RNA interference for Crop Protection and Production.

Jeremy Sweet

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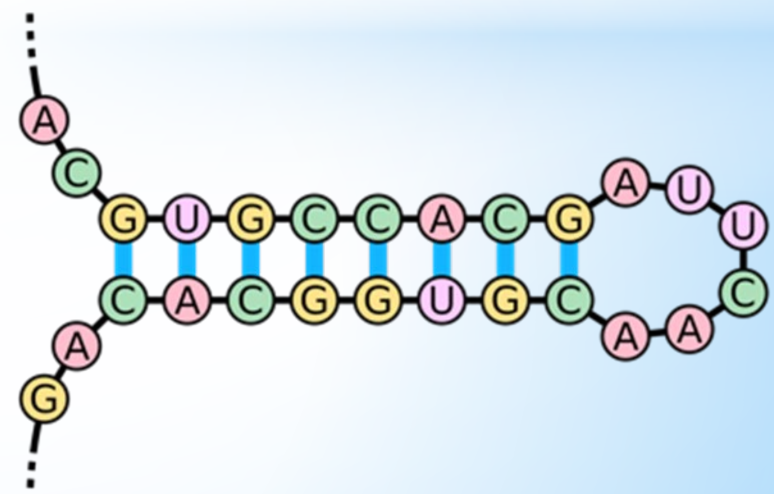
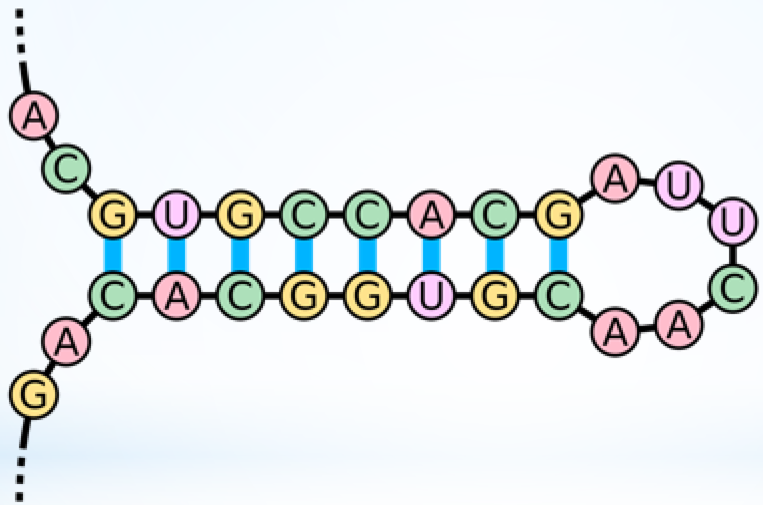


Plant Biologicals Network Symposium 17.11.22

RNAi a natural mechanism

RNAi inhibits gene expression (via mRNA) in a **sequence-specific manner** induced by **double strand RNA** (dsRNA)



Gene silencing can be partial or complete post transcriptional inhibition of gene activity

RNAi: A NATURAL MOLECULE IN PLANTS and TARGET ORGANISMS (PESTS/PATHOGENS)

- **Direct delivery by crop plant**

Host induced Gene Silencing (HIGS)*

Applications: a) Functional genomic studies,

b) improvement of important agronomic traits

c) Protection against pests/pathogens

- **Delivery via spray-application**

Spray-Induced Gene Silencing, (SIGS)**

Applications: Topical application to control pathogens and pests.

*Nowara et al. (2010) Plant Cell 22:3130
**Koch et al. (2016) PloS Pathogens

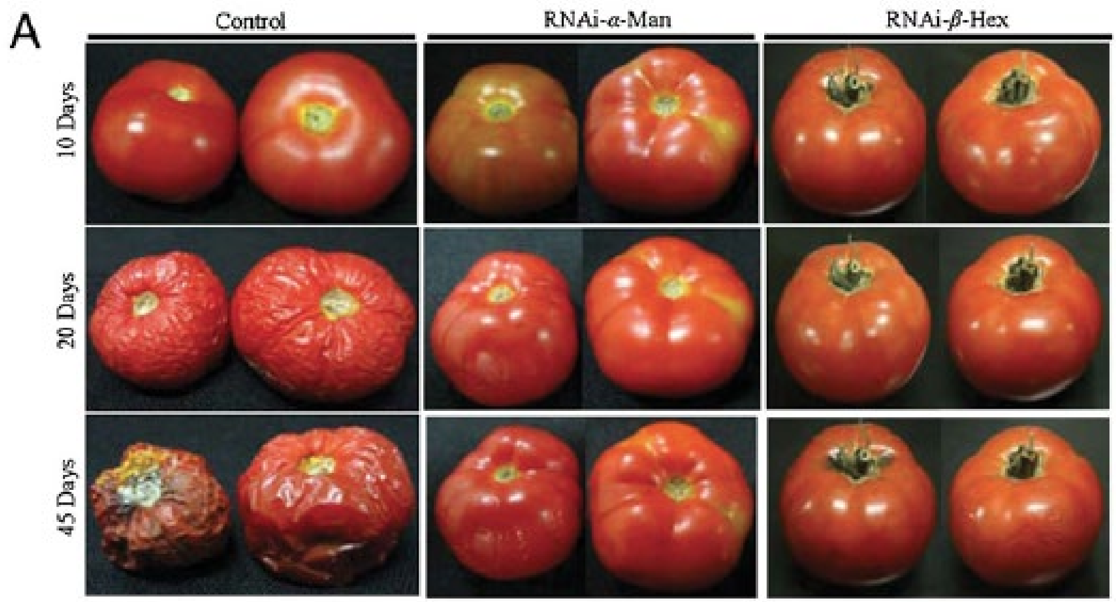
HIGS

ENHANCED SHELF LIFE (Meli et al. 2010)

Silencing of α-Man or β-Hex enhances tomato shelf life:
α-Man and β-Hex normally participate in the degradation of cell wall **N-glycoproteins** and the generation of free **N-glycans**, which further **stimulate ripening**

(A) RNAi (T0) and wild-type (control) fruits were harvested at pink stage and stored at room temperature(22-24°C in 55-60% relative humidity).

The progression of fruit deterioration was recorded by time-lapse photography. Time after harvest is specified by days.

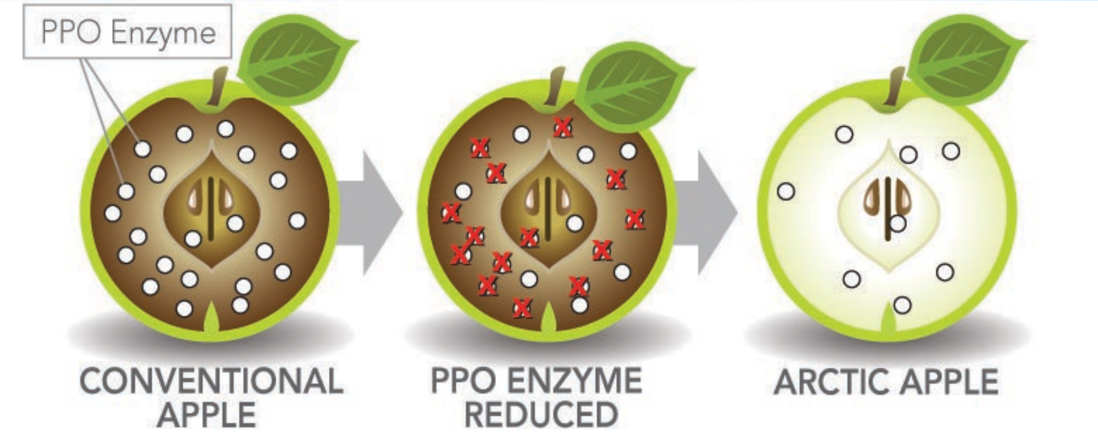


HIGS

ARCTIC APPLE -resist oxidative browning

Approved by the US Department of Agriculture (USDA).

RNAi apple with silenced **polyphenol oxidase gene**



Improving food/product quality

Examples:

- Change fatty acid profile in Soybean
- Modified starch Potato
- Tearless Onion
- Removing allergens: Peanut, apple, wheat

e.g. Francisco Barro[1], et al. Plant Biotechnol J . 2016 Mar;14(3):986-96. doi: 10.1111/pbi.12455. Epub 2015 Aug 24. Targeting of prolamins by RNAi in bread wheat: effectiveness of seven silencing-fragment combinations for obtaining lines **devoid of coeliac disease epitopes from highly immunogenic gliadins.**

RNAi for control of virus, fungal and bacterial pathogens

Virus control examples:

- **Crustacea** : shrimps, prawns
- **Gastropods** : shell fish : oysters etc..
- **Insects** : e.g. Bombyx silk worm, Bees
- **Birds and Mammals** : e.g. Avian flu,
- **Plants** : range of viruses in annual, herbaceous and woody plants . Particularly insect vectored viruses

Bacteria in a range of animal and plant species

e.g. Citrus Greening (Candidatus spp),

RNAi : Virus Protection

GM plants expressing double stranded RNA (dsRNA) which interferes with virus using plant RNA/DNA for replication. Examples:

- . Papaya ringspot,
- Plum Pox (Sharka) virus,

(both approved in USA ++)

Aphid transmitted viruses

PPV in apricot

HIGS

PRSV-RESISTANT TRANSGENIC PAPAYA

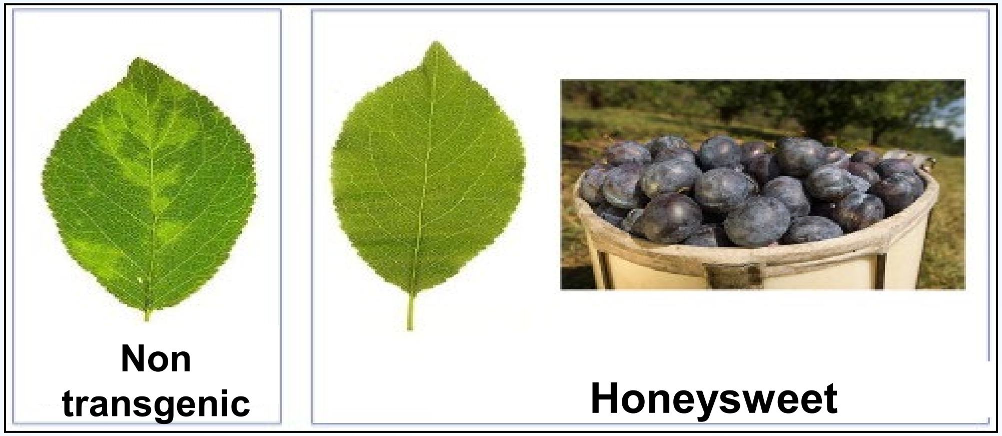
post-transcriptional gene silencing-mediated transgenic resistance with sequence homology between the transgene and the viral coat protein gene

Gonsalves 1998

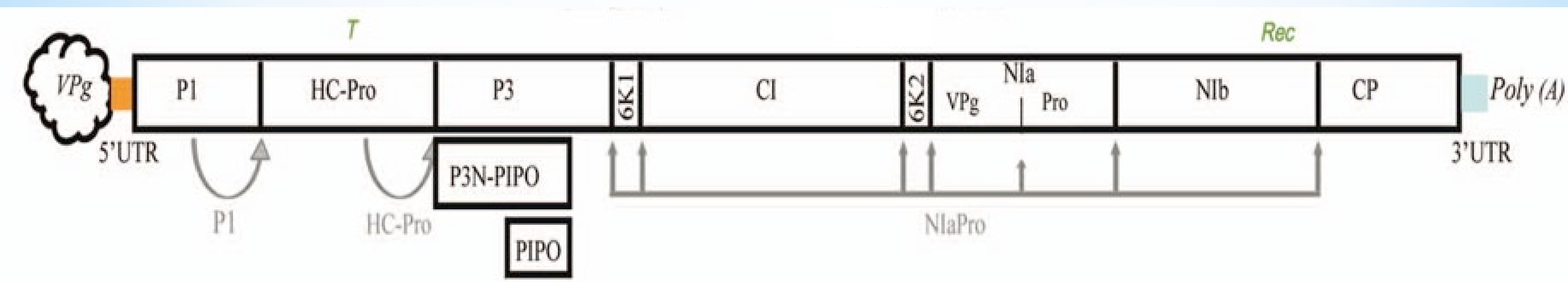
HIGS

PPV-SHARKA DISEASE: severe European disease in stone fruit.

Honey Sweet a new plum cultivar resistant to Plum Pox Virus Infection



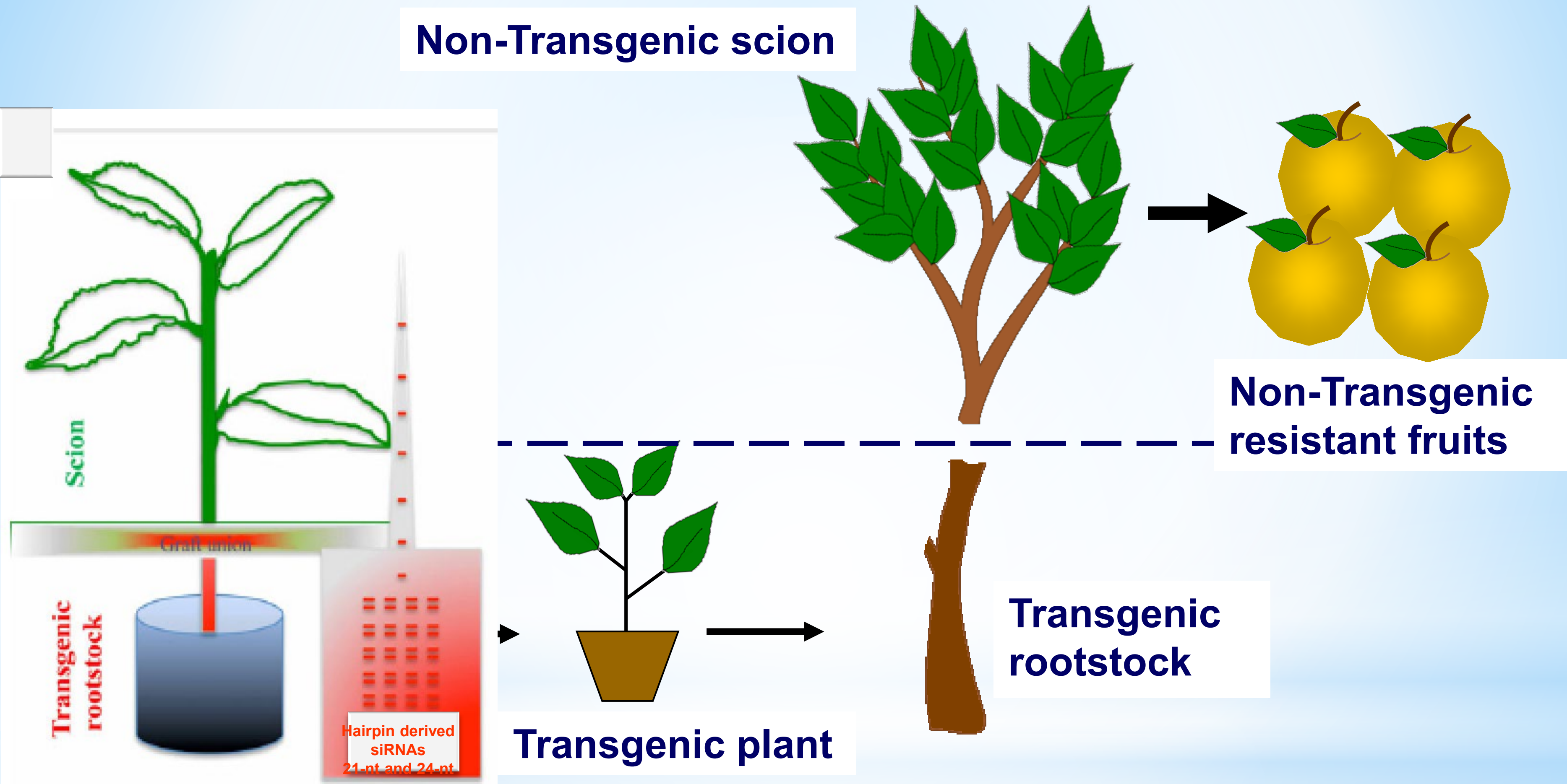
HAIRPIN RNAi GENE CONSTRUCT AGAINST PPV



Ravelonandro, M., Scorza, R., Bachelier, J. C., Labonne, G., Levy, L., Damsteegt, V., Callahan, A. M., and Dunez, J. 1997. Resistance of transgenic *Prunus domestica* to plum pox virus infection. Plant Dis. 81:1231-1235.

- Sequence homologous to part of P1 gene sequence of PPV strain M and D.

INDUCING RNAI PLANT RESISTANCE WITH MODIFIED ROOTSTOCKS



Transfer of virus resistance from rootstock to scion

Advantages in the use of a silenced rootstock:

- The scion maintains its genetic inheritance.
- There is not gene flow because pollen and seed are not genetically modified.

} PUBBLIC ACCEPTANCE

} LOW ENVIRONMENTAL RISK

Plant Fungal disease Control

homology-based gene silencing stimulated by transgenes (co-suppression), antisense, or dsRNAs has been demonstrated in several plant pathogenic fungi/oomycetes, including:

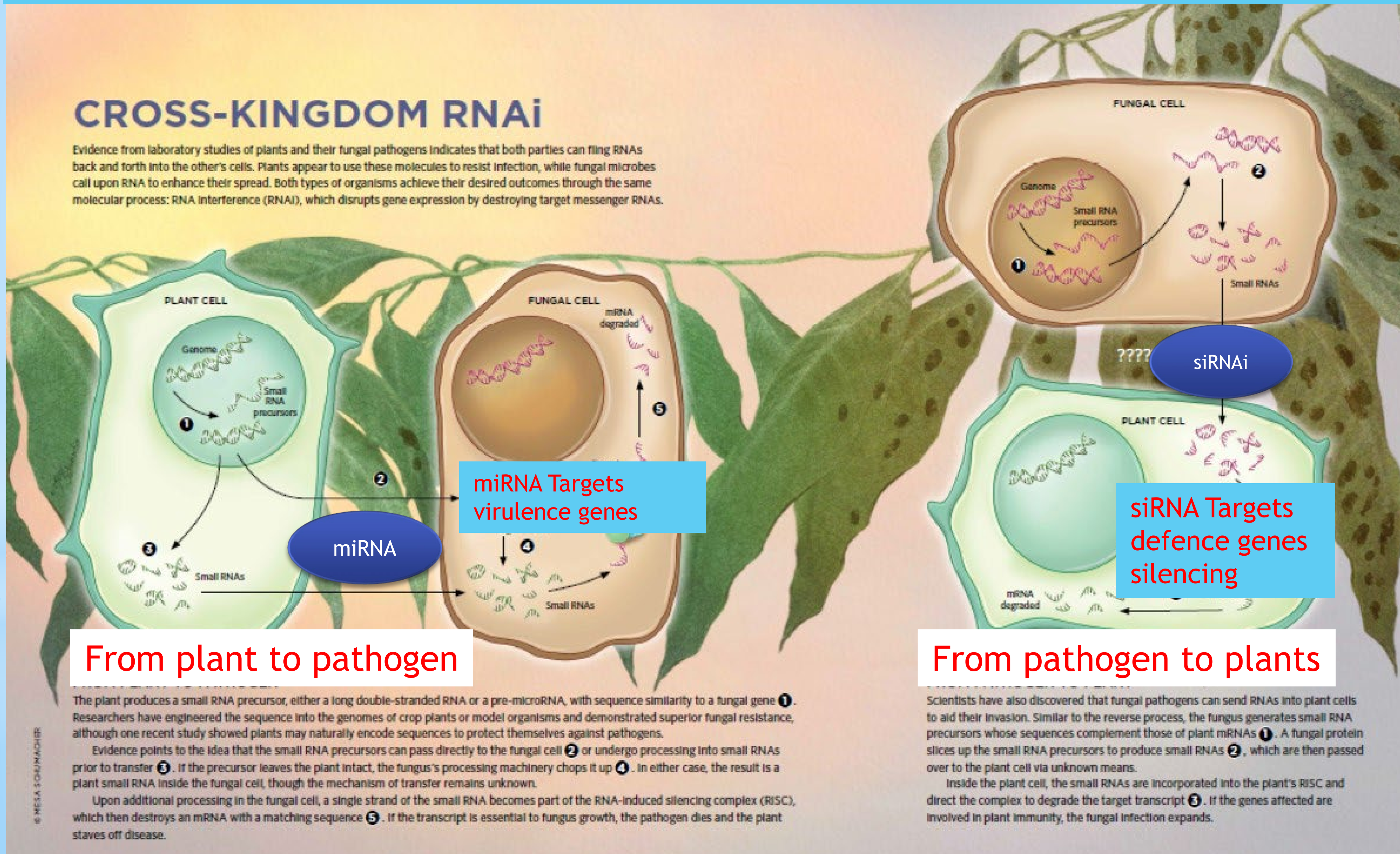
Botrytis : moulds
Fusarium :
Sclerotinia :
Phytophthora : Blight
Puccinia : Rusts
Mildews : Powdery and Downy
Venturia :

Acting via Susceptibility genes in plants and Pathogenicity genes in fungal pathogen

HOST-PATOGEN INTERACTION TO IDENTIFY GENES OF RESISTANCE, METHABOLITES AND RNA MOLECULES INDUCING RESISTENCE

Natural basis of RNAi-based crop protection

Bidirectional communication (Weiberg et al., 2013, Science)



CROSS-KINGDOM RNAi

Evidence from laboratory studies of plants and their fungal pathogens indicates that both parties can fling RNAs back and forth into the other's cells. Plants appear to use these molecules to resist infection, while fungal microbes call upon RNA to enhance their spread. Both types of organisms achieve their desired outcomes through the same molecular process: RNA interference (RNAi), which disrupts gene expression by destroying target messenger RNAs.

From plant to pathogen

The plant produces a small RNA precursor, either a long double-stranded RNA or a pre-microRNA, with sequence similarity to a fungal gene ❶. Researchers have engineered the sequence into the genomes of crop plants or model organisms and demonstrated superior fungal resistance, although one recent study showed plants may naturally encode sequences to protect themselves against pathogens.

Evidence points to the idea that the small RNA precursors can pass directly to the fungal cell ❷ or undergo processing into small RNAs prior to transfer ❸. If the precursor leaves the plant intact, the fungus's processing machinery chops it up ❹. In either case, the result is a plant small RNA inside the fungal cell, though the mechanism of transfer remains unknown.

Upon additional processing in the fungal cell, a single strand of the small RNA becomes part of the RNA-induced silencing complex (RISC), which then destroys an mRNA with a matching sequence ❺. If the transcript is essential to fungus growth, the pathogen dies and the plant staves off disease.

From pathogen to plants

Scientists have also discovered that fungal pathogens can send RNAs into plant cells to aid their invasion. Similar to the reverse process, the fungus generates small RNA precursors whose sequences complement those of plant mRNAs ❶. A fungal protein slices up the small RNA precursors to produce small RNAs ❷, which are then passed over to the plant cell via unknown means.

Inside the plant cell, the small RNAs are incorporated into the plant's RISC and direct the complex to degrade the target transcript ❸. If the genes affected are involved in plant immunity, the fungal infection expands.

Research on Invertebrate Pest Control

- **Crop pests** :

  Hemiptera, Homoptera, Coleoptora, Lepidoptera, Diptera, etc…..

  Mites , Nematodes,

Topical and oral exposure to mostly dsRNA,

HIGS

NEMATODE RESISTANCE

Root-knot nematodes

Ibrahim HMM, Alkharouf NW, Meyer SLF, Aly MAM, Gamal El- Din AY, Hussein EHA, Matthews BF: **Post- transcriptional gene silencing of root-knot nematode in transformed soybean roots**. Exp Parasitol 2011, 127:90-99.

HIGS

- **Smartstax amd Smartstax Pro maize for corn root worm (Diabrotica) control :** commercialised and cultivated in N America from 2022 and assessed by EFSA for **Import/Food/Feed** in EU.

- COMMISSION IMPLEMENTING DECISION (EU) 2018/2046 of 19 December 2018 authorising the placing on the market of products containing, consisting of or produced from genetically modified maize MON 87427 × MON 89034 × 1507 × MON 88017 × 59122, **contains DvSN17 (RNAi) and Cry toxins**

Research Article

SCI

Received: 8 July 2016 Revised: 2 February 2017 Accepted article published: 13 February 2017 Published online in Wiley Online Library: 17 March 2017

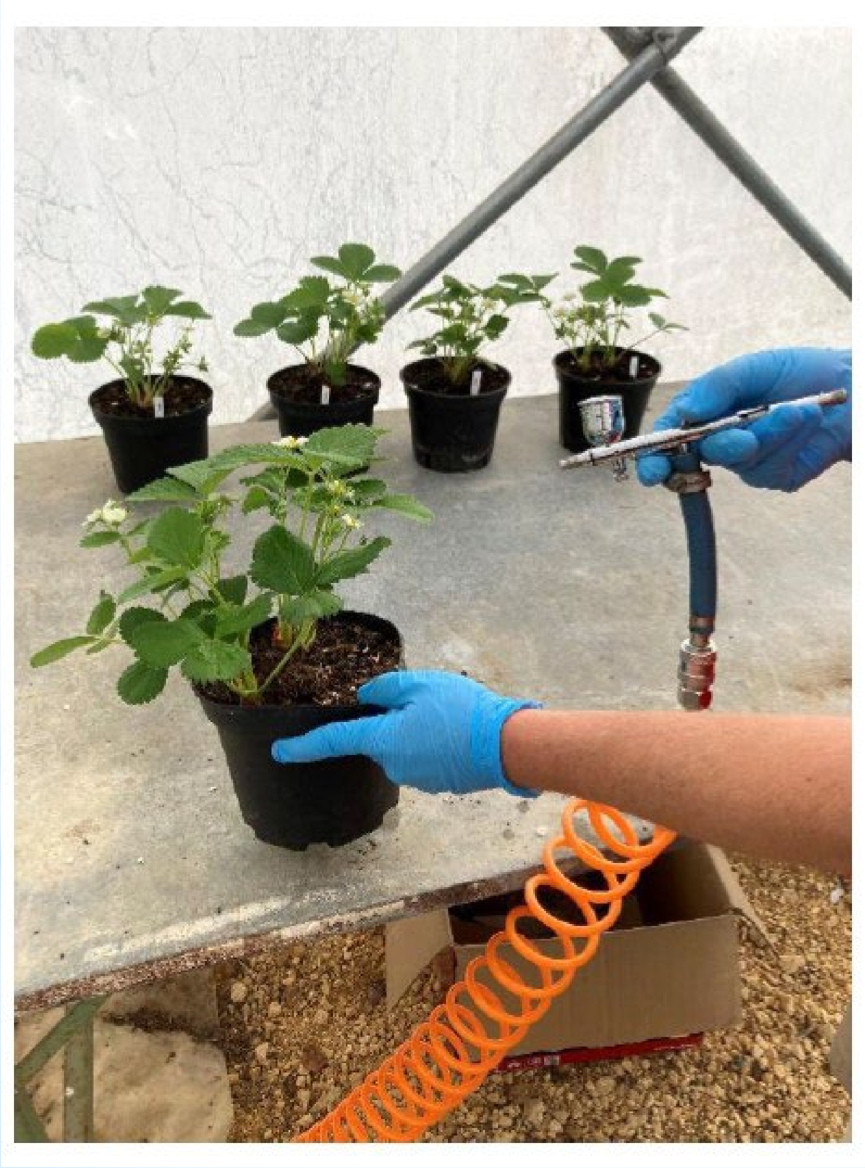
(wileyonlinelibrary.com) DOI 10.1002/ps.4554

Evaluation of SmartStax and SmartStax PRO maize against western corn rootworm and northern corn rootworm: efficacy and resistance management

Graham P Head,[a] Matthew W Carroll,[a*] Sean P Evans,[a] Dwain M Rule,[b] Alan R Willse,[a] Thomas L Clark,[a] Nicholas P Storer,[b] Ronald D Flannagan,[a] Luke W Samuel[a] and Lance J Meinke[c]

SIGS

Exogenous induction of gene silencing



Insect
Fungi
Bacteria
…
Control fruit ripening

Exogenous applications of dsRNA

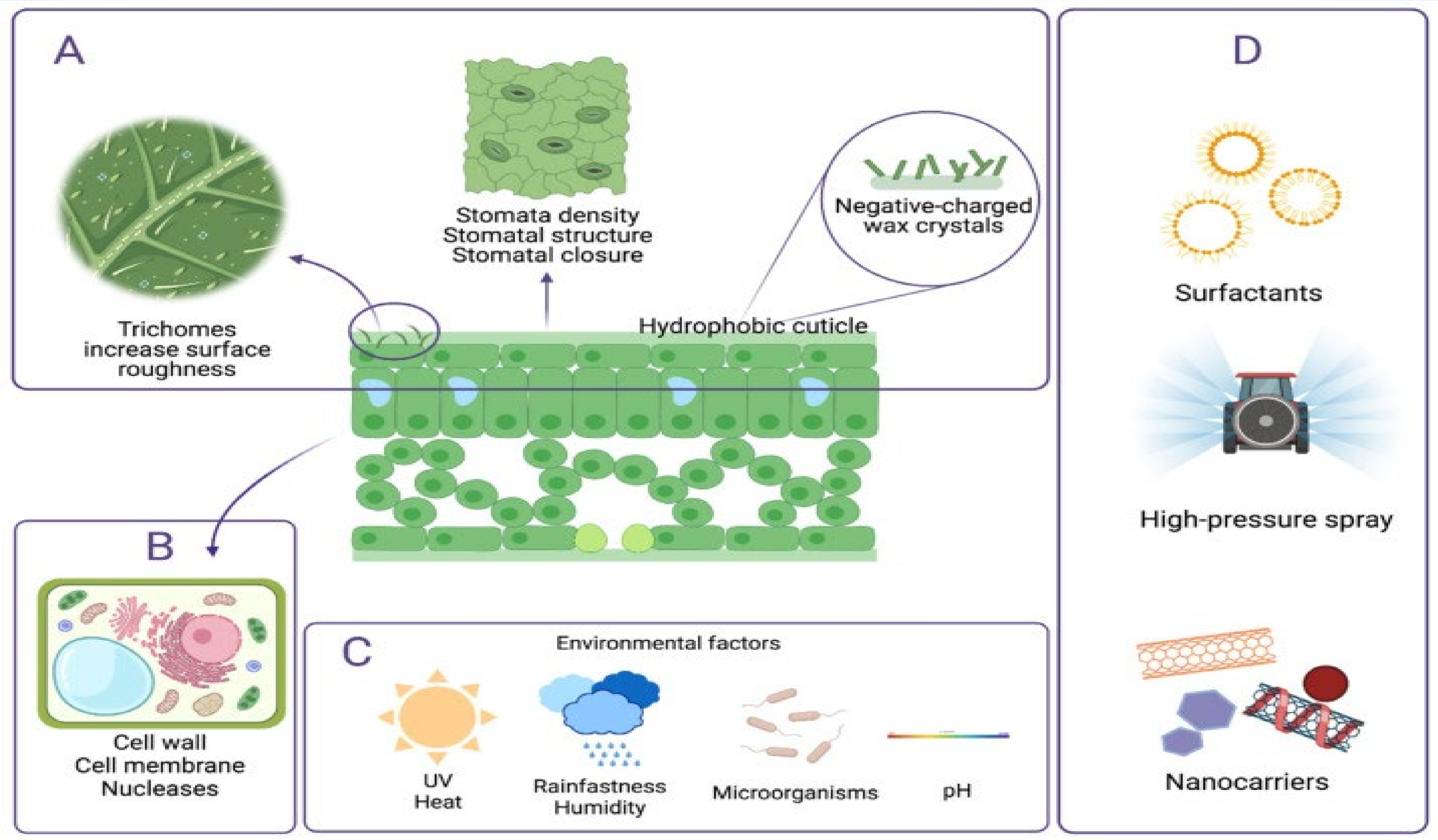
Apply synthesised dsRNA :-

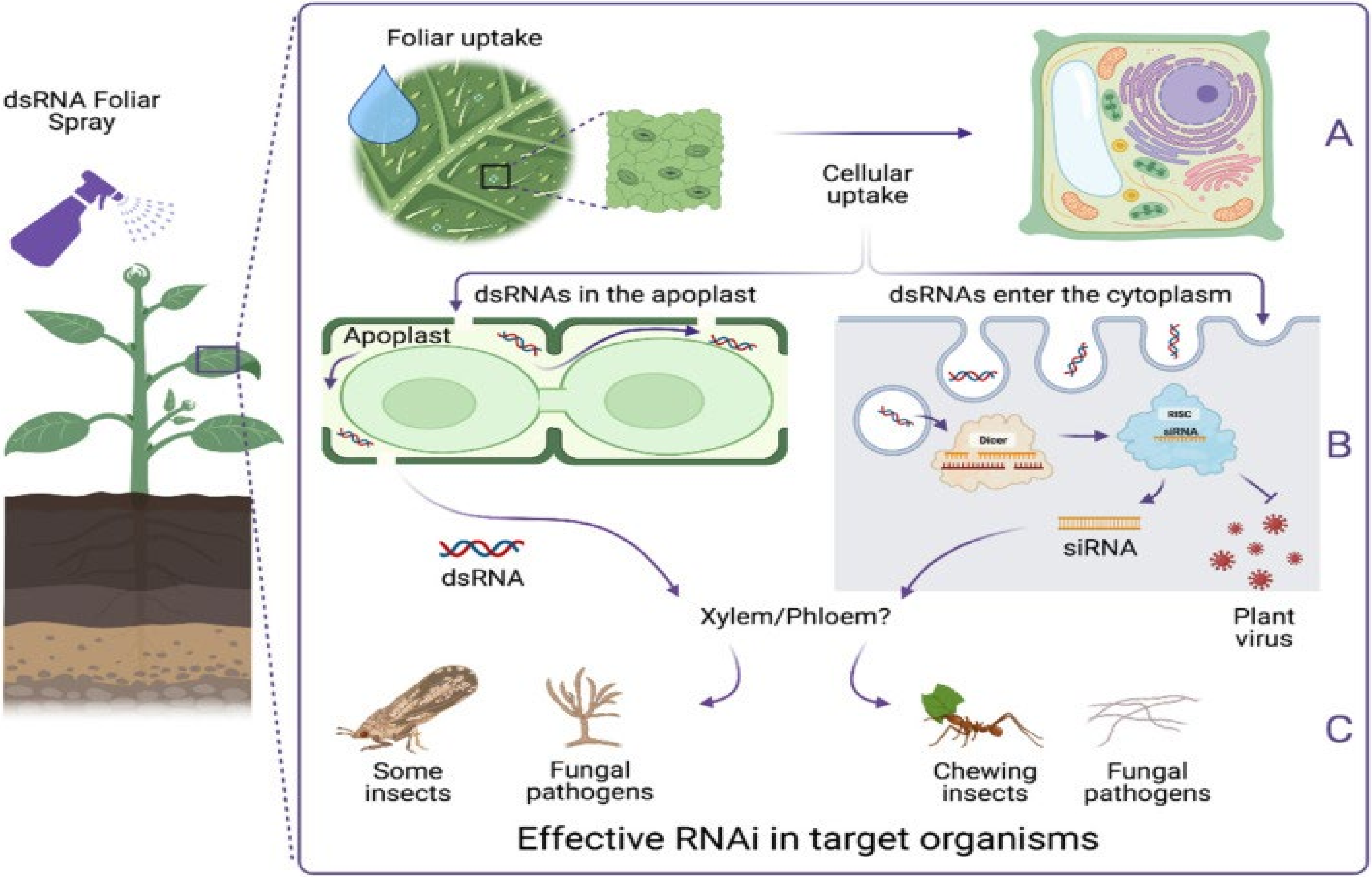
Spray Induced Gene Silencing : SIGS

Includes:

- Foliar and flower applications for arthropod pests, viruses, fungal infections and Herbicides.
- Arthropods : mostly ingestion of dsRNA applied to plants some direct uptake
- Fungi : direct uptake of applied dsRNA or uptake of dsRNA into epidermal cells and transfer to pathogen.
- Weeds : direct uptake
- Seed treatments for soil organisms (e.g. nematodes, fungi)

- RNA has high environmental sensitivity and low persistence
- Requires formulations to allow persistence, uptake, absorption, mobility and activity in target
- Formulations include nanoparticle carriers = bioclays





RNAi as a Foliar Spray: Efficiency and Challenges to Field Applications
Bao Tram Huang, Neena Mitter et al 2022

Diseases in horticultural plants: HIGS and SIGS

ARTICLES NATURE PLANTS DOI: 10.1038/NPLANTS.2016.151

HIGS

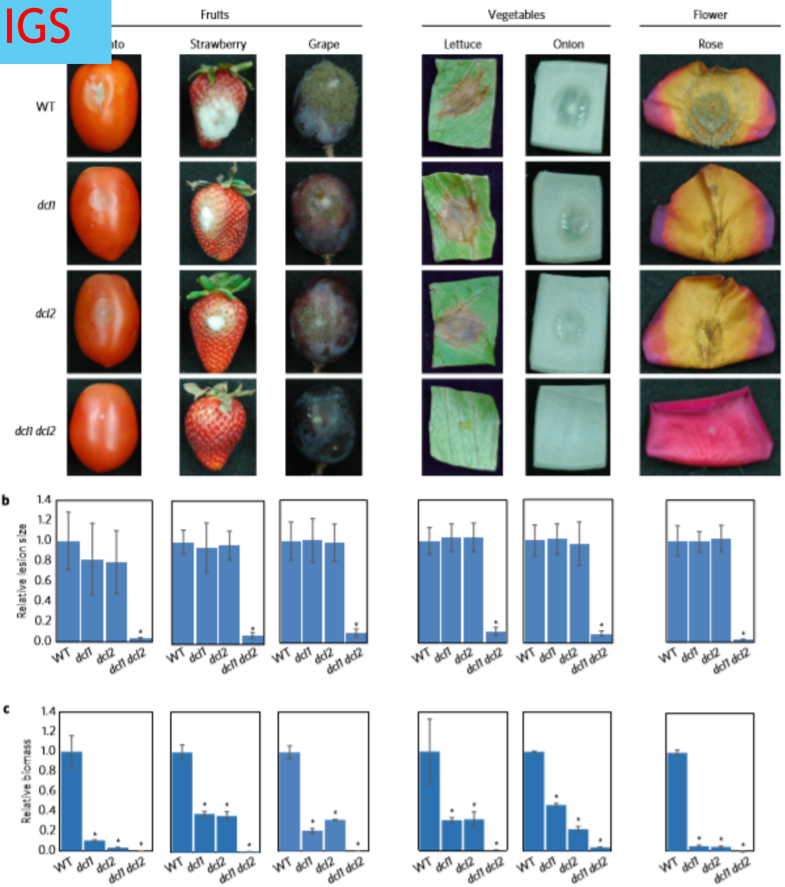


Figure 1 | *B. cinerea dcl1 dcl2* double mutant, but not the *dcl1* or *dcl2* single mutants, displays reduced virulence on fruits, vegetables, and flower petals. **a**, *B. cinerea dcl1 dcl2* double mutant shows compromised virulence on fruits (tomato, strawberry and grape), vegetables (lettuce and onion) and flower petals (rose), and *B. cinerea dcl1* and *dcl2* single mutants showed similar virulence as the WT strain. **b**, Relative lesion sizes of the infected plant samples were measured 3 days post inoculation (dpi) for lettuce, onion and strawberry and 5 dpi for tomato, grape and rose petal using ImageJ. Error bars indicate the standard deviations (s.d.) of ten samples. **c**, *B. cinerea* relative DNA content (relative biomass) was measured by quantitative PCR. Error bars indicate the s.d. of three technical replicates. Asterisks indicate statistically significant differences ($P < 0.01$). Similar results were obtained from at least three biological replicates.

ARTICLES NATURE PLANTS DOI: 10.1038/NPLANTS.2016.151

SIGS

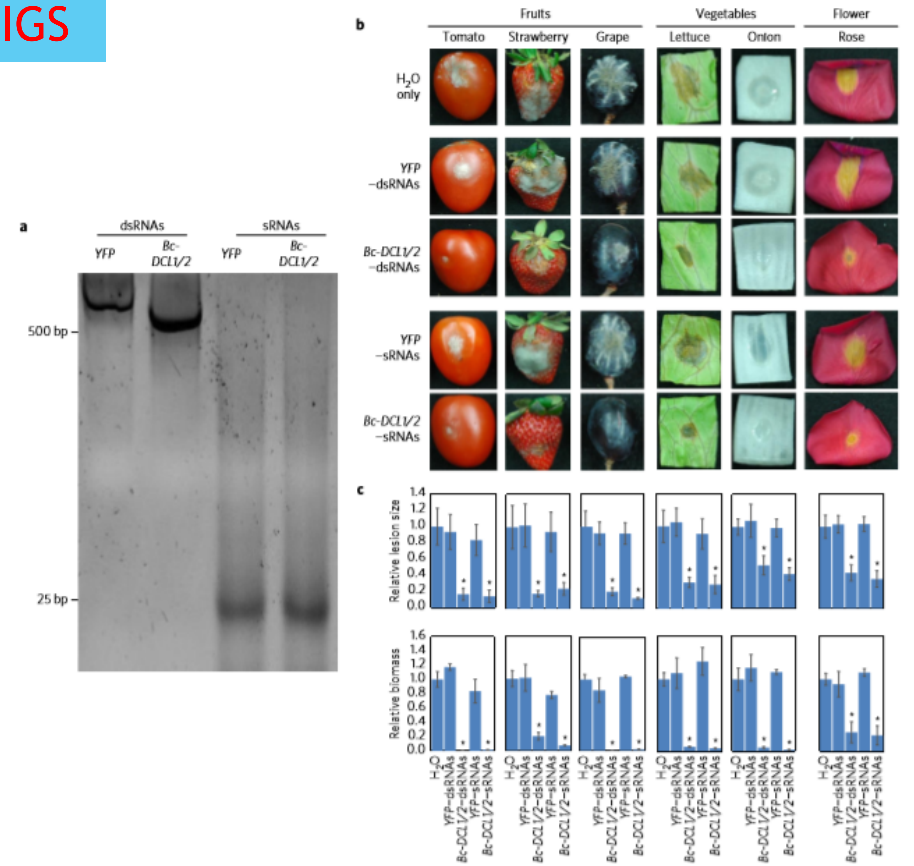


Figure 4 | Externally applied *Bc-DCL1/2*–sRNAs and –dsRNAs inhibited pathogen virulence on fruits, vegetables, and flower petals. **a**, *Bc-DCL1/2*–dsRNAs and –sRNAs, as well as *YFP*–dsRNAs and –sRNAs, were synthesized and processed, and 100 ng of RNAs was analysed on a native PAGE gel to check the quality. **b**, External application of *Bc-DCL1/2*–dsRNAs and –sRNAs (20 μl of 20 ng μl^{-1} synthetic RNAs) inhibits the virulence of *B. cinerea* on fruits (tomato, strawberry and grape), vegetables (lettuce and onion) and flower petals (rose) compared with the treatments using water, *YFP*–dsRNAs and –sRNAs. **c**, The relative lesion sizes and fungal biomass were measured at 3 dpi for lettuce, onion, rose and strawberry and at 5 dpi for tomato and grape fruits using ImageJ software and quantitative PCR, respectively. Error bars indicate the s.d. of ten samples and three technical repeats for the relative lesion sizes and relative biomass, respectively. Asterisks indicate statistically significant differences ($P < 0.01$). Similar results were obtained from three biological replicates.

GreenLight BIOSCIENCES™

Colorado Potato beetle (Leptinotarsa decemlineata)

Protect potatoes

RNA solution using foliar application; expected EPA approval this year; sales taking place ahead of the following growing season

The problem: Colorado potato beetle causes hundreds of millions[1] of damage a year and develops rapid resistance.

The solution:

Calantha ™

- On track to be cost competitive to other premium solutions
- Compatible with farmers' standard operating procedures
- Low risk for operators and consumers
- Low to no detectable residue

CPB has a long history of resistance development and has documented insensitivity to 54 different active ingredients in nearly all existing insecticide MoA groups.[2,3]

Potato fields protected in 2020 field trials	
Untreated, 30 days	9.9 g of GreenLight RNA per hectare

Sources: 1. Science Daily, 2020; 2. Alyokhin, A et al. 2008a. Colorado potato beetle resistance to insecticides. Am. J. Potato Res; 3. Whalon M. 2013. Arthropod pesticide resistance database

The problem:

Botrytis cinerea

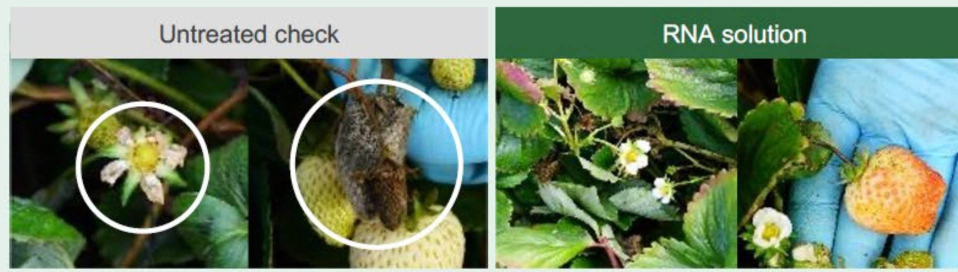
- Causes disease in more than 500 species of plants grown worldwide[3]
- It can result in up to 30% yield loss in fresh fruits and vegetables[4]
- Attacks food both in the field as well as after harvest
- Victims of botrytis include soft fruit such as strawberries and grapes, as well as onions, tomatoes, sweet potatoes, and other food crops

The solution:

Current strategies involve frequent spraying of these crops with traditional chemical-based pesticides, leaving significant residues.

GreenLight is developing an RNA anti-fungal solution that has undergone initial field trials in the U.S. and Europe.

Our testing shows a reduction in disease severity compared to untreated plants. We anticipate this product will be available in-season earliest 2026.



Sources: 1. Savary et al., 2019. The global burden of pathogens and pests on major food crops. Nat Ecol Evol; 2. Davies et. al., 2021. Evolving challenges and strategies for fungal control in the food supply chain, Fungal Biology Reviews; 3. Li Hua et. al., 2018. Pathogenic mechanisms and control strategies of *Botrytis cinerea* causing post-harvest decay in fruits and vegetables, Food Quality and Safety; 4. Dalphy O.C. Harteveld, Tobin L. Peever, Department of Plant Pathology, Washington State University

Fusarium : Cereal ear blight and grain infection

The solution:

Stackable dsRNA solutions for controlling Fusarium

- GreenLight leads aim to control mycotoxin production and reduce Fusarium growth
- **Lead sequences** suppress Fusarium head blight disease severity in wheat, reducing visual disease scale ratings from a median of 4.1 to a median of 2.1
- Under greenhouse conditions, GreenLight's dsRNA gives an average of 84% reduction in disease severity

Source: 1. Wang, H. et al. 2020. Horizontal gene transfer of Fhb7 from fungus underlies Fusarium head blight resistance in wheat. 2. Powell AJ,

GreenLight BIOSCIENCES™

Protect honeybees From Varroa parasitism

Proprietary solution testing in field trials; EPA submission planned this year

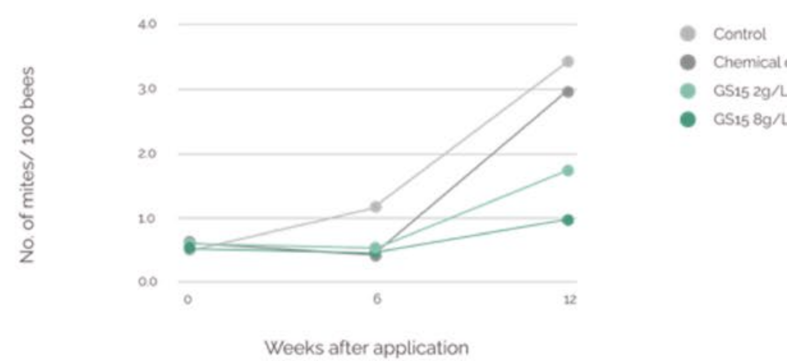
The problem: Honeybee colonies in the United States alone contribute to pollinating more than 100 crops annually worth ar estimated $18 billion[1]. But these colonies have been significantly threatened and diminished in the last decade or so by the *Varroa destructor* mite, which beekeepers worldwide say is the number one threat and can decimate whole colonies rapidly.

The solution:

Targeting Varroa mites

- GreenLight acquired Bayer's topical RNA intellectual property portfolio, which includes bee-health assets
- We combined that with our technology to develop an RNA-based treatment to combat the parasitic mites
- First field trials took place 4 months after after acquisition. We plan to launch it in 2024

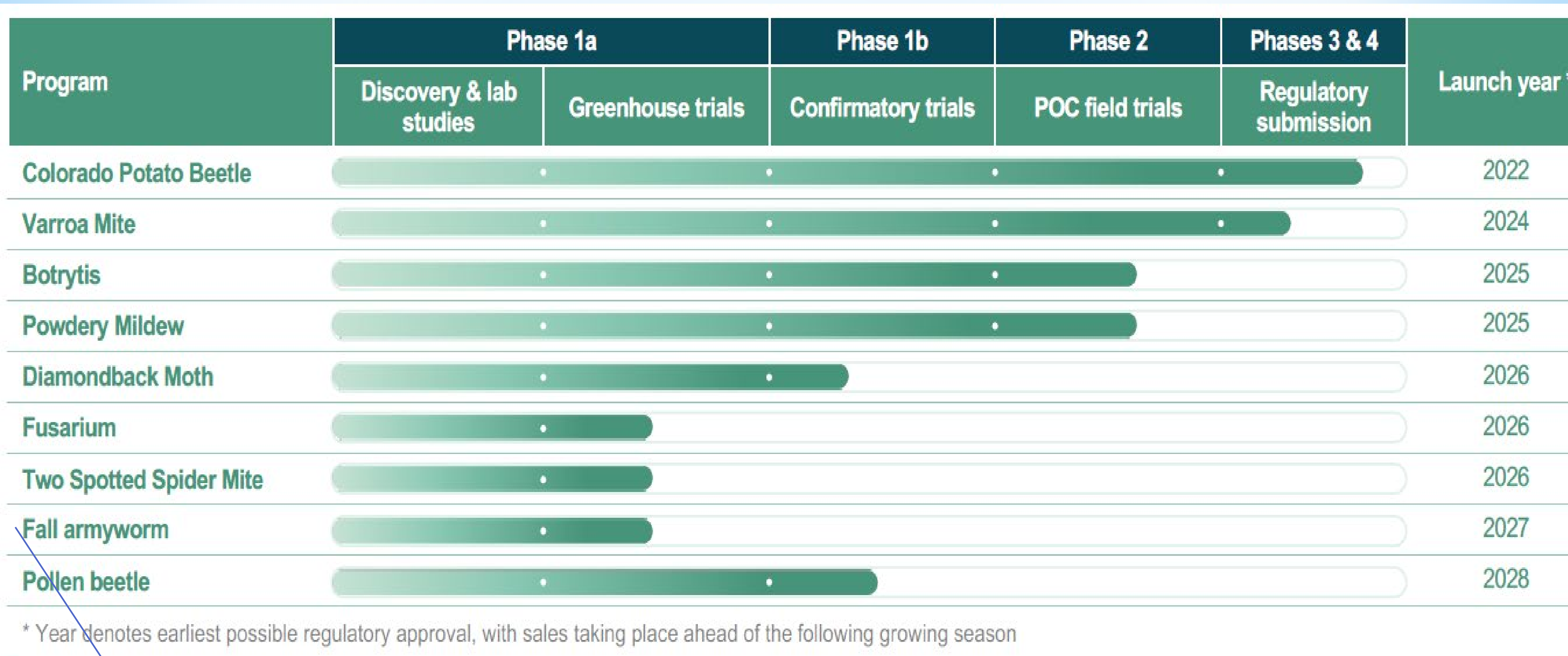
40% fewer Varroa mites in field trials at 12 weeks in hives with GS15, compared with leading chemical control product



Source: 1. United States Department of Agriculture, 2021.

GreenLight BIOSCIENCES™

SIGS development



+ Queensland Univ Australia

Also Bayer, Syngenta and other companies involved

Main Research and Biosafety Issues

- the mechanisms of activity and methods of delivery of ds/siRNAs to targets.
- miRNA interactions with RNAi mechanisms
- application bottlenecks depending on whether the target genes are *in planta* (i.e. viral or plant transcripts), or in fungi, insects, nematodes etc.
- Environmental stability of dsRNA
- Non-target effects in related and unrelated exposed species and food chain effects
- Off target effects : role of bioinformatics and omics for food and environment RA.
- Scale up : How to scale RNA production

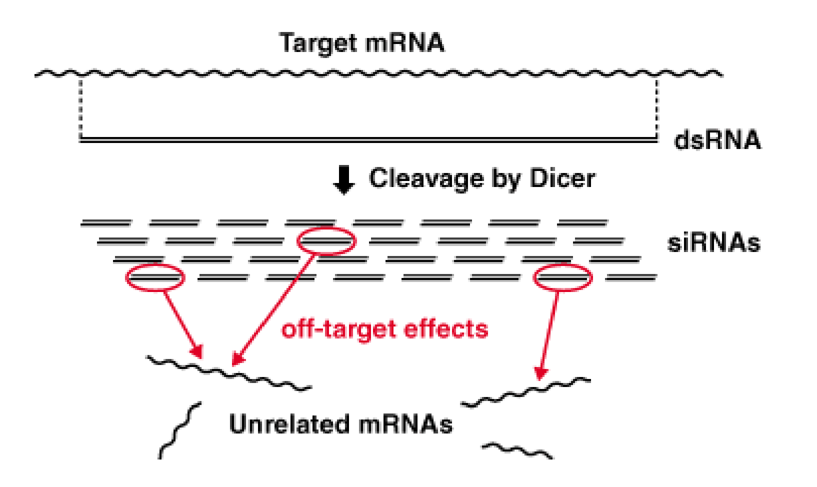
"The art and science of taking the RNA scale-up recipe from the lab to a manufacturing plant."

https://www.greenlightbiosciences.com/how-to-scale-rna-production/

Off target effects and effects on non-target organisms

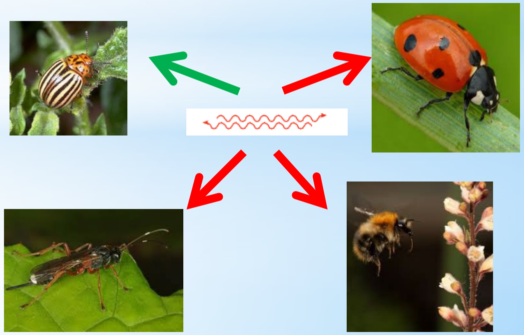
Off target effects

- Knockdown of a non-target gene
- Due to sequence homology
- e.g. conserved domains within protein families
- Important for research applications



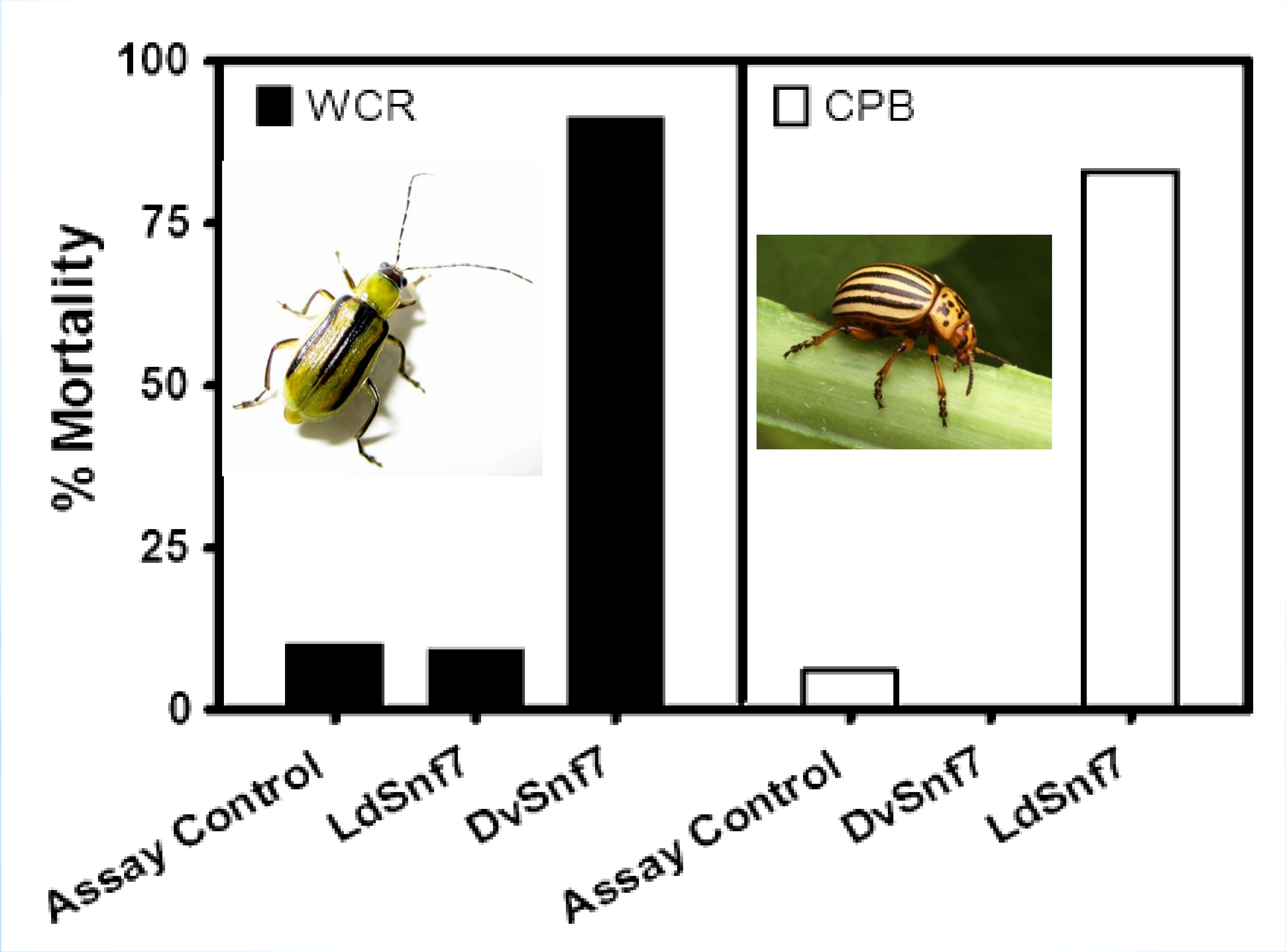
Silencing effects on non-target organisms

- Knockdown of any gene in non-target organism
- Due to sequence homology
- Important for risk assessment



Effects on non-target organisms and off target effects:

Corn/maize: MON87411; "Smart Stax Pro" : Chrysomelids



Bachman et al., 2013 – Transgenic Res

Bioinformatics:

- Bioinformatics is a **<u>useful tool in target design</u>** and to minimize potential risks
- siRNA sequence specificity requirements still uncertain
- Available sequence data is still limited
- Genbank 2022: 3200 insect genome sequences (several duplicates)
- i5k projects (BaylorCM/HGSC)

- However: unlikely to be very useful in risk assessment at this moment. But in future ... ?

Additional Data requirements for ERA of GM RNAi plants (and SIGS)

- Bioinformatics: useful guide but not definitive
- dsRNA persistence in the environment
- Routes and Level of Exposure to siRNAs and dsRNAs including amplification
- Potential Non and Off targets : requires information on sequence homology, mis-matches etc..
- EFSA Guidance on GM RNAi Plants

Risk assessment considerations for genetically modified RNAi plants: EFSA's activities and perspective April 2020
Frontiers in Plant Science 11 DOI: 10.3389/fpls.2020.0045

GMO regulations

Not operating in EU


LA TRIBUNE DES PLACEMENTS

Comment alléger ses impôts 1997

La Tribune

La France ouvre l'Europe aux cultures transgéniques

Les industriels optimistes... pour l'instant

La réforme du licenciement gêne Jospin

Le dollar tire Paris

SCIENTIFIC AMERICAN

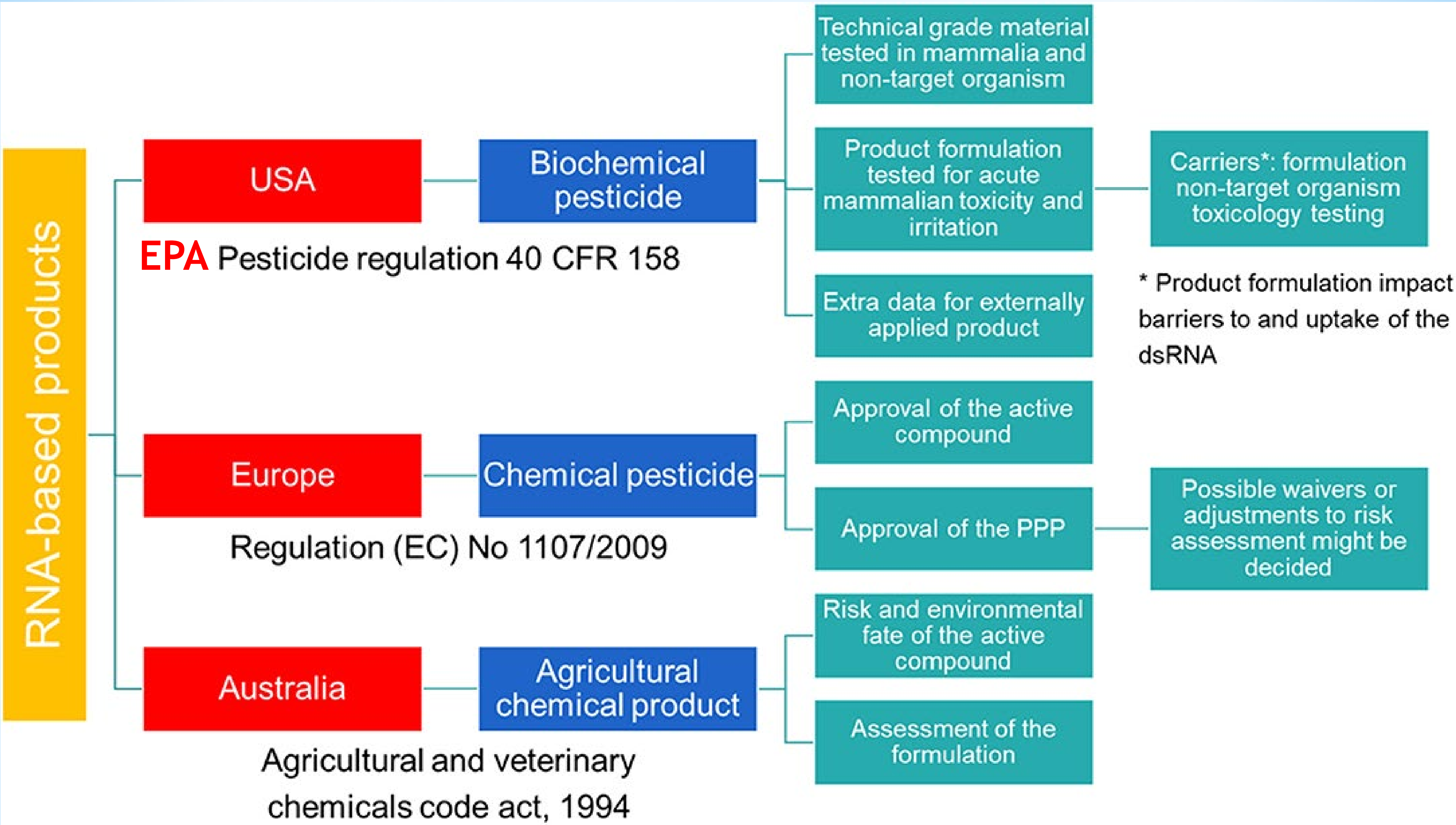
What Did COVID Teach Us about Preparing for a Plant Pandemic ?

The question is not whether we'll experience such an event; it's whether we'll be ready when it strikes

By Jonathan Margolis June 20, 2022

- All COVID vaccines (and many others) produced by GM technology.
- Some COVID vaccines based on RNA technology.
- We need to move focus of regulations away from process to product and how it will be used, since similar products can be made by many processes.

Regulation of Exogenous applications of dsRNA e.g SIGS



De Schutter et al. Front. Insect Sci., 05 January 2022

Regulation of Exogenous applications of dsRNA in EU

- **Assessed as chemical pesticides.**
- **At present no agreement on how they should be assessed and additional data requirements.**
- **Should they be classed differently : Biopesticides or bioactivator/stimulant ?**
- **OECD working group and others studying this.**

OECD (2020). *Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied ds-RNA-Based Pesticides*.

Szekacs et al. RNAi Based Pesticides. Front Pl Sci 2021; 12: 714116.

doi: 10.3389/fpls.2021.714116

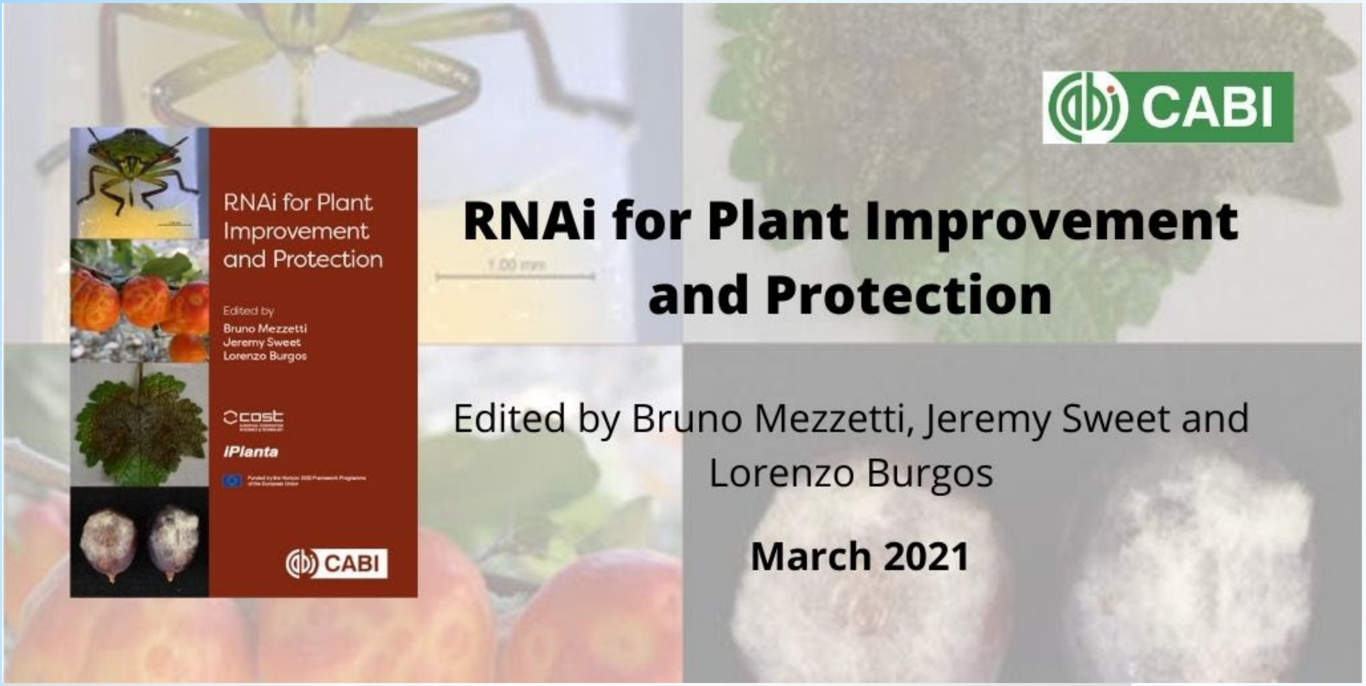
PMCID: PMC8358595

PMID: 34394170

Thanks to iPlanta contributors to this talk

https://www.youtube.com/watch?v=GPRyXVPsw38

www.***iplanta.univpm.it***. jeremysweet303@aol.com



Available as
E-Book – FREE !

Mange Tak

Thank you

jeremysweet303@aol.com

https://www.youtube.com/watch?v=GPRyXVPsw38