REVERSE GENETICS
ALTRI APPROCCI

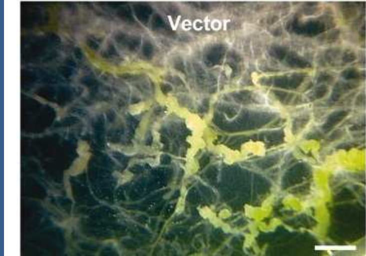
Applications of small RNA technologies

In plants, siRNA or miRNA-forming DNA can be introduced stably into the genome to selectively silence one or more genes.

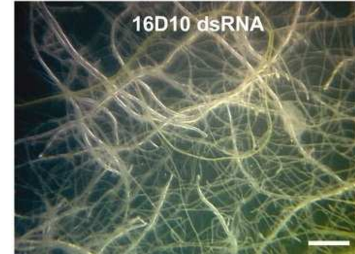
Gene silencing can eliminate allergens from peanuts.

Gene silencing can remove toxic compounds from cotton seed so they can be used as a food source.

Pest Control

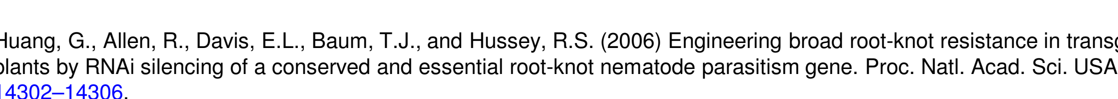


Control, infected by parasitic nematode



RNAi-inducing – no infection

Plants expressing dsRNA corresponding to insect or nematode genes are resistant to these pests! When ingested, the dsRNA induces gene silencing.


Huang, G., Allen, R., Davis, E.L., Baum, T.J., and Hussey, R.S. (2006) Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. Proc. Natl. Acad. Sci. USA 103: 14302–14306.

Approaches for RNAi-based Silencing (targeted)

- Antisense-mediated gene silencing
- Hairpin-loop transcript-based RNAi
- Artificial microRNA (amiR)
- VIGS (Virus-induced gene silencing)

Transgene Silencing

Inattivazione epigenetica dovuta a presenza di sequenze/geni espressi a livelli molto elevati (per esempio a causa di copie multiple nel genoma); dipende da interazione tra copie omologhe, i.e. **Homology-Dependent Gene Silencing** (HDGS)

Si distinguono due tipi di HDGS:

- **Transcriptional Gene Silencing (TGS)**
- **Post Transcriptional Gene Silencing (PTGS)**

- Entrambi i tipi di HDGS sono frequentemente associati con "sequence-specific *de novo* methylation"
- Entrambi i tipi di HDGS possono essere associati con la presenza di IRs
- HDGS puo' dare inattivazione sia in *cis* che in *trans*

Transcriptional Inactivation (methylation)

- **CIS Inactivation**

TGS

- Assenza sia dei trascritti maturi che dei precursori

- Lo stato silenziato è mantenuto ad ogni ciclo mitotico, ed è anche trasmesso alla progenie.

- A bassa frequenza, si osserva riattivazione spontanea (reversione) del locus silenziato.

- Geni (endogeni e/o trans) silenziati sono caratterizzati da pattern alterato di metilazione (strategia tipicamente vegetale)

- Struttura cromatinica alterata (come in Drosphila e lievito)

PTGS

PTGS = Post-Transcriptional Gene Silencing

PTGS is a sequence-specific RNA degradation process that targets foreign RNA (details to follow).

- This includes:
  - viral RNA
  - transposon RNA
  - dsRNA, etc.

- **Why is PTGS significant with respect to plant viruses?**
  - PTGS is a mechanism that plants have developed for protection from virus infection (i.e., the plant PTGS system degrades viral RNA)

PTGS

PTGS come soppressione dell'espressione genica x degradazione del (trans)gene RNA

- Avviene nel citoplasma, ed è gene-specifico
- Il livello di trascrizione è inalterato (run-on)
- Rappresenta un meccanismo di difesa dalle infezioni virali

Geni (endogeni e/o trans) silenziati sono caratterizzati da:

- pattern alterato di metilazione (strategia tipicamente vegetale)
- struttura cromatinica alterata (come in Drosphila e lievito)

Post-transcriptional gene silencing across kingdoms.

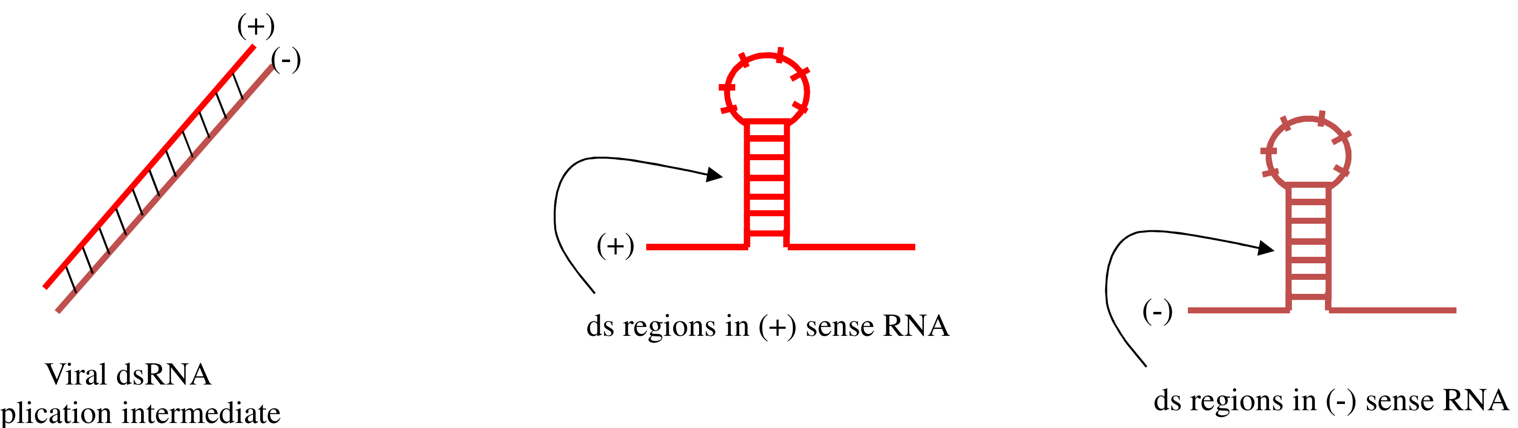
Kingdom	Species	Phenomenon	Trigger
Fungi	*Neurospora*	Quelling	Transgenes
Plants	*Petunia*, *Nicotiana*, *Arabidopsis*, tomato, rice, potato, etc.	PTGS, co-suppression	Transgenes, viruses (dsRNA form)
Animals			
Invertebrates	*C. elegans*	RNAi	dsRNA
	Drosophila	RNAi	dsRNA
	Paramecium	Co-suppression	Transgenes
	Planaria	RNAi	dsRNA
	Hydra	RNAi	dsRNA
	T. brucei	RNAi	dsRNA
Vertebrates	Zebrafish	RNAi	dsRNA
	Mouse	RNAi	dsRNA

RNAi = RNA interference

Note different terms used for same phenomenon:
PTGS = quelling = co-suppression = VIGS = RNA interference

What triggers (induces) the PTGS response when a virus infects a plant?

- Inducers of PTGS:
    1. Viral dsRNA (the double-stranded replication intermediate that arises during virus infection)
    2. Viral plus sense RNA (the double-stranded regions)
    3. Viral minus sense RNA (the double-stranded regions)



The PTGS pathway has two distinct phases:

1. **Initiation** – the viral RNA triggers the PTGS system to degrade viral RNA into small pieces (called siRNA or small interfering RNA)

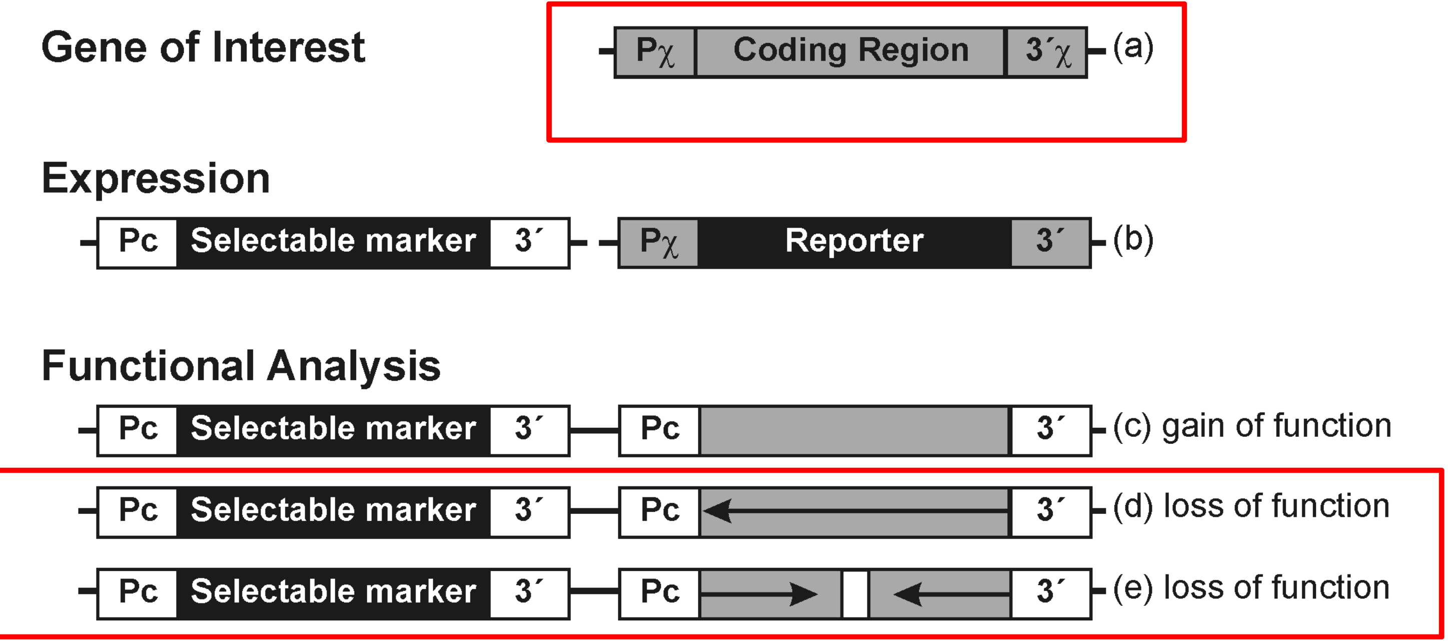
2. **Maintenance-**the siRNA binds to complementary regions in viral RNA and this is either:

   a. degraded by a complex called RISC (RNA-Induced Silencing Complex)
   or

   b. or is used to make more viral RNA via the host RNA dependent RNA polymerase. The resulting dsRNA then feeds back into the system at the point where dsRNA is degraded to siRNA and the cycle continues to repeat.

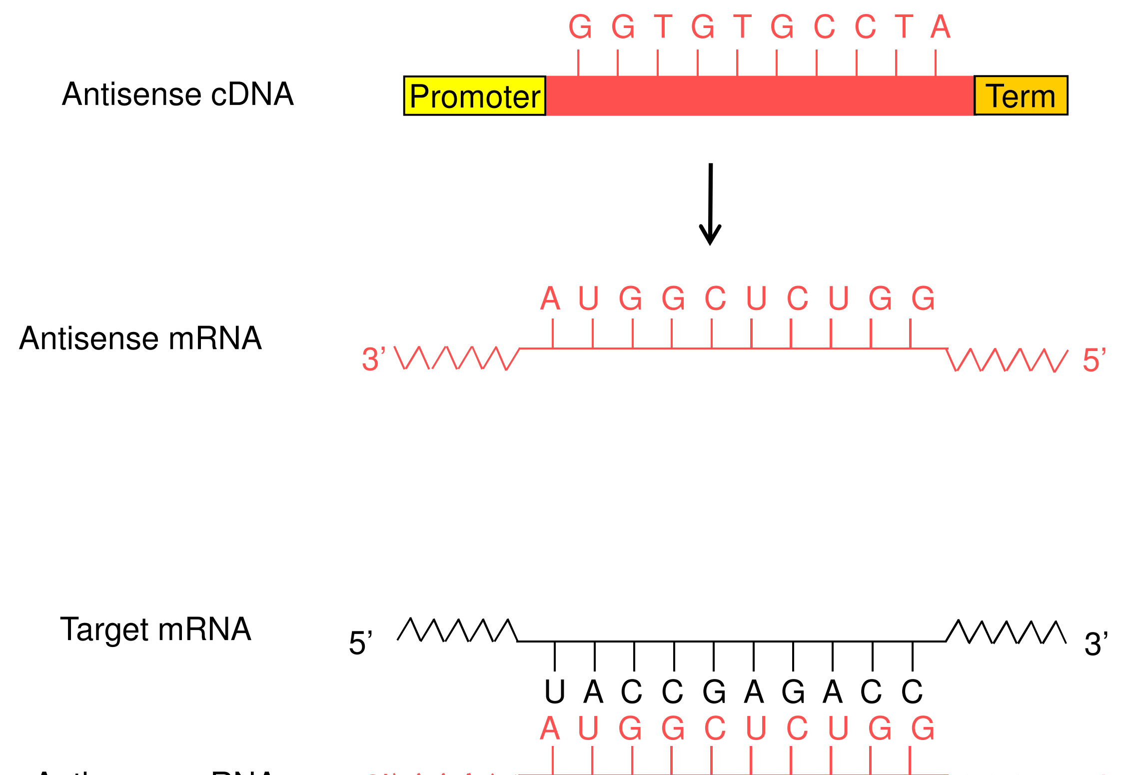
Figure 9.1

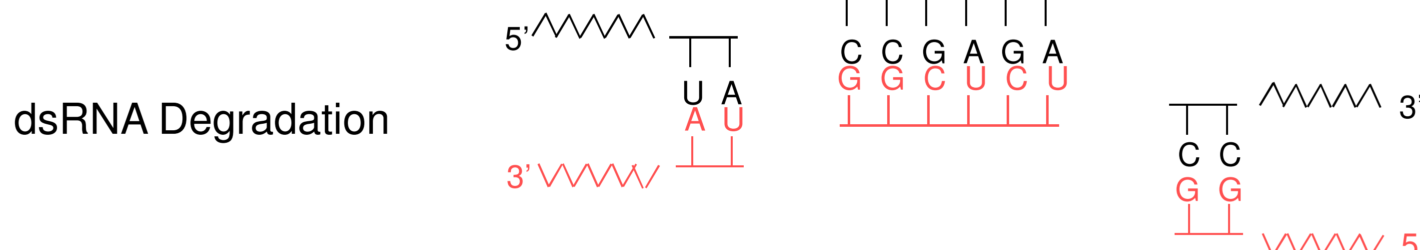
Types of cassettes



Antisense-mediated gene silencing

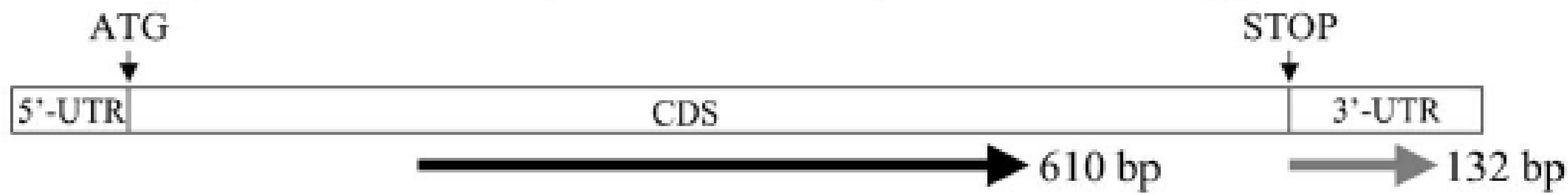
To reduce the expression of a target gene by expression of sequences complementary to the target sequence.


G G T G T G C C T A
Antisense cDNA
Promoter
Term
A U G G C U C U G G
Antisense mRNA
3'
5'
Target mRNA
5'
3'
U A C C G A G A C C
A U G G C U C U G G
Antisense mRNA
3'
5'

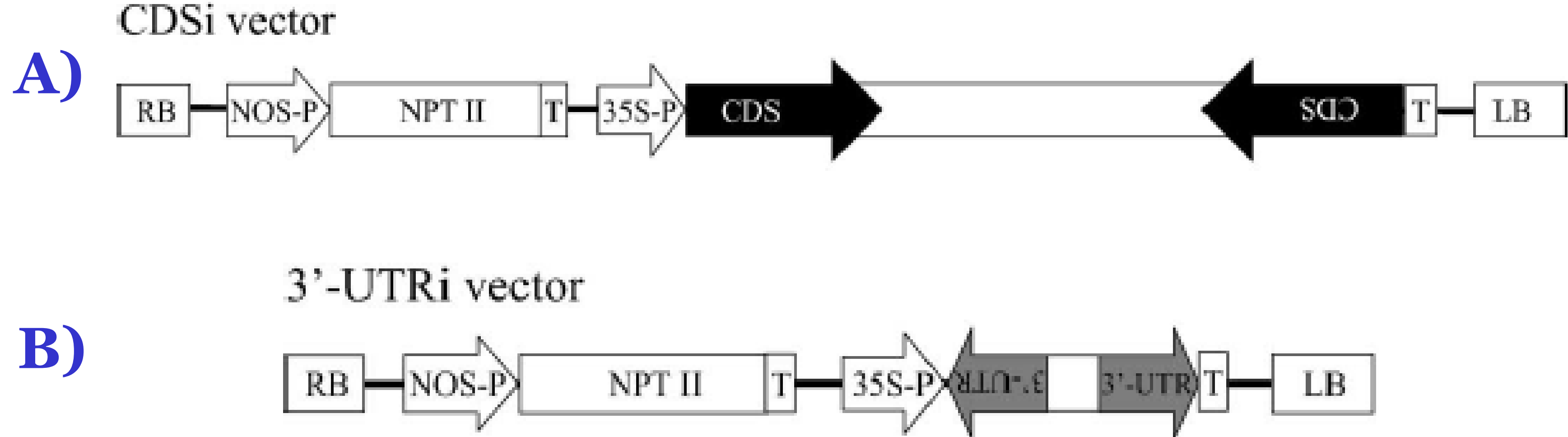

dsRNA Degradation
5'
U A
A U
3'
C C G A G A
G G C U C U
3'
C C
G G
5'

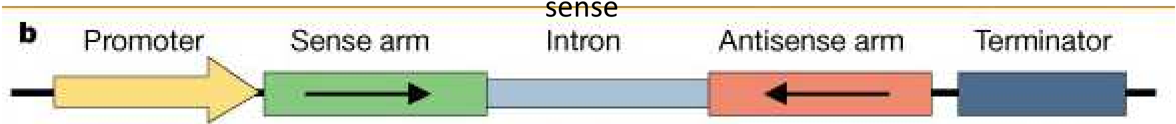
Hairpin loop constructs

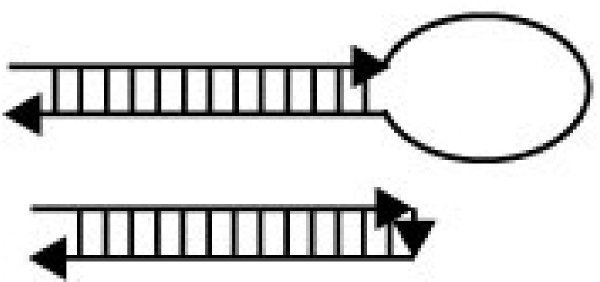
Il cDNA della Calcone sintasi



I costrutti per l'RNAi




sense
b
Promoter
Sense arm
Intron
Antisense arm
Terminator



VIGS (Virus Induced Gene Silencing)

- Vettori virali che portano un frammento del gene target per generare un dsRNA che ne induce il silenziamento

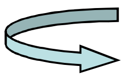
- Possibilità di silenziare un gene specifico senza trasformare geneticamente la pianta studiata

- Permette di vincere il problema della ridondanza funzionale legato al knock-down di un gene target

Constructs for RNAi

- Hairpin Loop
- miRNA artificiali

- **Virus a DNA** **:** derivato del *Cabbage leaf curl geminivirus* (CbLCV)



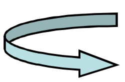
Svantaggi:

-difficoltà nell'inserire il virus nella pianta tramite bombardamento di particelle

-Dimensioni limitate dell'inserto

2. **Virus a RNA** **:** ***T**obacco **R**attle **V**irus* (TRV), RNA1 e RNA2

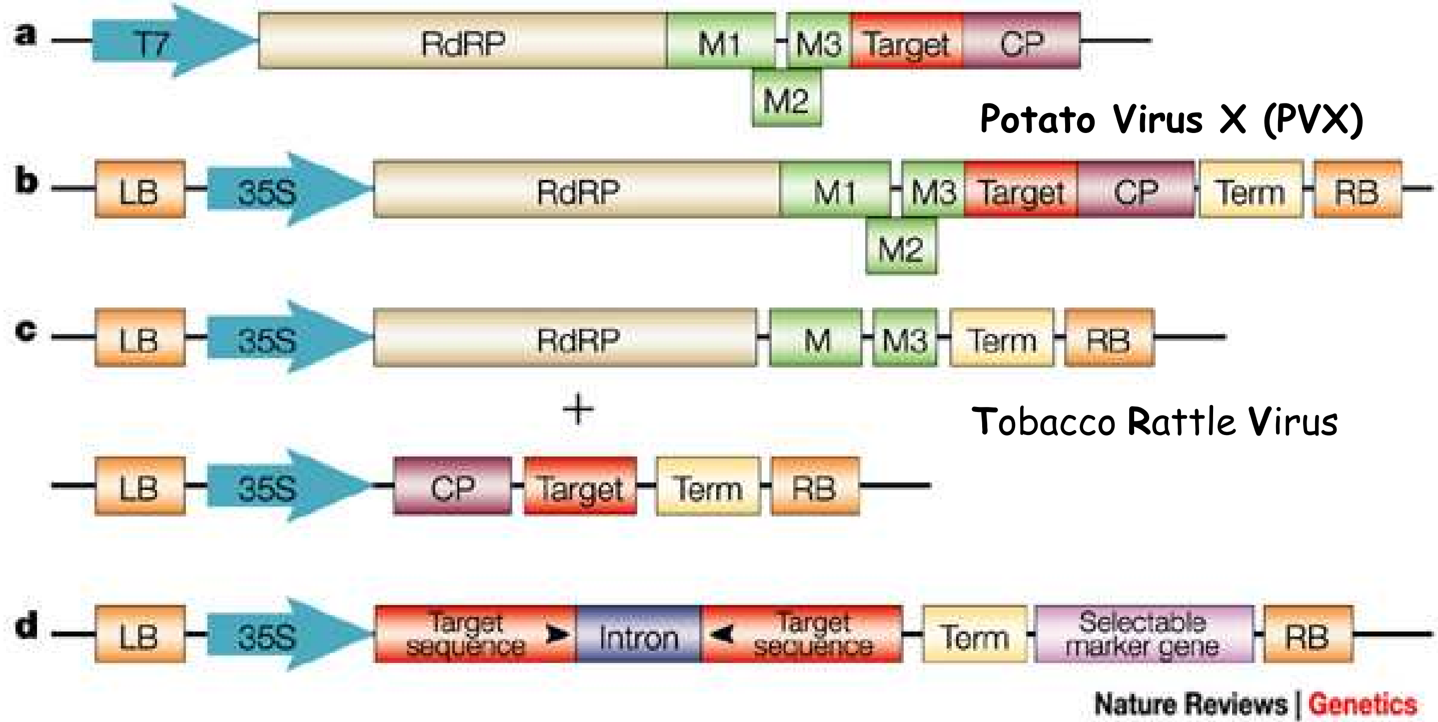
Vantaggi:



-In natura infetta più di 100 specie, in laboratorio più di 400

-Capacità di mediare VIGS in assenza di sintomi indotti dal virus

-Capacità di veicolare gli inserti nei punti di crescita della pianta (meristemi)



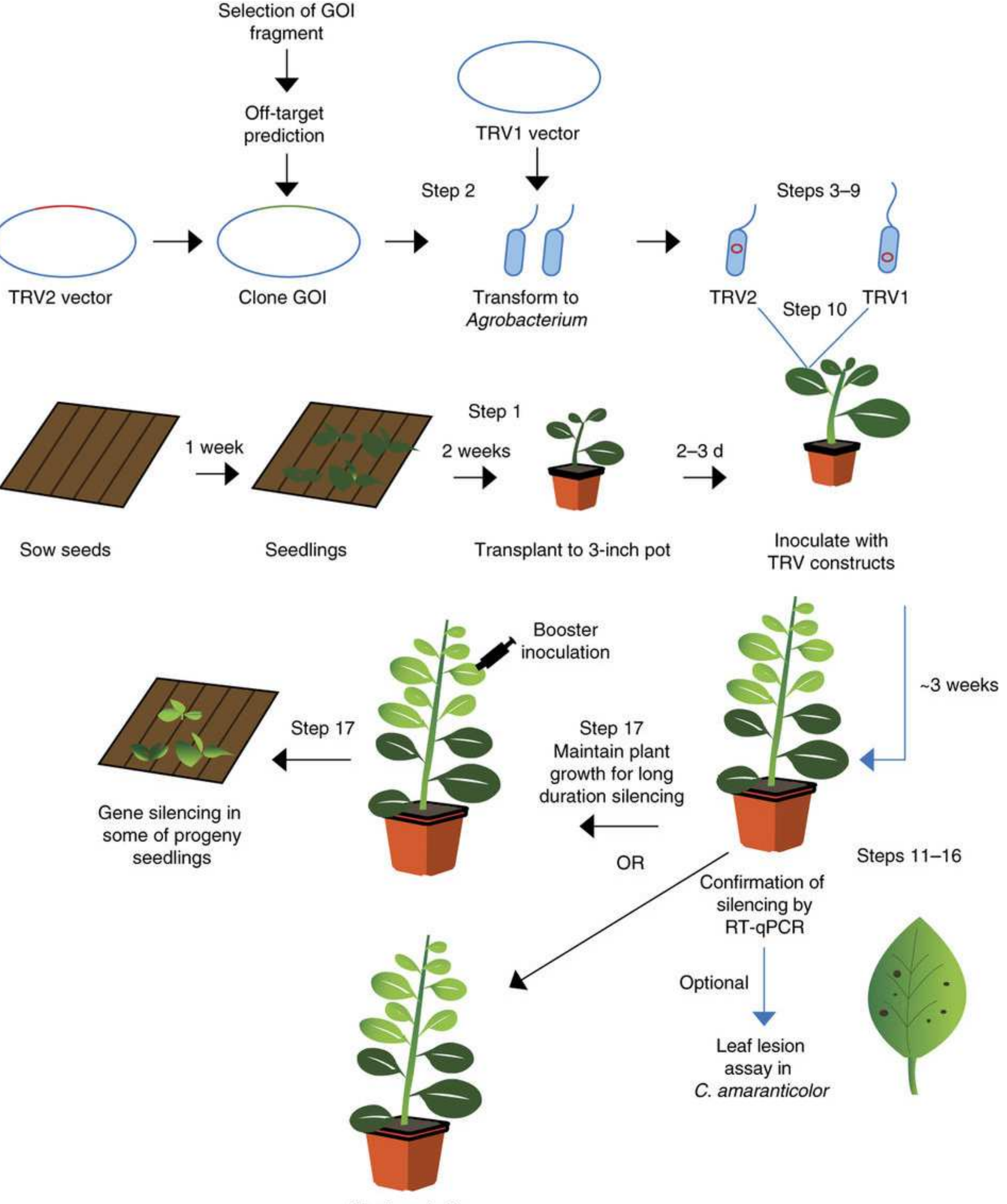
DNA constructs for RNA-mediated gene silencing. **a** | A DNA plasmid that can be propagated in *Escherichia coli* from which infectious potato virus X (PVX) RNA can be transcribed *in vitro*, using T7 polymerase. The PVX cassette contains sequence derived from the gene to be targeted. **b** | A transferred (T)-DNA plasmid that is propagated in *Agrobacterium*. When this plasmid-carrying *Agrobacterium* is inoculated onto a plant, it transfers the DNA between its left (LB) and right (RB) borders into the plant's cells. The region between the borders contains the viral sequences shown in part **a**, but in this vector, the T7 promoter has been replaced with the cauliflower mosaic virus promoter. This enables the transferred DNA to be transcribed by the plant's endogenous transcription machinery to generate infectious PVX (plus insert sequence) RNA. In amplicon transgene vectors, a selectable maker gene is also present between the left and right borders of this plasmid, enabling plants to be stably transformed with the transferred DNA. **c** | The tobacco rattle virus (TRV) virus-induced gene-silencing (VIGS) system. Two T-DNA plasmids that encode the TRV genome (one encoding TRV RNA1 and the other encoding TRV RNA2, which carries the inserted target sequence) are propagated separately in *Agrobacterium* and used to co-infect plant tissue. **d** | A typical T-DNA plasmid for the expression of hairpin RNAs (hpRNAs). This plasmid can be transiently introduced into plants by bombardment or stably introduced by agroinfiltration. A generic silencing precursor construct (pHANNIBAL) that enables hpRNA vectors to be easily constructed has different multiple cloning sites either side of the intron to enable the rapid insertion of target sequences in forward and reverse orientations. 35S, CaMV 35S promoter; CP, coat protein; M1,2,3, movement proteins 1, 2, 3; RdRP, RNA-dependent RNA polymerase; T7, T7 promoter; Term, transcription termination sequence.

V I G S

• Non necessita di trasformazione genetica delle piante
• Consente lo studio del silenziamento di *geni letali*
• Supera il problema della ridondanza genica attraverso al silenziamento di famiglie geniche

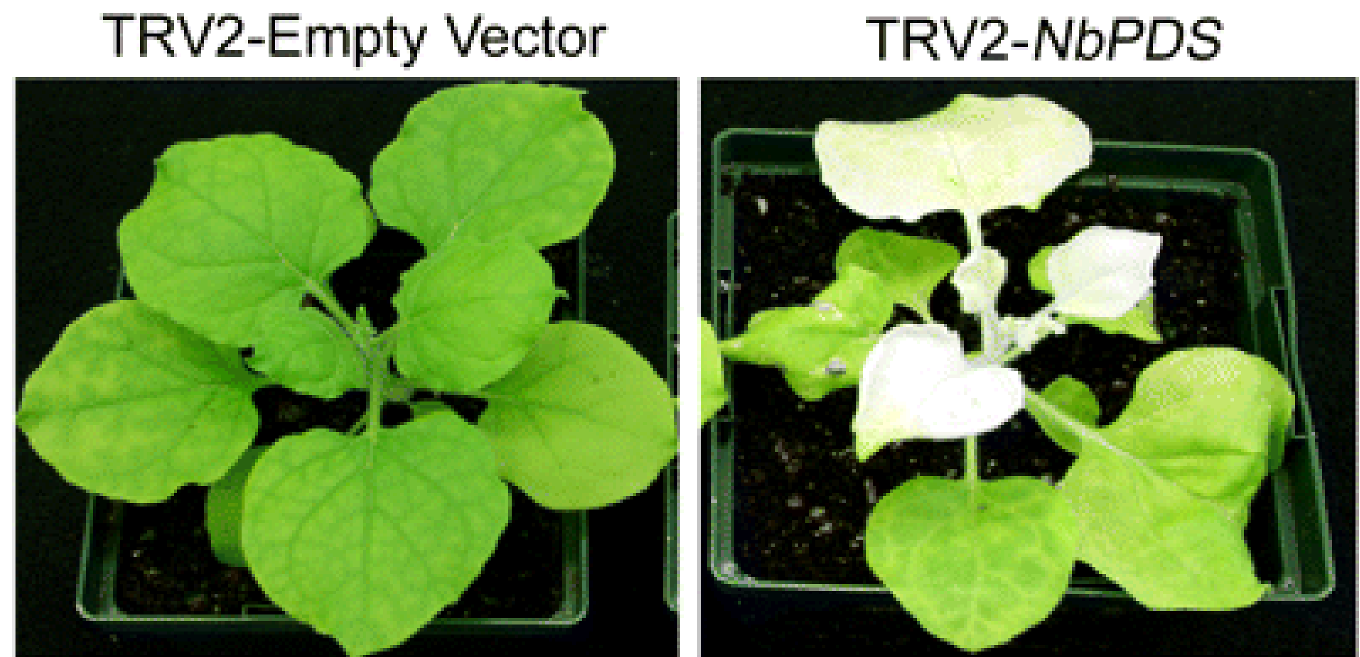
Svantaggi

• Distorsione fogliare dovuta all'infezione
• Necrosi tissutale dovuta all'infezione
• Bassa efficienza d'infezione degli *apici meristematici*



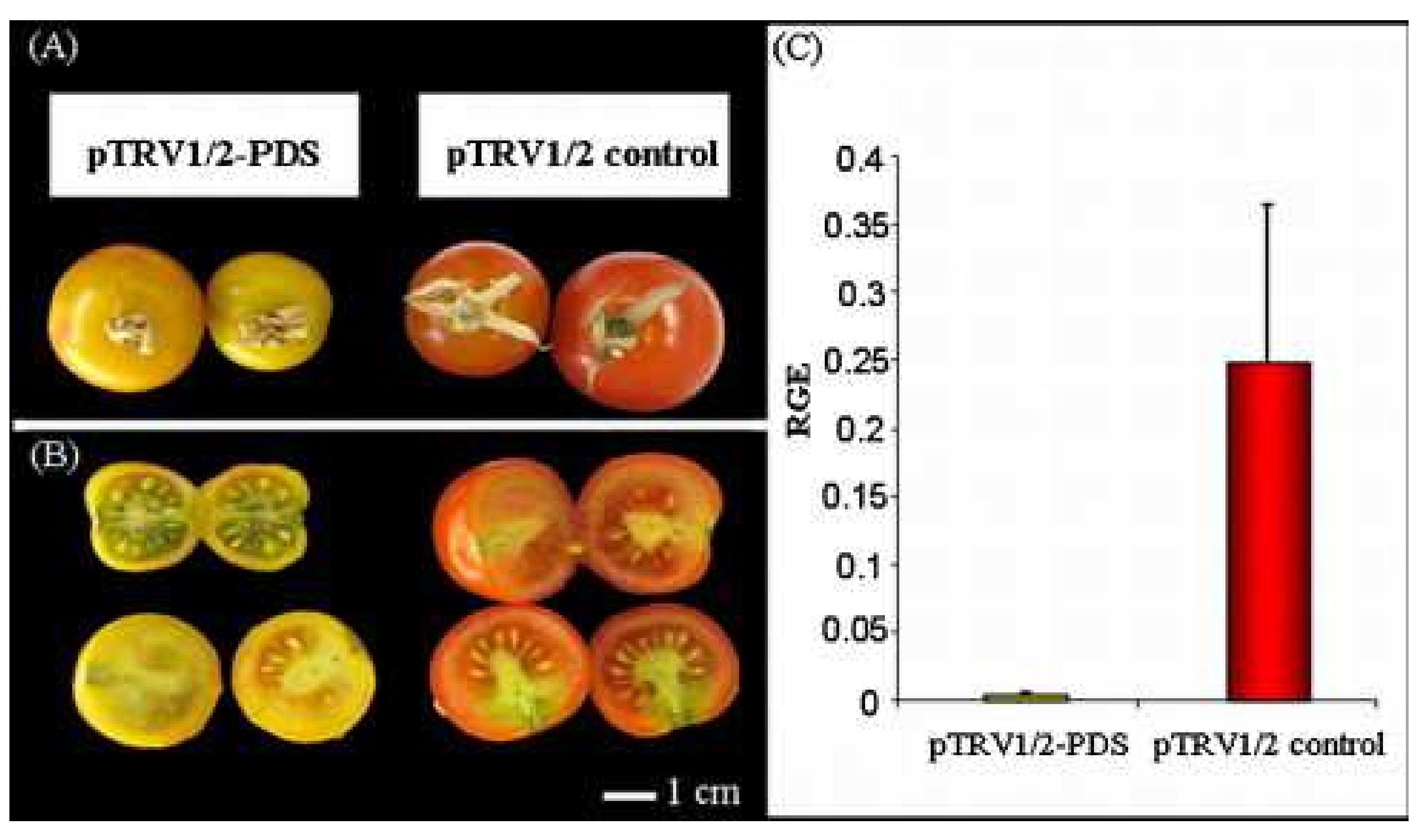
Selection of GOI fragment
Off-target prediction
TRV1 vector
Step 2
Steps 3–9
TRV2 vector
Clone GOI
Transform to *Agrobacterium*
TRV2
Step 10
TRV1
Step 1
1 week
2 weeks
2–3 d
Sow seeds
Seedlings
Transplant to 3-inch pot
Inoculate with TRV constructs
Booster inoculation
~3 weeks
Step 17
Step 17
Maintain plant growth for long duration silencing
OR
Gene silencing in some of progeny seedlings
Steps 11–16
Confirmation of silencing by RT-qPCR
Optional
Leaf lesion assay in *C. amaranticolor*
Plant analysis

VIGS del gene FITOENE DESATURASI (PDS) in *Nicotiana benthamiana*



N.B.: PDS serve alla sintesi dei carotenoidi -> l'assenza di carotenoidi porta a photobleaching (sbiancamento per stress ossidativo)

VIGS del gene FITOENE DESATURASI (PDS) in frutti di pomodoro



Some Uses of PTGS in Agriculture

- **Functional genomics/identification of gene function**
  - Expression of specific genes with unknown function can be silenced via *Agrobacterium* or a viral vector (i.e., VIGS) and the effect on the plant can be analyzed.
- **Overexpression of genes (use of viral suppressor)**
  - Sometimes difficult to express high levels of proteins due to PTGS. Viral suppressors of gene silencing can be used to inhibit PTGS and therefore achieve high level expression
- **Inhibiting virus expression**
  - Transform plant with a virus gene. The plant then becomes resistant to virus infection via PTGS pathway

Table 2. Use of RNAi for virus resistance in plants

Name of virus	Family	Region targeted	Results	System used	Genome	Refs
Potato virus Y	*Potyviridae*	HC-Pro	Immunity	Potato	RNA	[5]
Mungbean yellow mosaic India virus (MYMIV)	*Geminiviridae*	Bidirectional promoter	Recovery from infection	*Vigna mungo* (black gram)	DNA	[23]
African cassava mosaic virus (ACMV)	*Geminiviridae*	Replication-associated protein gene	Reduced virus accumulation	Tobacco protoplast	DNA	[26]
Tomato yellow leaf curl Sardinia virus	*Geminiviridae*	Replication-associated protein gene	Poor resistance	Tomato	DNA	[27]
Pepper mild mottle virus (PMMoV)	*Tobamoviridae*	Arbitrary sequence	Block in viral infectivity	Tobacco	RNA	[11]
Tobacco etch virus (TEV)	*Potyviridae*	Arbitrary sequence	No viral-specific symptoms appeared	Tobacco	RNA	[11]
Alfalfa mosaic virus (AMV)	*Bromoviridae*	Arbitrary sequence	Recovery from infection	Tobacco	RNA	[11]
Beet necrotic yellow vein virus (BNYVV)	*Benyviridae*	Coat protein	Tolerance	Tobacco	RNA	[28]
Tobacco mosaic virus (TMV)	*Tobamoviridae*	Replication-associated protein	Inhibition of TMV replication	Tobacco	RNA	[52]

Abbreviation: HC-PRO, helper-component proteinase gene.

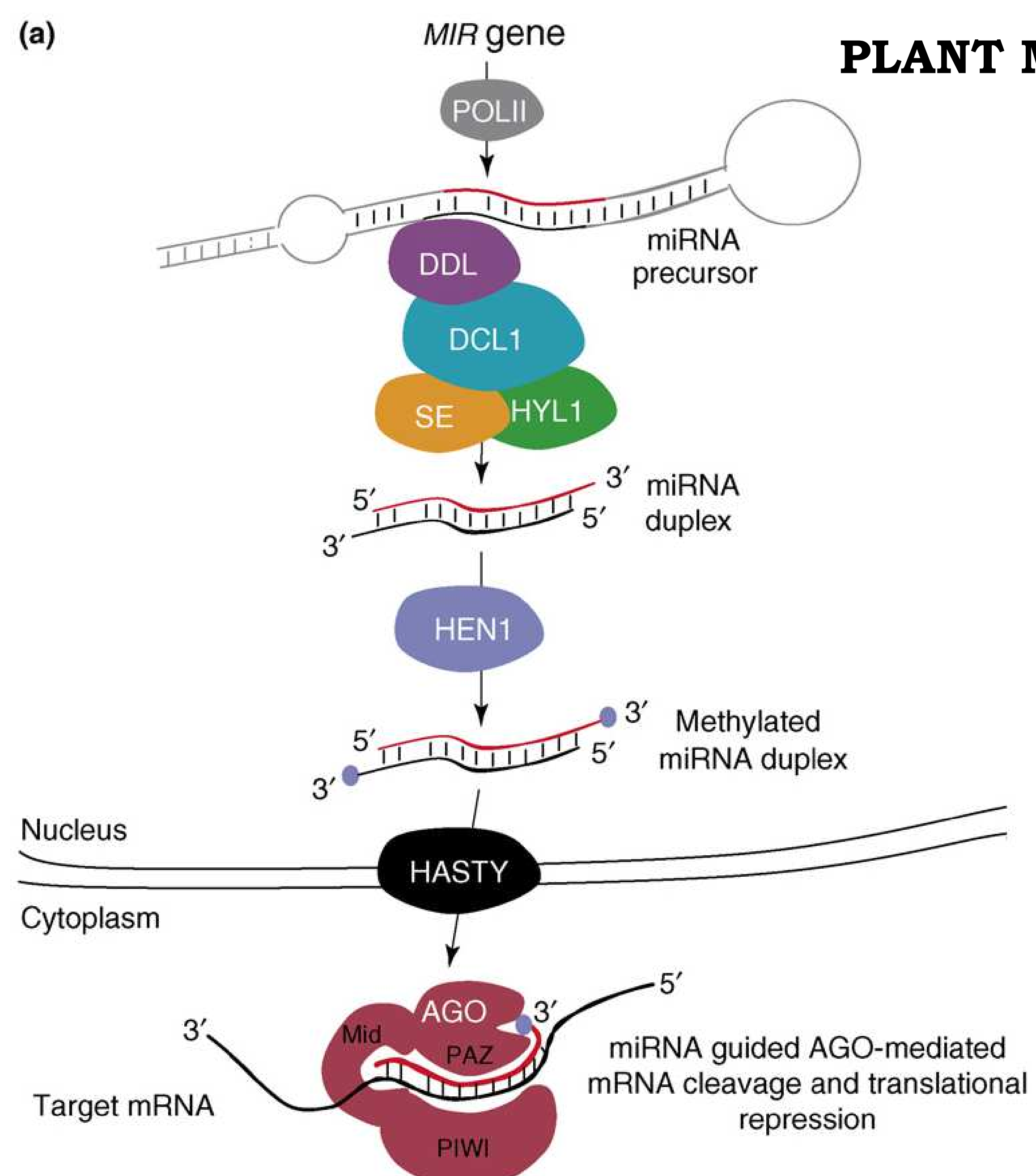
Table 1. Use of RNAi in metabolic engineering of plants

Trait	Target gene	Host plant	Potential benefit	Refs
Enzymatic browning	Polyphenyl oxidase gene	Potato	Extended storage life	[7]
Increased stearic acid and oleic acid content of seed oil	ghSAD-1 and ghFAD2-1 genes	Cotton	Useful for cooking applications without the need for hydrogenation	[13]
Reduced caffeine production	CaMxMt 1 gene	Coffee bean plant	Decaffeinated coffee	[15]
Reduced or absent petals	BP1 gene	Oilseed rape	Improved photosynthesis	[16]
Non-narcotic alkaloid production	Codeine reductase (COR) gene	Opium poppy	–[a]	[17]
Increased carotenoid and flavonoid content	DET1 gene	Tomato	Consumer health benefits	[18]
Flower colour	CHI gene	Tobacco	–[a]	[49]
Maize quality	Starch branching enzyme	Maize	Up to 50% increase in amylose content	[50]
Allergy	Lol p1 and Lol p2	Ryegrass (*Lolium* spp.)	Hypo-allergic ryegrass	[14]
Reduced ethylene sensitivity	1-Aminocyclo propane-1-carboxylate oxidase	Tomato	Longer shelf life (slower ripening)	[51]
Increased arsenic uptake	ACR2 gene	*Arabidopsis*	Phytoremediation of soils	[19]

[a]No direct benefit because these experiments were designed to demonstrate 'proof of concept'.

SILENZIAMENTO INDOTTO DA microRNA ARTIFICIALI

PLANT MICRO-RNA BIOGENESIS



Allison C Mallory, Taline Elmayan and Herve´ Vaucheret, Current Opinion in Plant Biology 2008

The Plant Cell, Vol. 18, 1121–1133, May 2006, www.plantcell.org © 2006 American Society of Plant Biologists

RESEARCH ARTICLES

Highly Specific Gene Silencing by Artificial MicroRNAs in *Arabidopsis*

Rebecca Schwab, Stephan Ossowski, Markus Riester, Norman Warthmann, and Detlef Weigel[1]
Department of Molecular Biology, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany

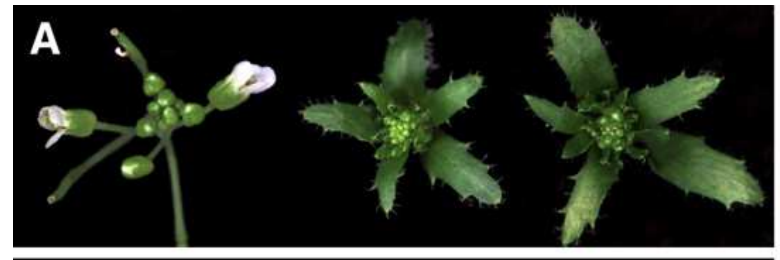
Compared with conventional RNAi, amiRNAs offer several advantages:

1) miRNA precursors generally generate only a single effective small RNA of known sequence. By contrast, several siRNAs with undefined 5’ and 3’ ends are produced as a silencing trigger from hairpin constructs. Therefore, potential off-targets of amiRNAs can be more accurately predicted than those of longer hairpin constructs.

2) because of their exquisite specificity, amiRNAs can possibly be adapted for allele-specific knockouts.

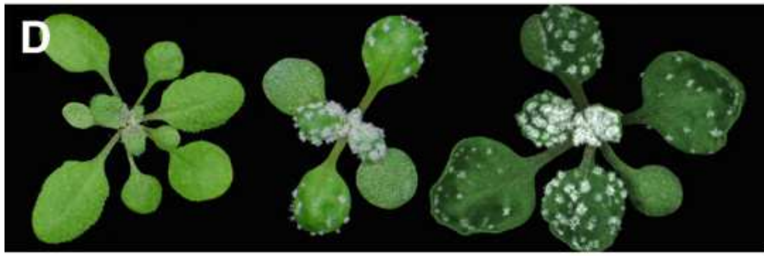
3) as with natural miRNAs, amiRNAs are likely to be particularly useful for targeting groups of closely related genes, including tandemly arrayed genes. Approximately 4000 genes in Arabidopsis are found in tandem arrays (Arabidopsis Genome Initiative, 2000), and no convenient tool exists for their knockout.

PHENOTYPES OF amiRNA OVEREXPRESSERS

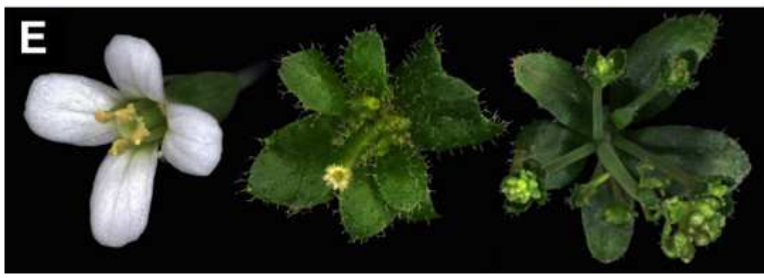


(A) Inflorescences. From left to right: the wild type, lfy-12, and amiR-lfy-1 (MIR172a backbone) overexpresser.

(B) Seedlings. From left to right: the wild type, gun4-1, and amiR-white-1 (MIR172a backbone) overexpresser. Bleaching of cotyledons is more pronounced in the amiR-white plants than in gun4-1, consistent with the more severe molecular profile of the amiR-white overexpressers.



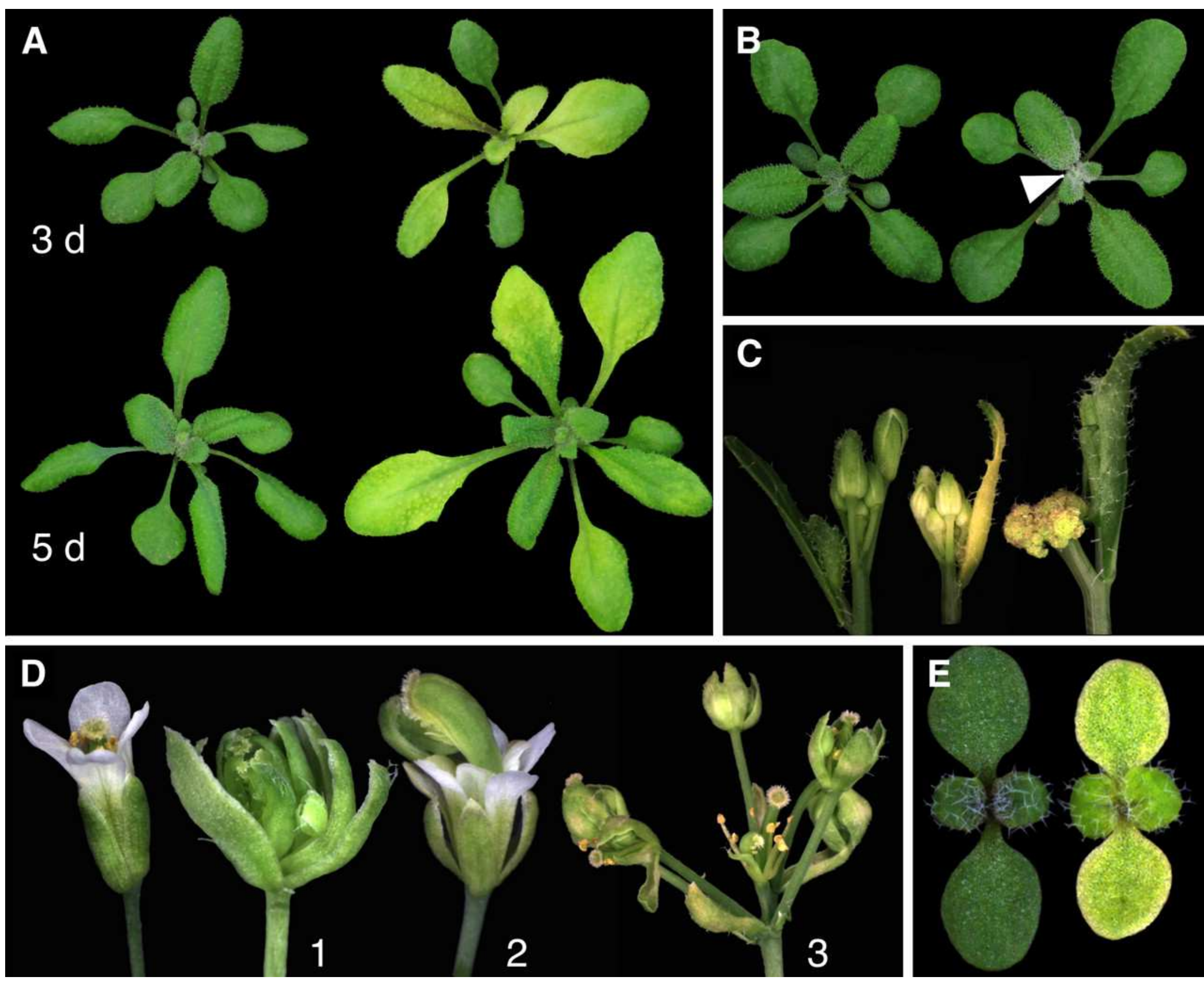
(D) Leaf rosettes. From left to right: the wild type, try cpc double mutants, and amiR-trichome (MIR319a backbone) overexpresser. Clustered trichomes are evident even at low magnification.



(E) Flowers. From left to right: the wild type, weak amiR-mads-2 (MIR319a backbone) overexpresser, and strong amiR-mads-2 (MIR319a backbone) overexpresser. In the strong line, secondary inflorescences replace the central gynoecium.

Schwab R. et.al. Plant Cell 2006:18:1121-1133

INDUCIBLE AND TISSUE-SPECIFIC EXPRESSION OF AMIRNAs



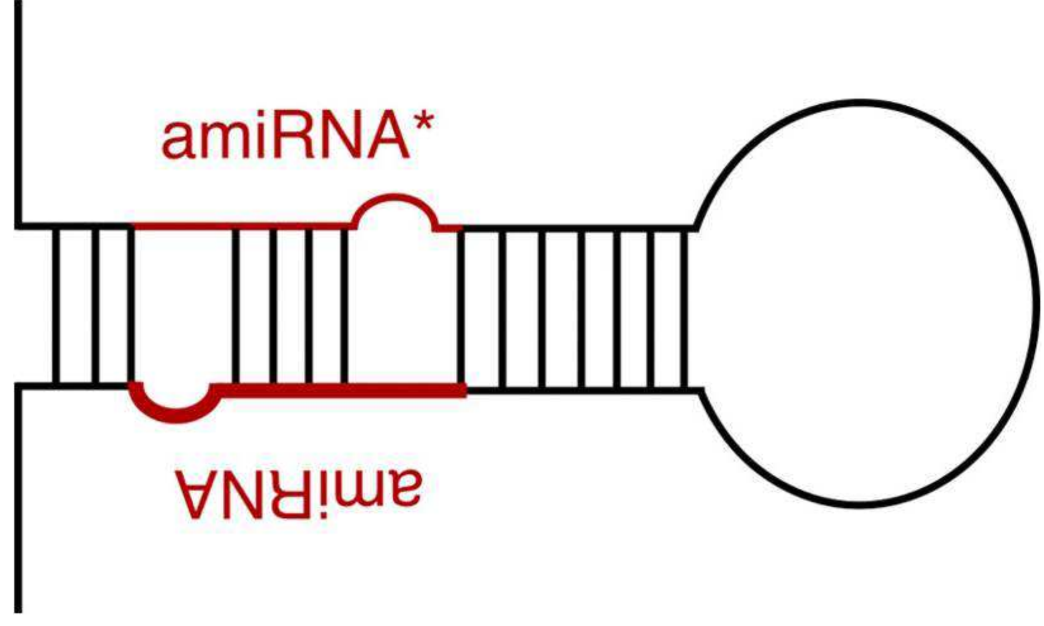
Schwab R. et.al. Plant Cell 2006:18:1121-1133

ENGINEERING OF AMIRNAs

miR319 (BACKBONE):

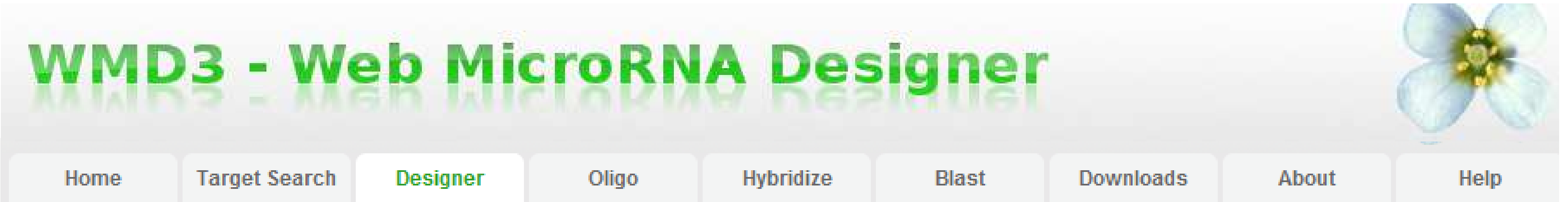
caaacacacgctcggacgcatattacacatgttcatacacttaatactcgctgttttgaatt
gatgttttaggaatatatatgt**ag****agagagcttccttgagtccat****tcacaggtcgtgatatgattaatta**
gcttccgactcattcatccaaataccgagtcgccaaaattcaaactagactcgttaaatgaatgaatgatgcg
gtagacaaattggatcattgattctcttga**ttggactgaagggagctccct****ct**ctcttttgtatccaatt
ttcttgattaatctttcctgcacaaaaacatgcttgatccactaagtgacatatatgctgcc
ttcgtatatatagttctggtaaaattaacattttgggtttatctttatttaaggcatcgcca
tg

miRNA319
miRNA319*



Schwab R. et.al. Plant Cell 2006:18:1121-1133

ENGINEERING OF AMIRNAS



Designer

Input Examples: A.thaliana Multi A.thaliana Single O.sativa Single G.max Single P.trichocarpa Single

Target genes:		Help
Genome:	Arabidopsis thaliana cDNA (TAIR8)	Help
Minimum number of included targets:		Help
Accepted off-targets:		Help
Description:		Help
Email:		Help

http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home;project=stdwmd

ARTIFICIAL MICRORNA SELECTION CRITERIA

There are still some criteria, which have to be considered when choosing the final amiRNA. Most of them have been implemented into the ranking process, and they should be considered here again, especially when multiple genes are targeted simultaneously.
We prefer (not require):

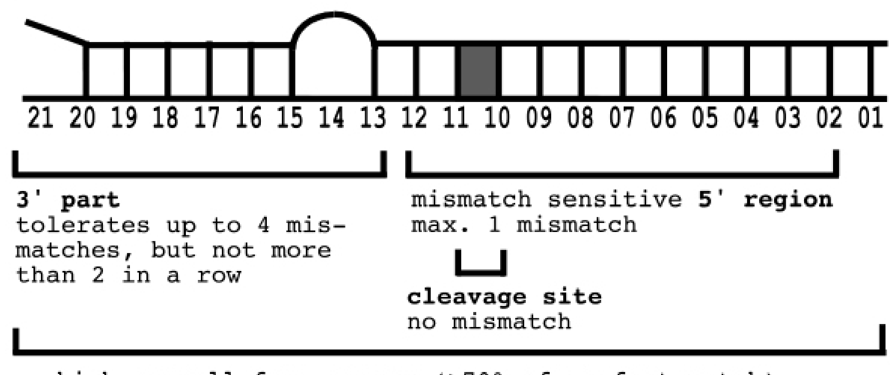
1. No mismatch between positions 2 and 12 of the amiRNA for all targets.
   Mismatches are not allowed for the target gene that is used as a template, but they might come up for additional intended targets since the target determinants allow for one mismatch.

2. One (or two) mismatches at the amiRNA 3? end (pos.18-21).
   There is no evidence for transitive formation of secondary siRNAs from amiRNA targets, but if there was, this mismatch should reduce the process.

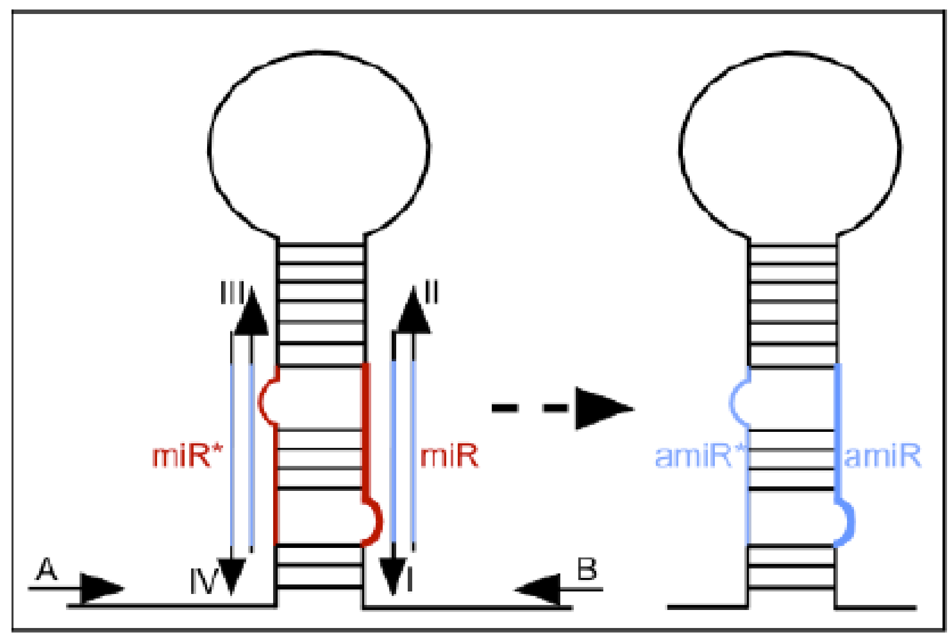
3. Similar mismatch pattern for all intended targets.
   There is no evidence that the pattern of mismatches matters, but similar patterns definitely don't hurt.

4. Absolute Hybridization energy between -35 and -38 kcal/mole.
   These are the values observed for most endogenous miRNA targets. We don?t consider amiRNAs which pair to intended targets with energies higher than -30 kcal/mole.

5. Target site position.
   There is no evidence that the position of the target site in the target transcript has an effect on effectiveness, but target sites in most endogenous miRNA targets are found towards the 3' end of the coding regions. Examples in the 3'UTR are also not uncommon.



CLONING STRATEGY



I: microRNA forward
II: microRNA reverse
III: microRNA* forward
IV: microRNA* reverse

	forward oligo	reverse oligo	template
(a)	A	IV	pRS300
(b)	III	II	pRS300
(c)	I	B	pRS300
(d)	A	B	(a)+(b)+(c)

Schwab R. et.al. Plant Cell 2006:18:1121-1133

DNA CLONING USING IN VITRO SITE-SPECIFIC RECOMBINATION

The Gateway reactions:

1)

P1 ccdB P2
Donor vector

+

B1 B2
attB PCR product

BP Clonase™ II

L1 L2
Entry Clone

2)

attL1 attL2 Entry clone Kan[R]

\+

attR1 ccdB attR2 Destination vector Amp[R]

LR clonase

BP clonase

attB1 attB2 Expression clone Amp[R]

\+

attP1 attP2 Donor vector Kan[R]

pMDC7
13228 bp

G10-90
Xba I (161)
Hin dIII (115)
Bam HI (167)
Bam HI (109)
Bam HI (271)
Pst I (99)
Sac I (593)
Sma I (680)
Hin dIII (83)
Bam HI (792)
Hin dIII (971)
Xba I (1089)
Nco I (1139)
RB
Bgl II (1223)
XVE
Pst I (1277)
Pst I (1448)
Sac I (1748)
Eco RI (1756)
Hin dIII (1825)
Hin dIII (2178)
Bam HI (2514)
Cla I (10059)
Eco RI (2769)
Pst I (2863)
Nco I (2878)
hygromycin resistance
Pst I (3852)
lexA -46 35S promoter
LB
Xho I (4200)
Asc I (4206)
Eco RI (6621)
attR1
Bam HI (4418)
Sac I (6619)
Eco RI (4668)
chloramphenicol resistance
Kpn I (6613)
Nco I (4969)
ccdB
Bgl II (5115)
Eco RI (6451)
Pst I (5794)
Bam HI (5121)
Sma I (5539)
attR2
Pac I (5941)
Kpn I (6441)
Spe I (5944)
T3A

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An Arabidopsis Mitogen-Activated Protein Kinase Kinase Kinase Gene Family Encodes Essential Positive Regulators of Cytokinesis

Patrick J. Krysan,[1,2] Peter J. Jester, Jennifer R. Gottwald, and Michael R. Sussman
Biotechnology Center, University of Wisconsin–Madison, 425 Henry Mall, Madison, Wisconsin 53706

Hormone Sensitivity

The results described above demonstrate that the *ANP* genes are involved in the control of cellular growth and division. Therefore, we tested the sensitivity of the *anp2 anp3* plants to the phytohormones abscisic acid, auxin, brassinosteroids, cytokinin, ethylene, and gibberellin using agar plate assays. Vertically oriented plates were grown either in the dark for 3 days or in constant light for 1 week. During growth in the light, the plants were observed daily. None of these exogenous hormone treatments "rescued" the mutant phenotype of the double mutants. In addition, all of the mutants displayed a level of sensitivity to each hormone treatment that was equivalent to that of the

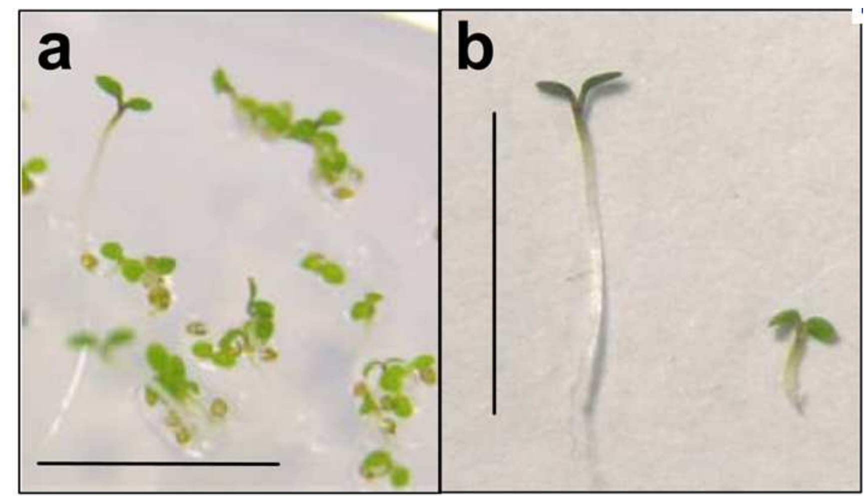
wild type (i.e., the degree of growth inhibition or stimulation was the same; data not shown). Furthermore, we observed no qualitative changes in growth that were unique to the mutant genotypes under the various hormone treatments.

A

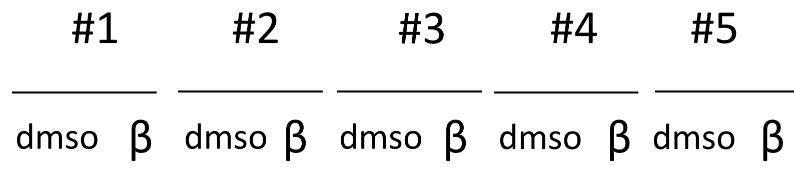
Genome-Wide Gene Expression Analysis

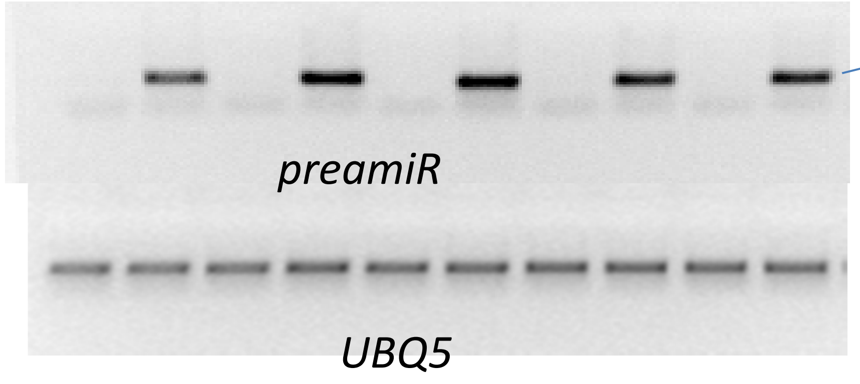
To gain additional insight into the signaling pathways affected by the *ANP* mutations, we used the Affymetrix Arabidopsis Gene Chip to compare the RNA levels for 8200 genes in *anp2 anp3* double-mutant plants and wild-type plants. Plants were grown in soil for 11 days under constant light, and the aerial tissue then was harvested and used for RNA analysis. Overall, RNA levels increased threefold or greater for 211 genes, whereas levels decreased threefold or greater for 30 genes (see supplemental material at www.biotech.wisc.edu/krysan/). The most striking result from the gene chip experiment was that a number of pathogen- and stress-related genes were upregulated in the *anp2 anp3* plants. These genes include numerous disease-resistance genes, chitinases, glucanases, peroxidases, glutathione *S*-transferases, and several heat shock–related genes

TRANSGENIC PLANTS SELECTON AND ANALYSIS



Hygromycin resistance





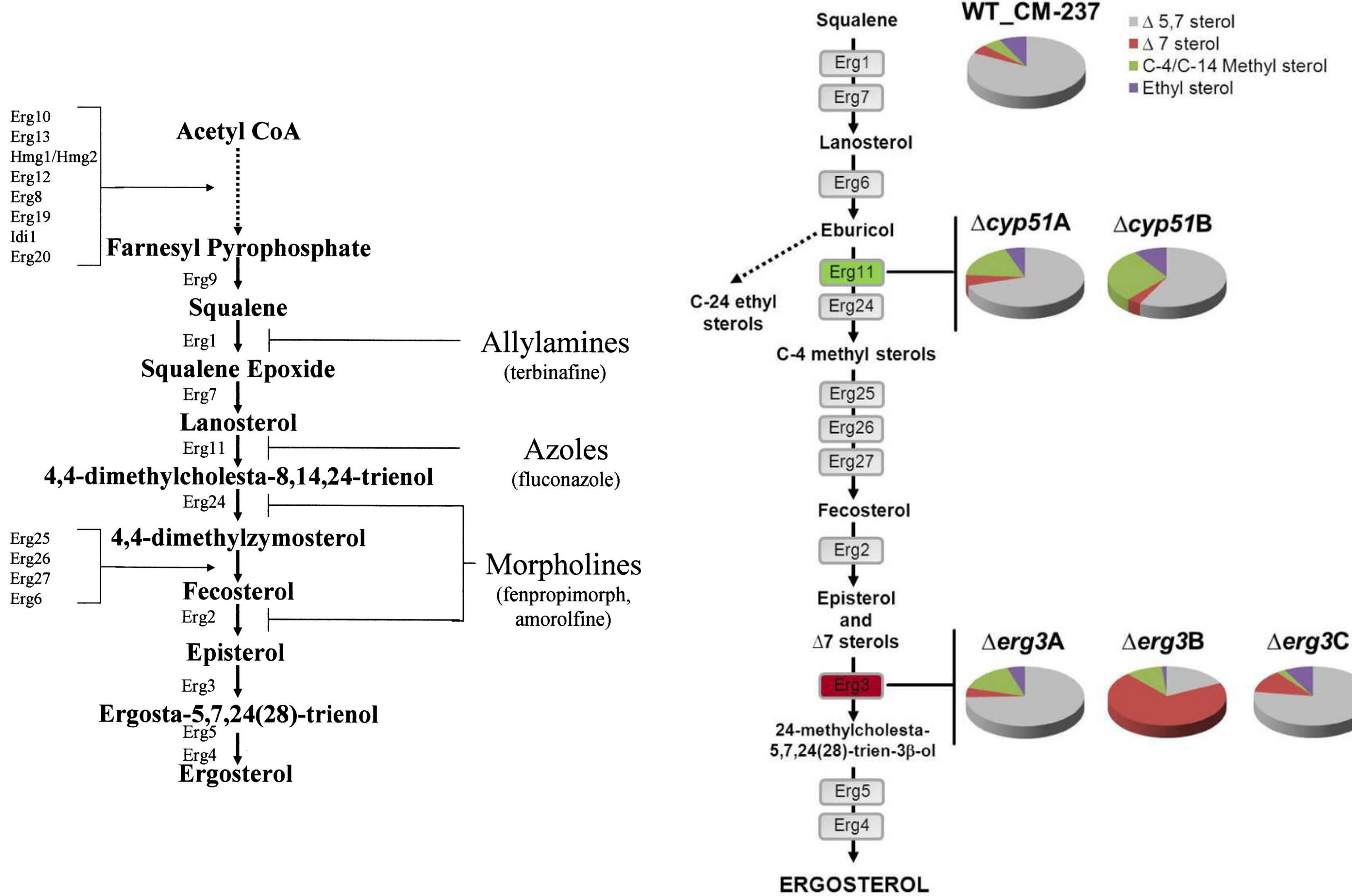
miR PRECURSOR (miR319 BACKBONE):

CAAACACACGCTCGGACGCATATTACACATGTTC
ATACACTTAATACTCGCTGTTTTGAATTGATGTTTT
AGGAATATATATGT**AGCAAGTAGTCGTGATTTGA**
ATTTCACAGGTCGTGATATGATTCAATTAGCTT
CCGACTCATTCATCCAAATACCGAGTCGCCAAA
ATTCAAACTAGACTCGTTAAATGAATGAATGAT
GCGGTAGACAAATTGGATCATTGATTCTCTTTG
ATATTCAATTCACGACTACCTGCTCTCTTTTGTA
TTCCAATTTTCTTGATTAATCTTTCCTGCACAAAAA
CATGCTTGATCCACTAAGTGACATATATGCTGCC
TTCGTATATATAGTTCTGGTAAAATTAACATTTTG
GGTTTATCTTTATTTAAGGCATCGCCATG

amiR * - CAAGTAGTCGTGATTTGAATT
amiR – TATTCAATTCACGACTACCTG

INDUCIBILE PTGS

SILENZIAMENTO DI GENI DI PATOGENI INDOTTO DA RNAi IN PIANTA



Erg10
Erg13
Hmg1/Hmg2
Erg12
Erg8
Erg19
Idi1
Erg20
Acetyl CoA
Farnesyl Pyrophosphate
Erg9
Squalene
Erg1
Allylamines
(terbinafine)
Squalene Epoxide
Erg7
Lanosterol
Erg11
Azoles
(fluconazole)
4,4-dimethylcholesta-8,14,24-trienol
Erg24
Erg25
Erg26
Erg27
Erg6
4,4-dimethylzymosterol
Morpholines
(fenpropimorph, amorolfine)
Fecosterol
Erg2
Episterol
Erg3
Ergosta-5,7,24(28)-trienol
Erg5
Erg4
Ergosterol
Squalene
Erg1
Erg7
Lanosterol
Erg6
Eburicol
C-24 ethyl sterols
Erg11
Erg24
C-4 methyl sterols
Erg25
Erg26
Erg27
Fecosterol
Erg2
Episterol and Δ7 sterols
Erg3
24-methylcholesta-5,7,24(28)-trien-3β-ol
Erg5
Erg4
ERGOSTEROL
WT_CM-237
Δ 5,7 sterol
Δ 7 sterol
C-4/C-14 Methyl sterol
Ethyl sterol
Δcyp51A
Δcyp51B
Δerg3A
Δerg3B
Δerg3C

A)

Clone sequences of CYP51A (294nt)

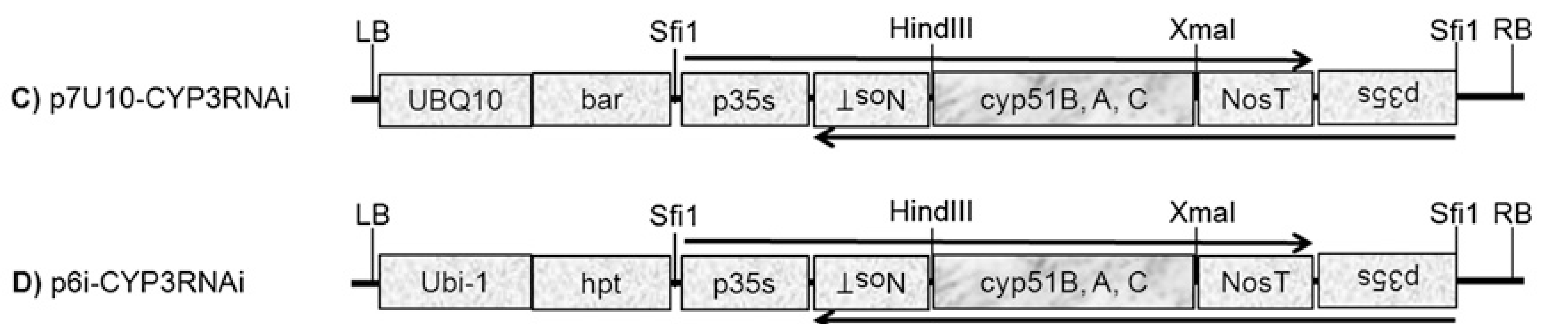
CGGTCCATTGACAATCCCCGTCTTTGGTAGCGATGTCGTATACGATTGTCCCAACTCGAAGCTCATGGAACAAAAGAAGT
TTGTCAAGTTTGGCCTTACGCAAAAAGCACTCGAGTCACACGTCCAGTTAATCGAGCGAGAGGTTCTTGACTACGTCGA
AACTGATCCATCCTTTTCTGGCAGAACTAGCACCATCGATGTCCCCAAGGCAATGGCTGAGATAACAATCTTTACTGCCT
CACGTTCTTTGCAGGGTGAGGAAGTTCGGAGAAAACTCACTGCCGAGTTTGCTGC

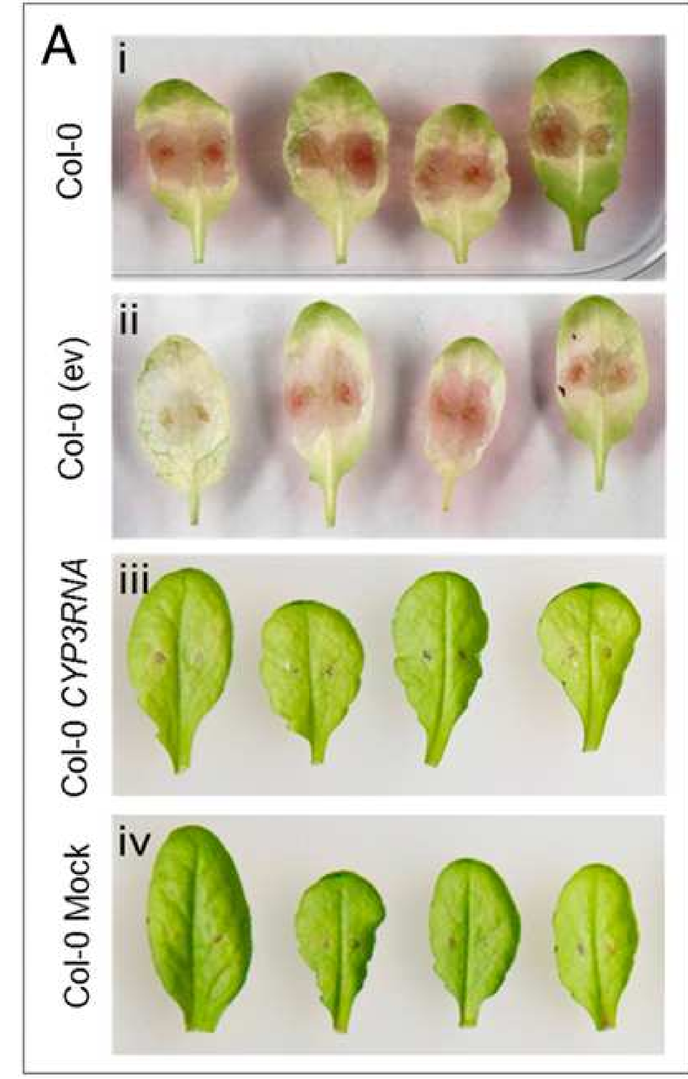
Clone sequences of CYP51B (220nt)

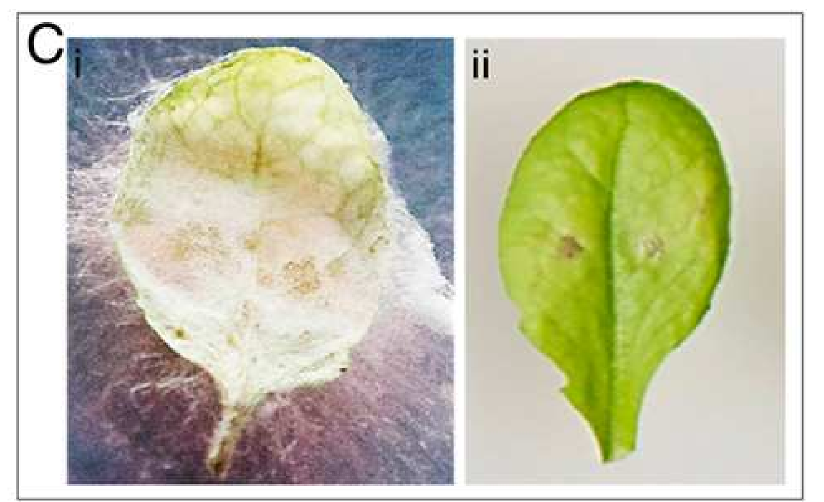
CAGCAAGTTTGACGAGTCCCTGGCCGCTCTCTACCACGACCTCGATATGGGCTTCACCCCCATCAACTTCATGCTTCAC
TGGGCCCCTCTCCCCTGGAACCGTAAGCGCGACCACGCCCAGCGCACTGTTGCCAAGATCTACATGGACACTATCAAG
GAGCGCCGCGCCAAGGGCAACAACGAATCCGAGCATGACATGATGAAGCACCTTATGAACTCT

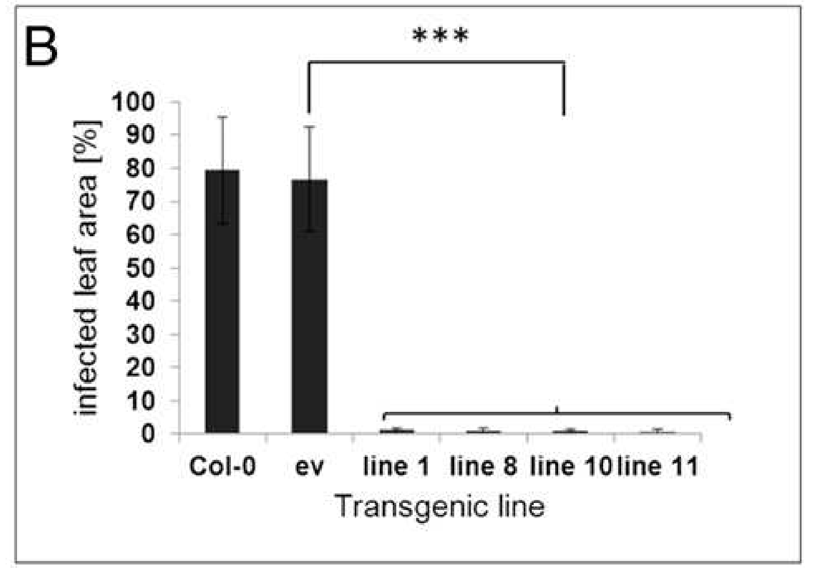
Clone sequences of CYP51C (238nt)

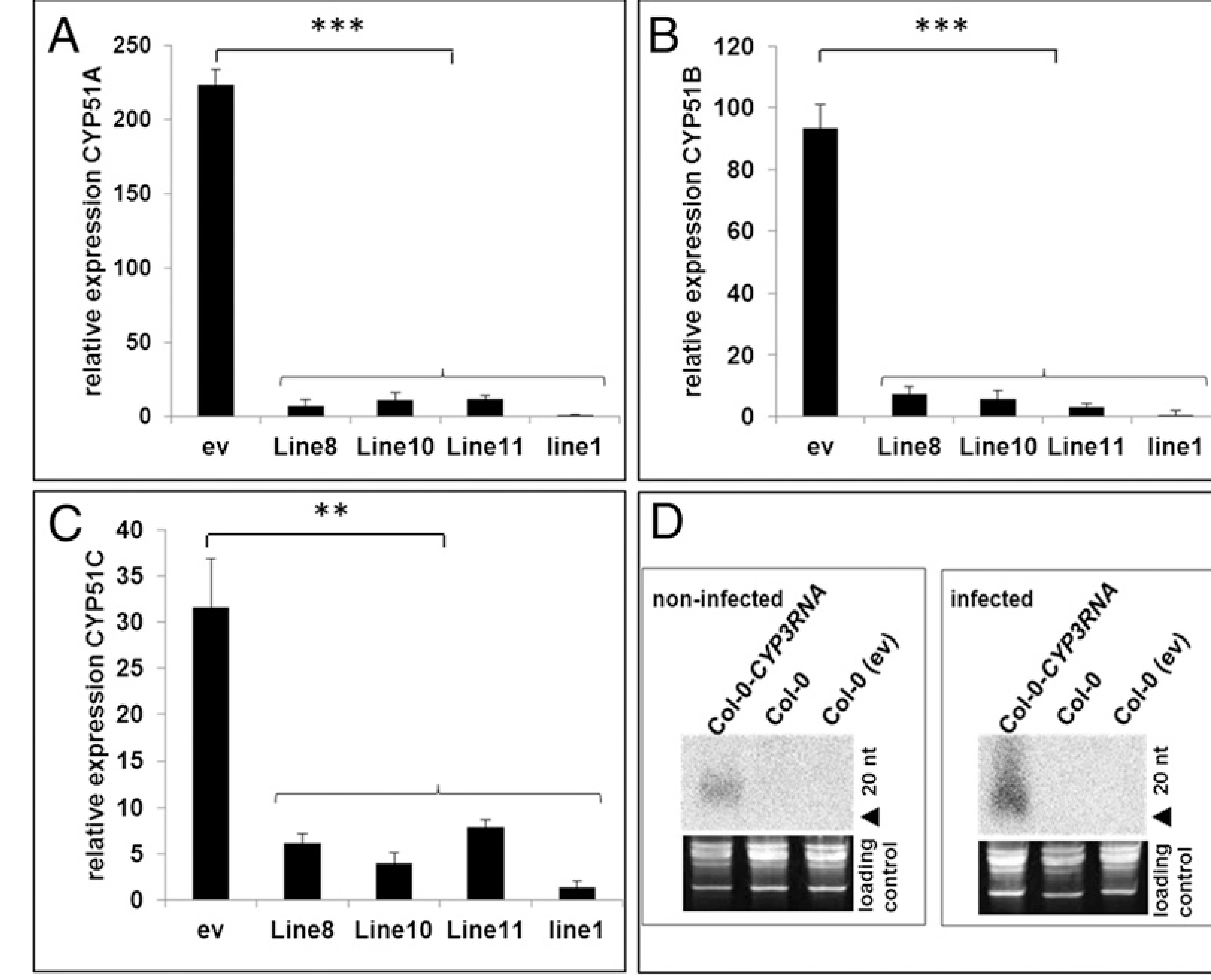
ATTGGAAGCACCGTACAATATGGCATCGACCCGTACGCTTTTTTCTTCGACTGCAGAGATAAATACGGCGACTGCTTTAC
CTTTATTCTCCTTGGCAAATCAACGACTGTCTTTCTTGGTCCCAAGGGCAATGACTTTATCCTCAACGGCAAACACGCCG
ATCTCAACGCCGAGGACGTTTATGGGAAACTTACCACGCCCGTGTTTGGTGAGGAGGTTGTTTATGACTGCTCCAATG

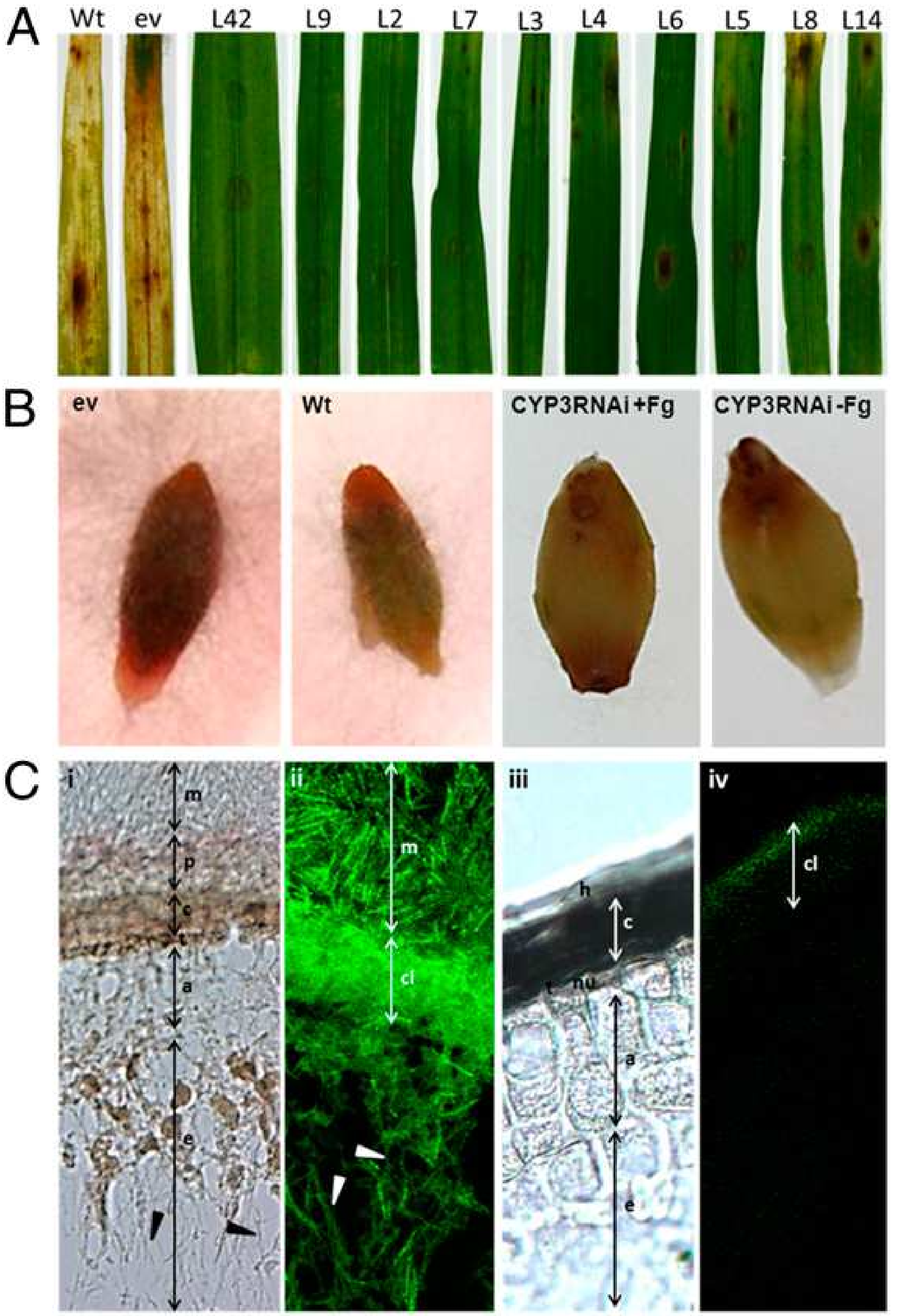



A
i
Col-0
ii
Col-0 (ev)
iii
Col-0 CYP3RNA
iv
Col-0 Mock


B
***
infected leaf area [%]
100
90
80
70
60
50
40
30
20
10
0
Col-0
ev
line 1
line 8
line 10
line 11
Transgenic line


C
i
ii


A
relative expression CYP51A
250
200
150
100
50
0
***
ev
Line8
Line10
Line11
line1
B
relative expression CYP51B
120
100
80
60
40
20
0
***
ev
Line8
Line10
Line11
line1
C
relative expression CYP51C
40
35
30
25
20
15
10
5
0
**
ev
Line8
Line10
Line11
line1
D
non-infected
Col-0-CYP3RNA
Col-0
Col-0 (ev)
20 nt
loading control
infected
Col-0-CYP3RNA
Col-0
Col-0 (ev)
20 nt
loading control


A
Wt
ev
L42
L9
L2
L7
L3
L4
L6
L5
L8
L14
B
ev
Wt
CYP3RNAi +Fg
CYP3RNAi -Fg
C
i
m
p
c
t
a
e
ii
m
cl
iii
h
c
t
nu
a
e
iv
cl

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]*

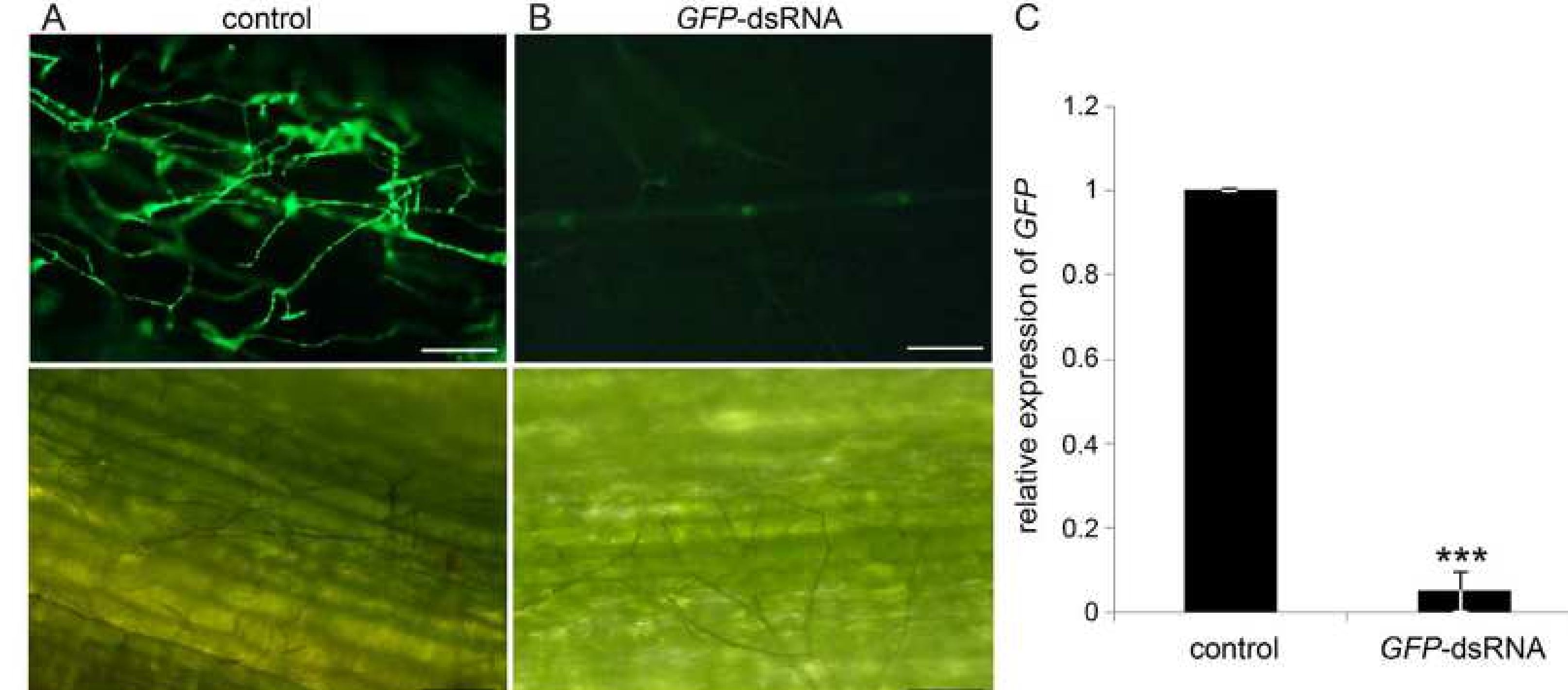


Fig 1. (A-C) Spray-induced gene silencing (SIGS) of *GFP* expression in *Fusarium graminearum* strain Fg-IFA65$_{GFP}$. Detached second leaves of three-week-old barley plants were locally sprayed with Tris-EDTA (TE, **A**, control) or *GFP*-dsRNA **(B)**. Forty-eight hours after spraying, distal, non-sprayed leaf segments were drop-inoculated with Fg-IFA65$_{GFP}$ (20 µL of a solution containing 2 x 10^4 conidia mL^{-1}). *GFP* silencing efficiency was visualized 6 dpi using confocal microscopy. **(C)** *GFP* transcripts were quantified by qPCR at 6 dpi. The reduction in fungal *GFP* expression on leaves sprayed with *GFP*-dsRNA and infected with Fg-IFA65$_{GFP}$ compared with TE-sprayed controls was statistically significant (***$P < 0.001$; Student´s *t* test). Bars represent mean values ± SDs of three independent experiments. Scale bars represent 100 µm.

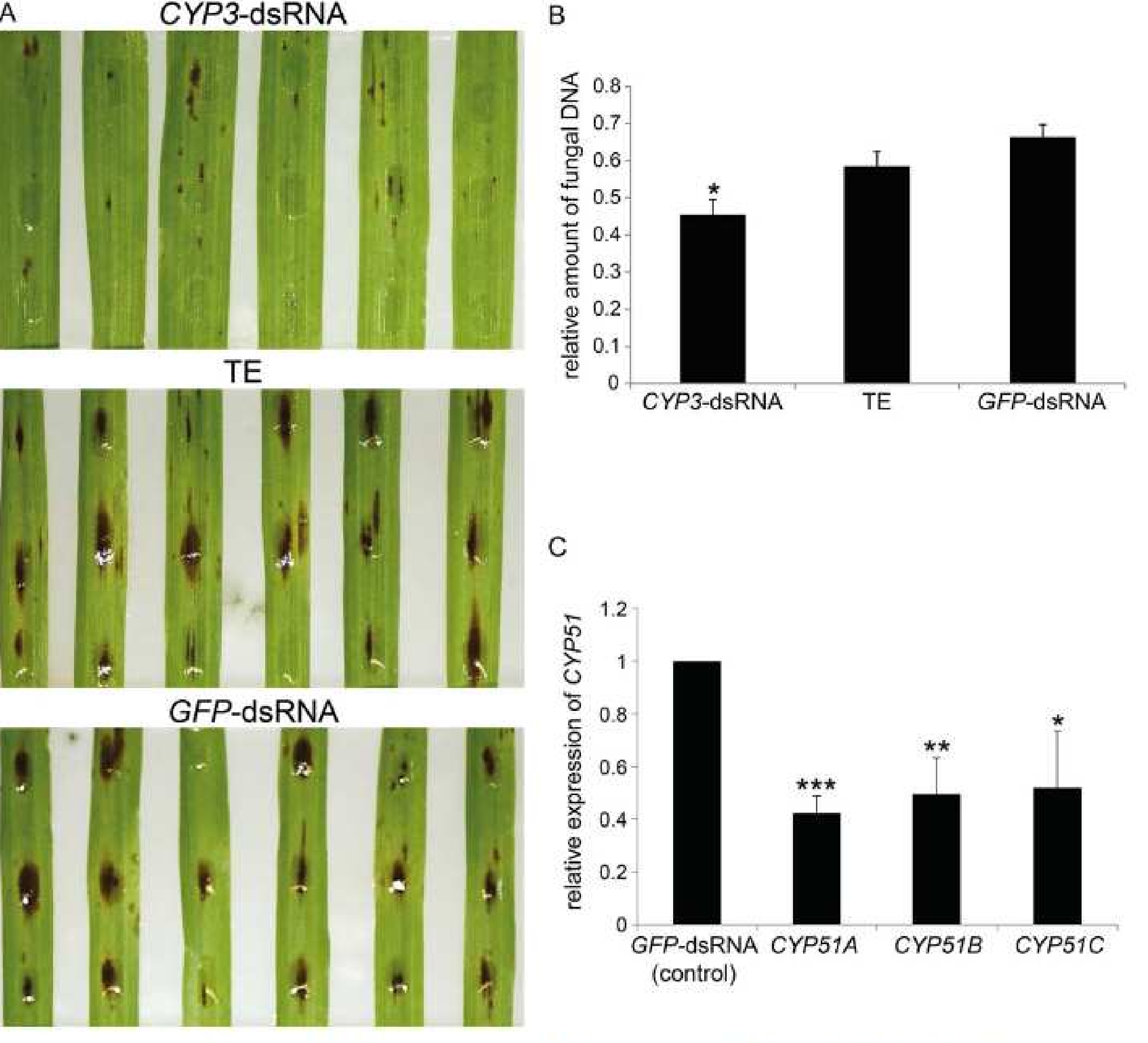


Fig 2. (A-C) SIGS-mediated control of *F. graminearum* on leaves sprayed with *CYP3*-dsRNA. (A) Detached second leaves of three-week-old barley were sprayed evenly with *CYP3*-dsRNA, TE (mock control), and *GFP*-dsRNA (negative control), respectively. After 48 hours, leaves were drop-inoculated with 2×10^4 conidia mL^{-1} of Fg-IFA65 onto the sprayed area and evaluated for necrotic lesions at 6 dpi. **(B)** The relative amount of fungal DNA at 6 dpi as measured by qPCR was reduced in *CYP3*-dsRNA-treated leaves compared to control leaves. Bars represent mean values ± SDs of three independent experiments. The reduction of fungal growth on *CYP3*-dsRNA vs. TE- or *GFP*-dsRNA-sprayed leaves was statistically significant (*P < 0.05; Student´s t test). **(C)** Gene-specific qPCR analysis of fungal *CYP51A*, *CYP51B*, and *CYP51C* transcripts at 6 dpi (corresponding to 8 d after spraying). The reduction in fungal *CYP51* gene expression on *CYP3*-dsRNA-sprayed leaves as compared with *GFP*-dsRNA-sprayed controls was statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001; Student´s t test).

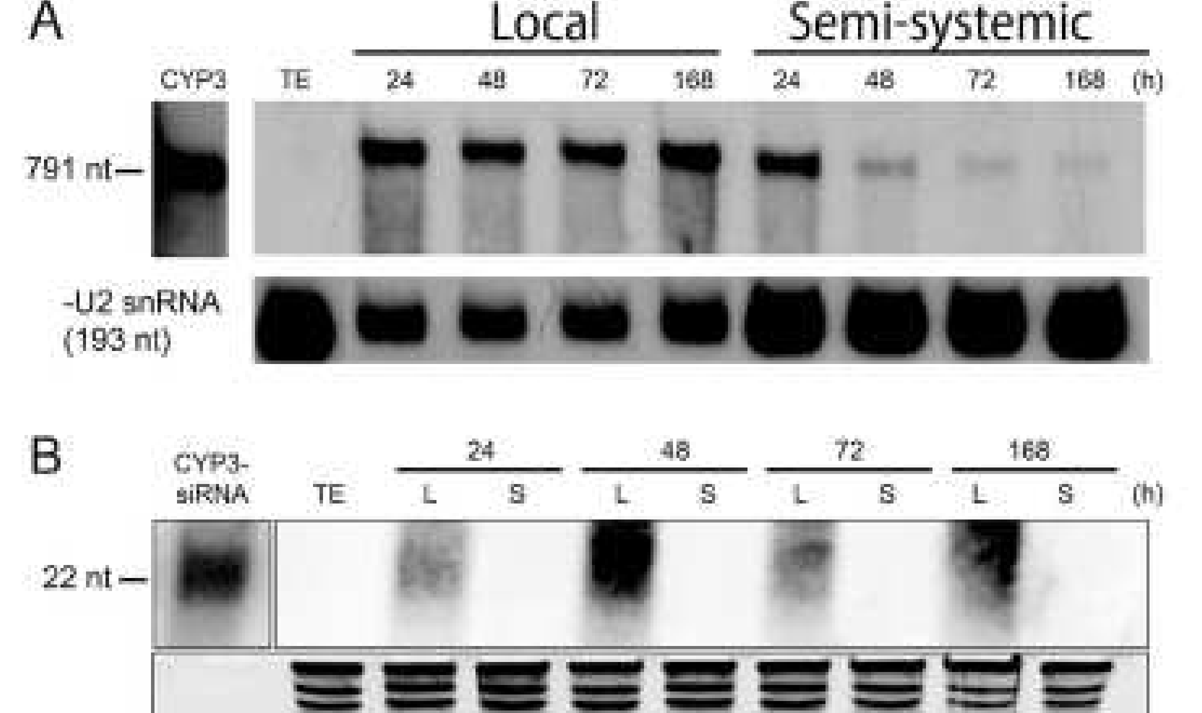


Fig 4. (A, B) Northern gel blot analysis of *CYP3*-dsRNA and *CYP3*-dsRNA-derived siRNA accumulation in local and distal (semi-systemic) barley leaf areas. (A) Detection of 791 nt long *CYP3*-dsRNA precursor in pooled leaf tissue from non-infected leaves using [α-32P]-dCTP labeled *CYP3*-dsRNA as probe. Local (L) and distal (semi-systemic [S]) leaf segments were sampled separately at the indicated times after spraying with *CYP3*-dsRNA. No signal was detected in samples from TE-sprayed plants. **(B)** Recording *CYP3*-dsRNA-derived small RNAs in local and distal (semi-systemic) leaf areas using [α-32P]-dCTP labeled *CYP3*-dsRNA as probe. In this experiment, small RNAs could not be detected in distal (non-sprayed) tissues. siRNA generated *in vitro* by a commercial Dicer preparation from *CYP3*-dsRNA was used as positive control. No signal was detected in samples from TE-sprayed plants. Ethidium bromide-stained rRNA served as the loading control. Signals originate from the same membrane but different exposure times.