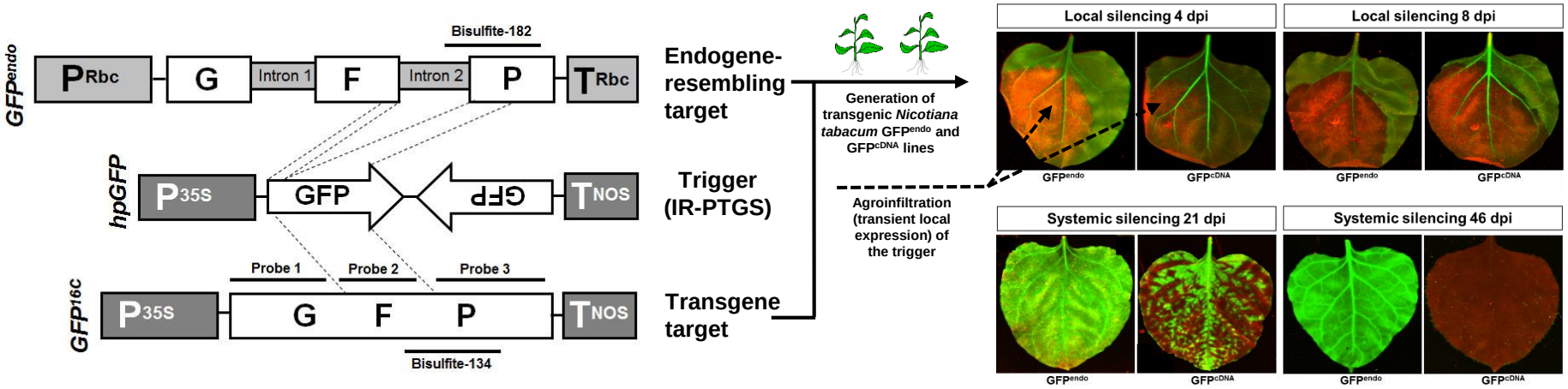
Local silencing of endogenous sequences is feasible but initiation of systemic silencing is problematic.

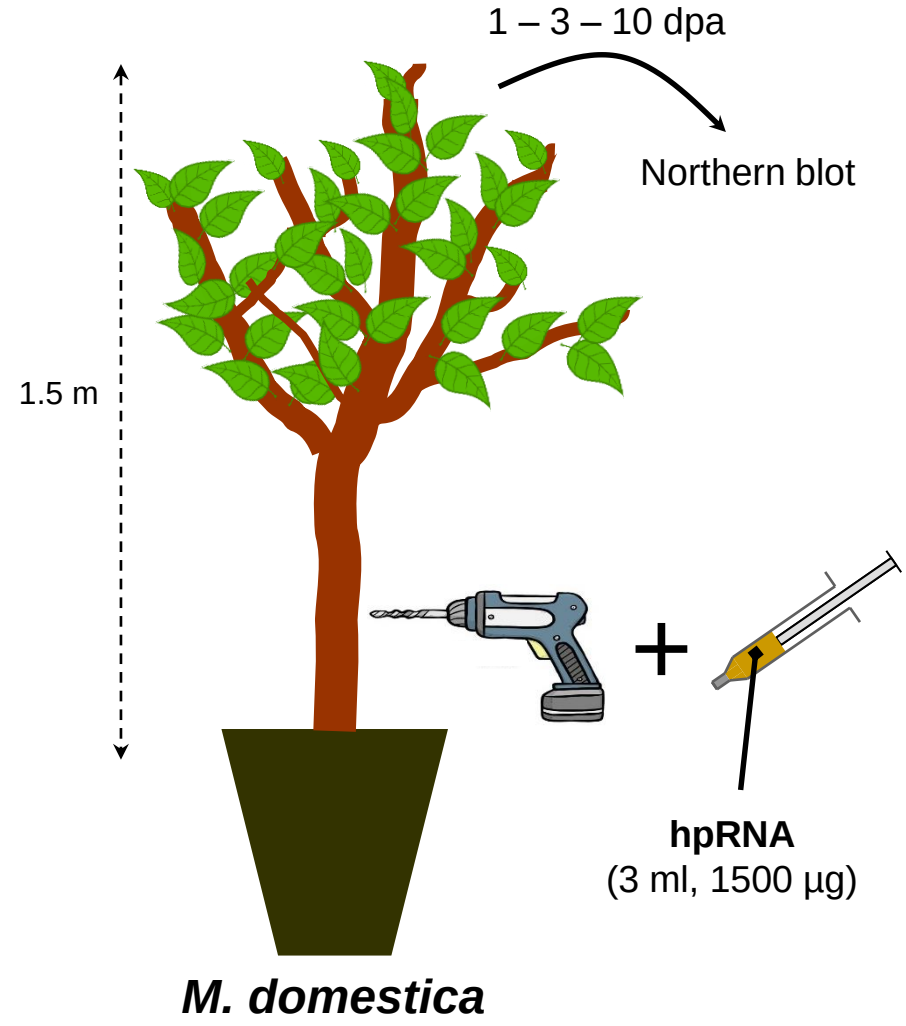


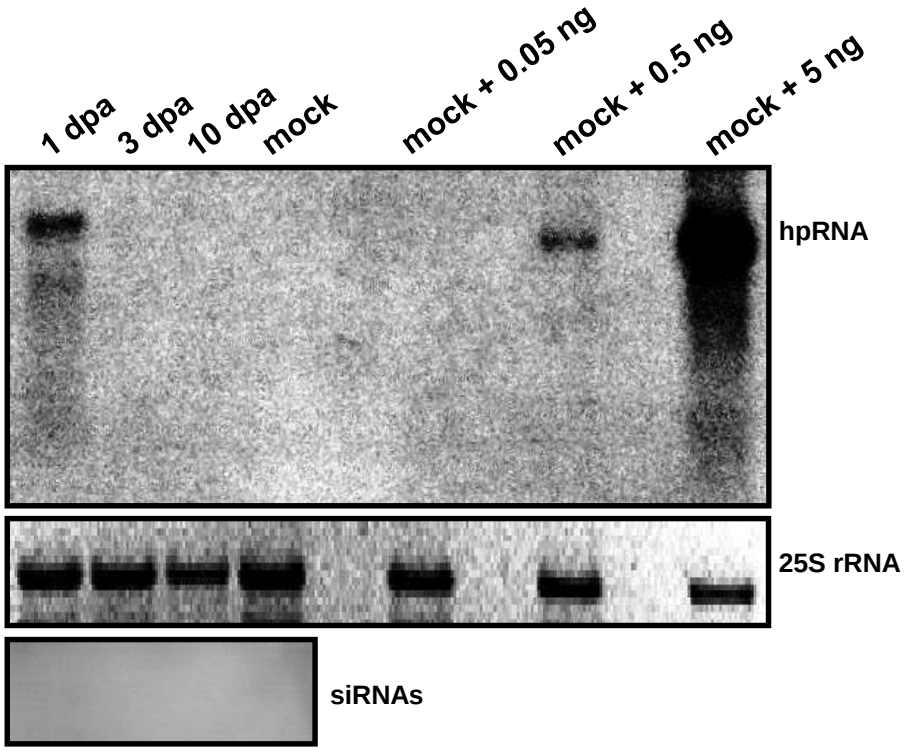
In contrast to disclosures in the Monsanto patent, we were not able to achieve systemic silencing of the PDS gene in *Nicotiana tabacum* and *N. benthamiana* (neither by spraying of siRNAs nor by agroinfiltration by IR-transgene constructs).

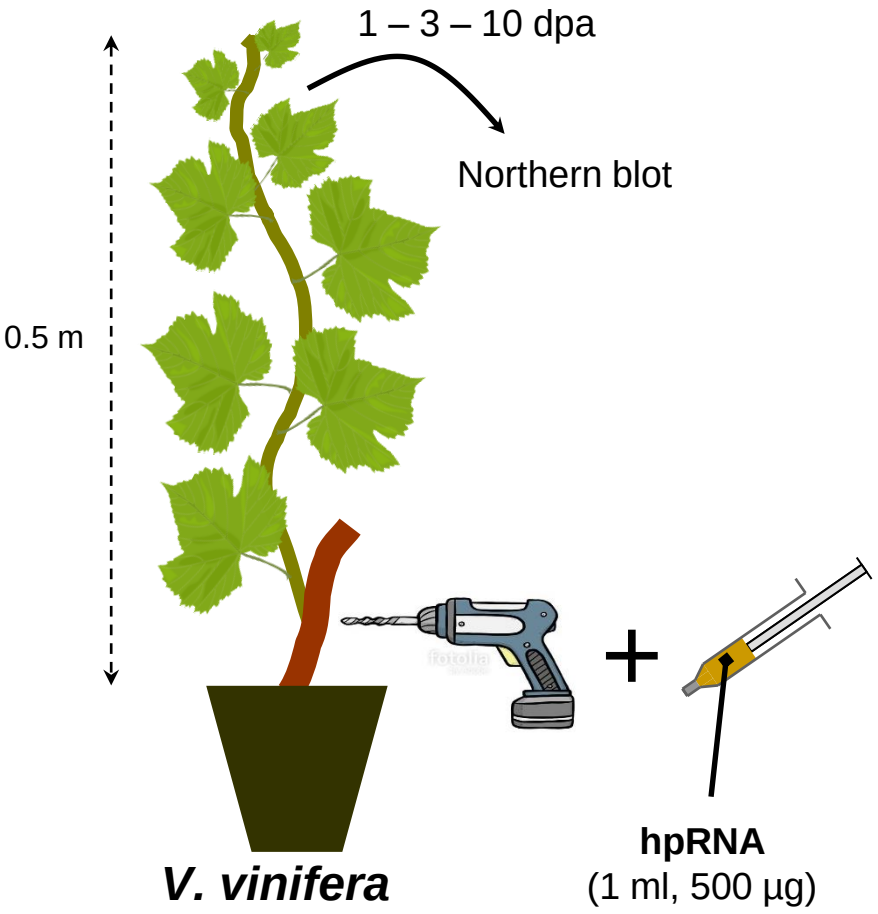
Dadami *et al.*, (2014). An endogene-resembling transgene is resistant to DNA methylation and systemic silencing. RNA Biol. 11: 934-941.
Dadami *et al.*, (2013). An endogene-resembling transgene delays the onset of silencing and limits siRNA accumulation. FEBS Letters 587: 706–710.

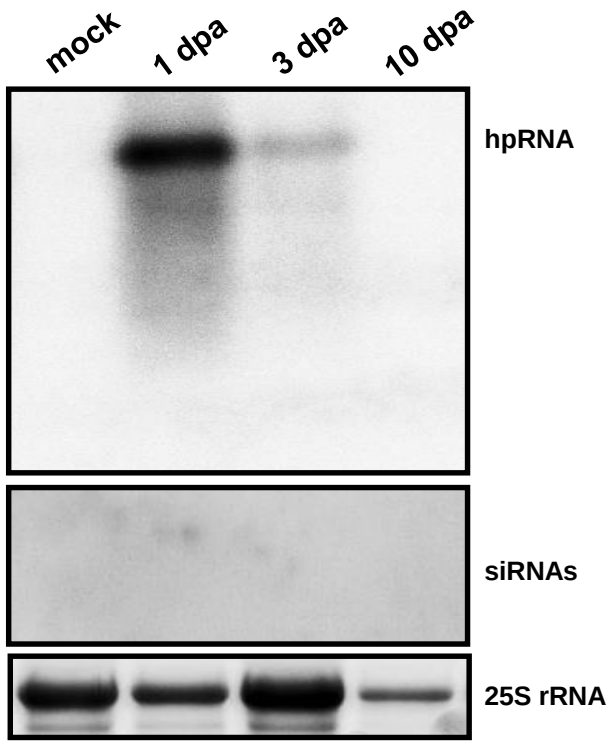
Own work on delivery of RNA molecules into plant II

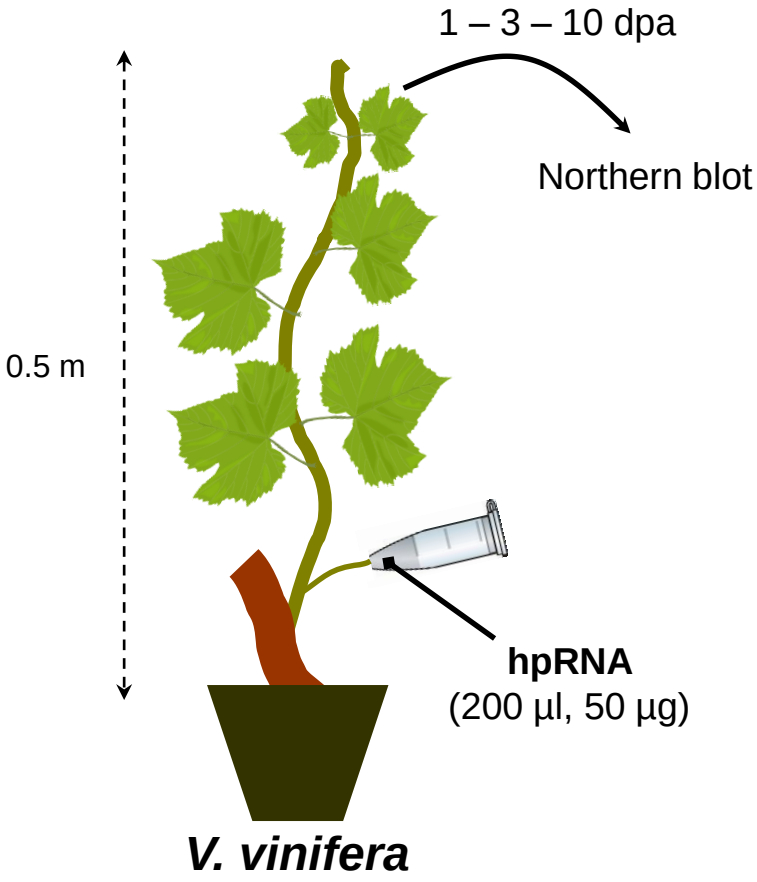
Dalakouras et al. (2018) Delivery of hairpin RNAs and small RNAs into woody and herbaceous plants by trunk injection and petiole absorption.
Front Plant Sci, 9,1253.

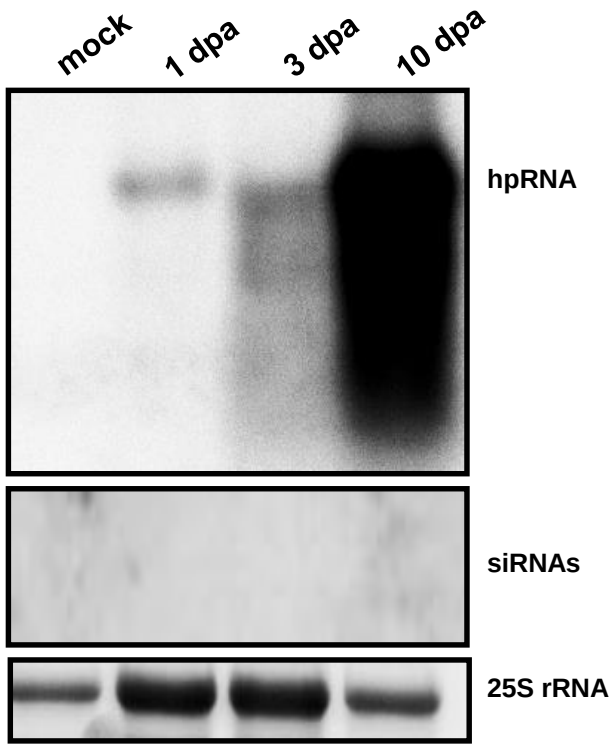


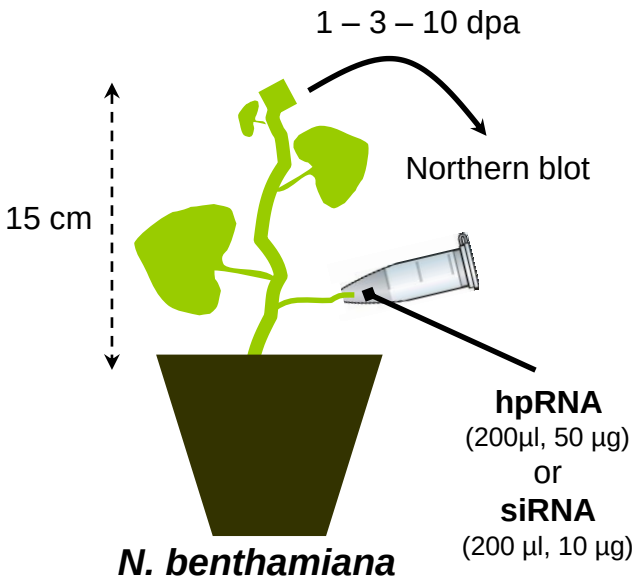


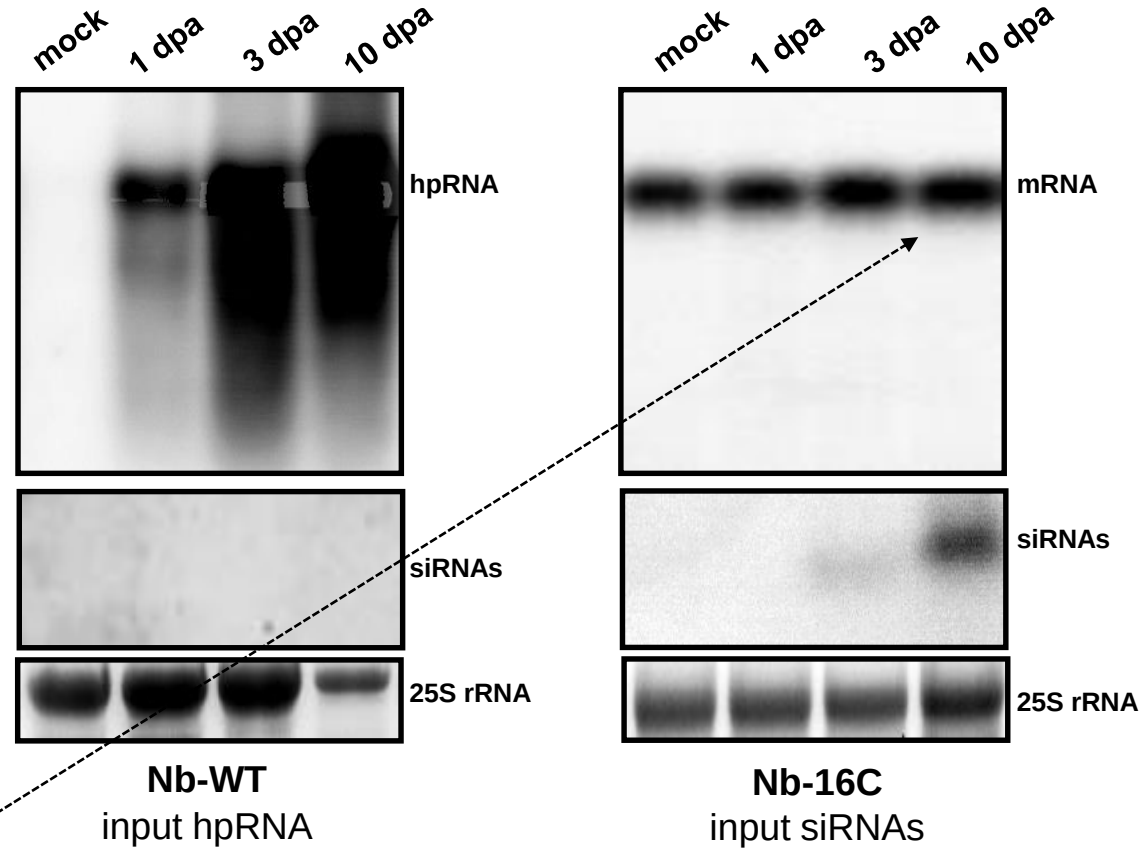

1 – 3 – 10 dpa
Northern blot
0.5 m
V. vinifera
hpRNA
(1 ml, 500 µg)

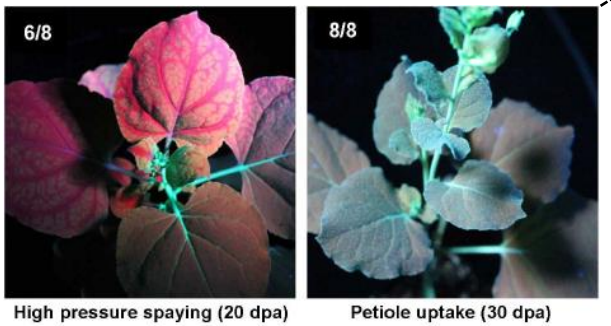

mock
1 dpa
3 dpa
10 dpa
hpRNA
siRNAs
25S rRNA


1 – 3 – 10 dpa
Northern blot
0.5 m
hpRNA
(200 µl, 50 µg)
V. vinifera


mock
1 dpa
3 dpa
10 dpa
hpRNA
siRNAs
25S rRNA







Note: siRNA application did not results in GFP silencing in the *N. benthamiana* 16C line.

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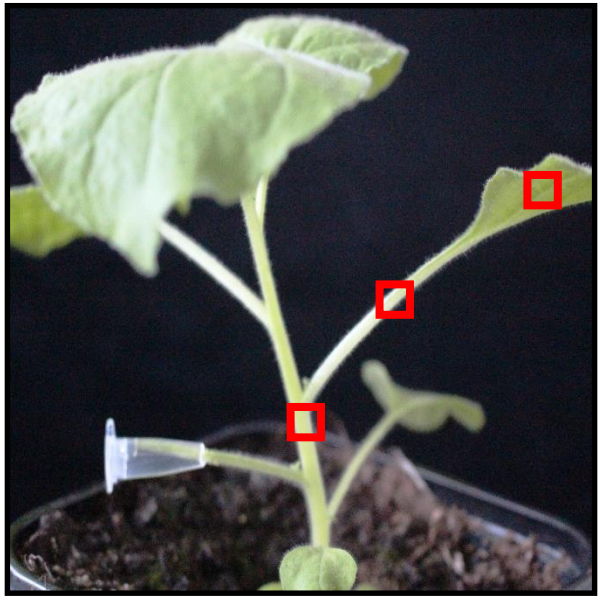
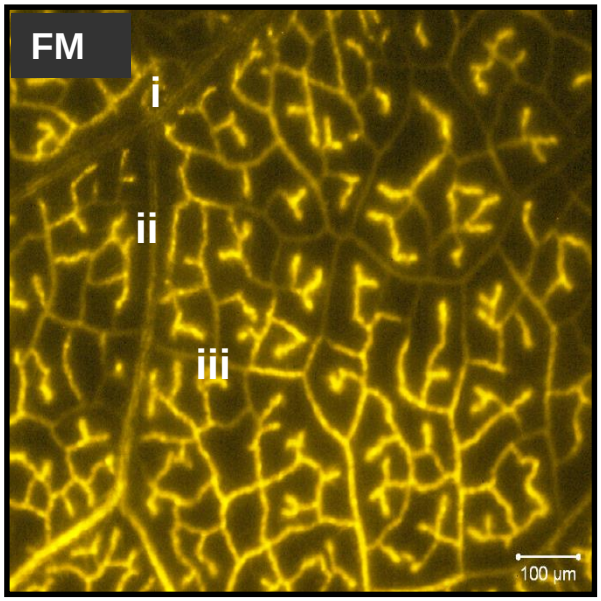
Delivery of CY3-labeled 22-nt siRNA by petiole absorption to *N. benthamiana*.

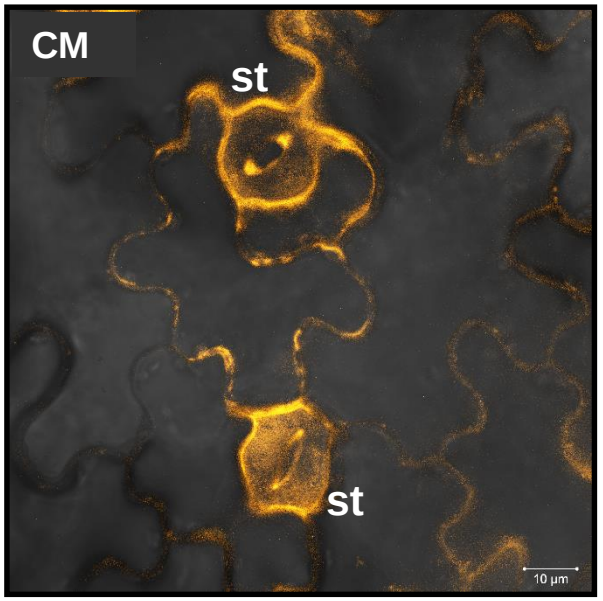


N. benthamiana

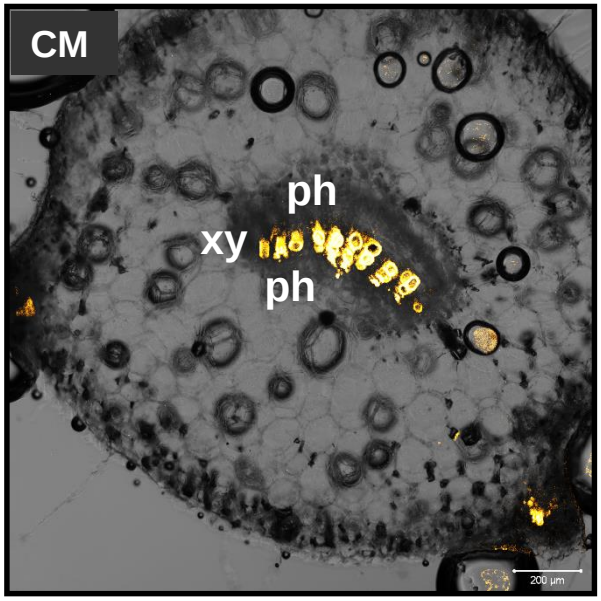
FM = Fluorescence microscopy
CM = Confocal microscopy
i, ii,iii = Class I to class III veins
st = Stomata
xy = Xylem
ph = Phloem



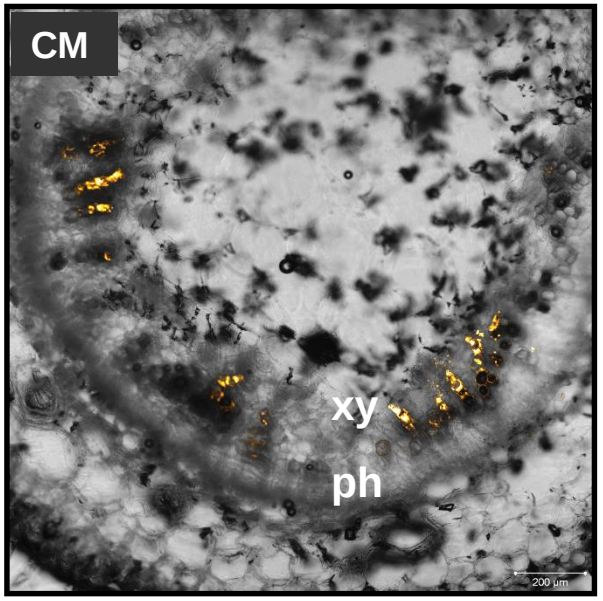
Leaf (abaxial), 1 dpa



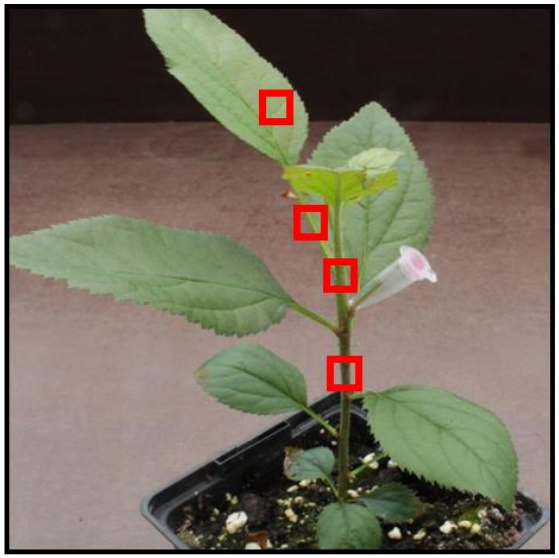
Leaf (abaxial), 1 dpa



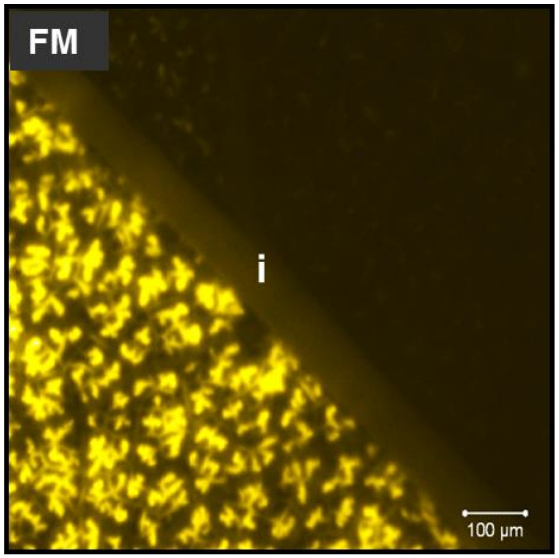
Petiole, 1 dpa



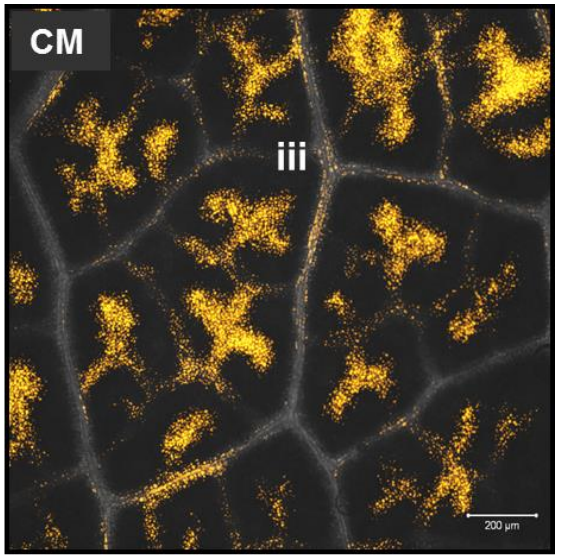
Stem, 1 dpa



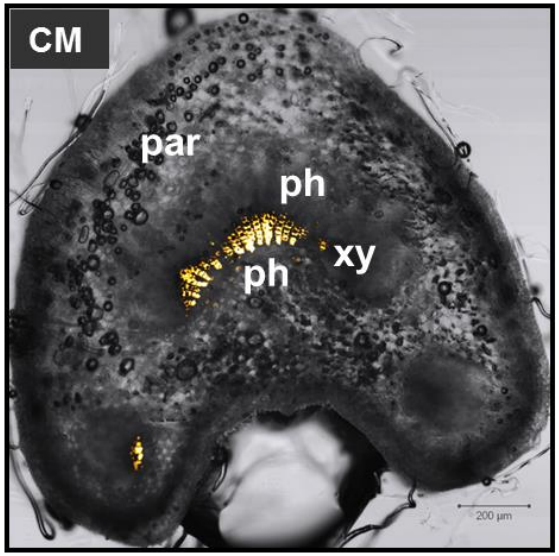
M. domestica



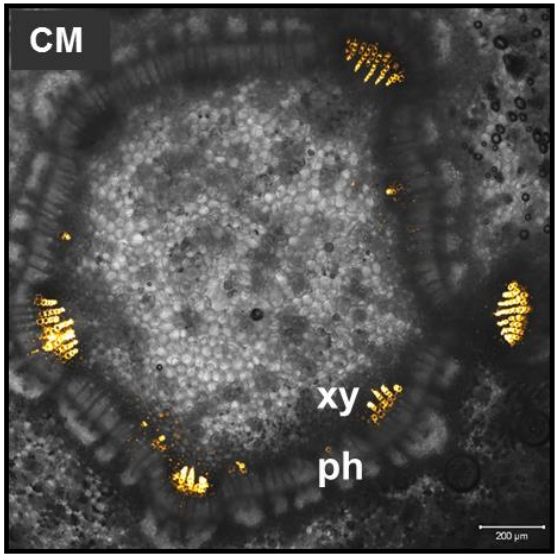
Leaf (abaxial, 1 dpa)



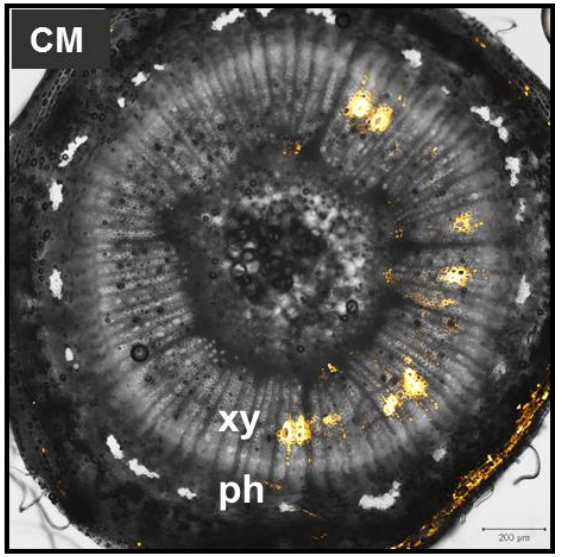
Leaf (abaxial, 1 dpa)



Petiole (1 dpa)



Upper stem (1 dpa)



Lower stem (1 dpa)

Delivery of CY3-labeled 22-nt siRNA by petiole absorption to young apple tree.

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Benefits and problems of RNAi exploitation

- CRISPR/Cas is restricted to single cells, RNAi can act on whole plants.

- RNAi may have great impact in pest management against chewing and/or xylem sap-feeding vectors and eukaryotic pathogens that reside in the xylem.

- Penetration of the cell wall (high pressure spraying) is required to target RNA into the plant cell.

- Inactivation of endogenous sequences appears to be limited (no herbicide), intronless endogenes might be candidates for RNAi targeting.

- RNA application is not useful to mediate virus resistance.

- Waiting for EU regulation on RNA application (you never know, see CRISPR/Cas).

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The 'RNA-mediated gene silencing' lab

Current lab members

Baßler, Alexandra

Dalakouras, Athanasios (left end of June)

Veli Vural Uslu (will start in September)

Wassenegger, Michèle

Schwind, Nora (parental leave)

Collaboration

Manfred Heinlein
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SIXTH FRAMEWORK PROGRAMME

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SIXTH FRAMEWORK PROGRAMME

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