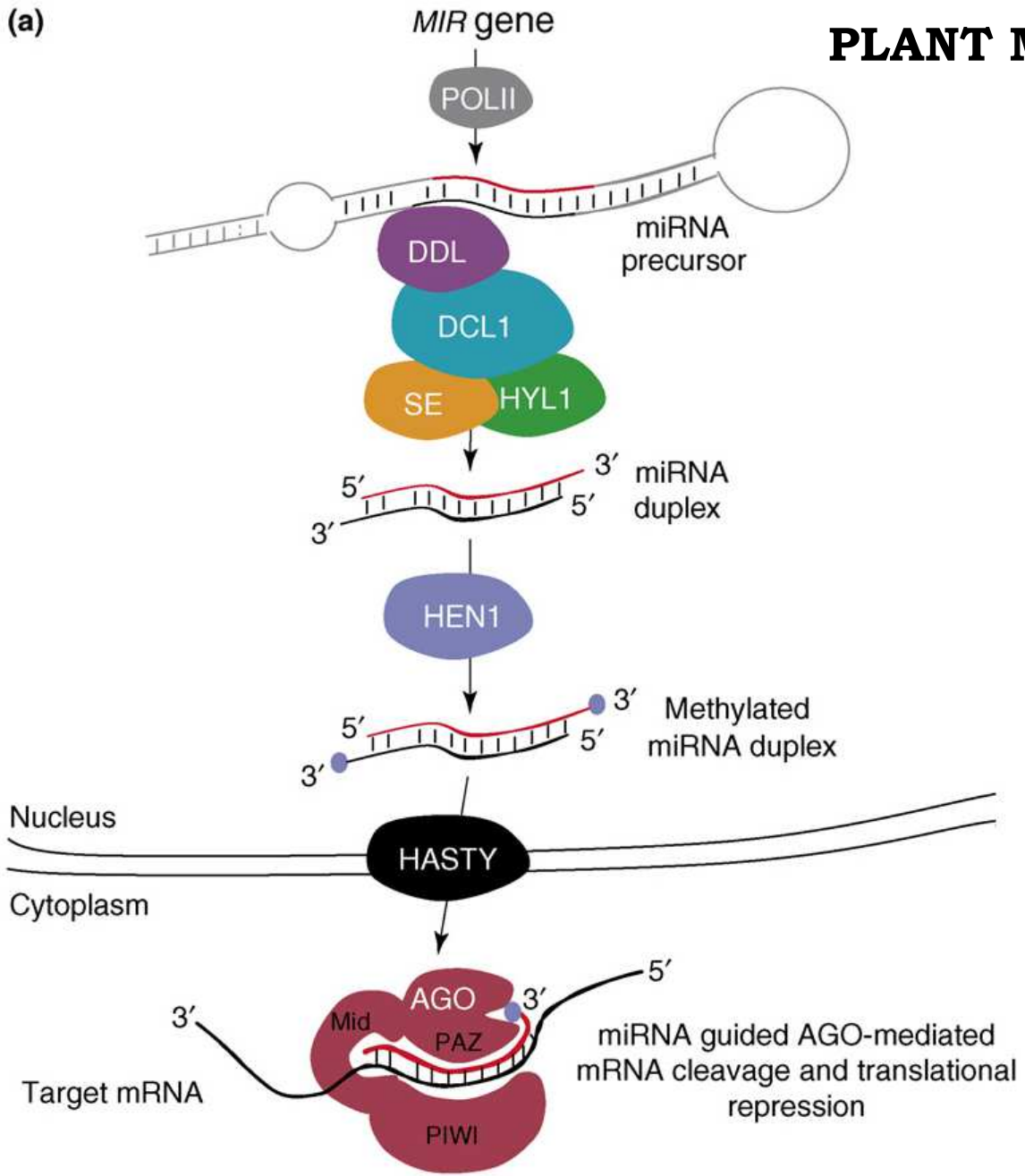
SILENZIAMENTO INDOTTO DA microRNA ARTIFICIALI

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

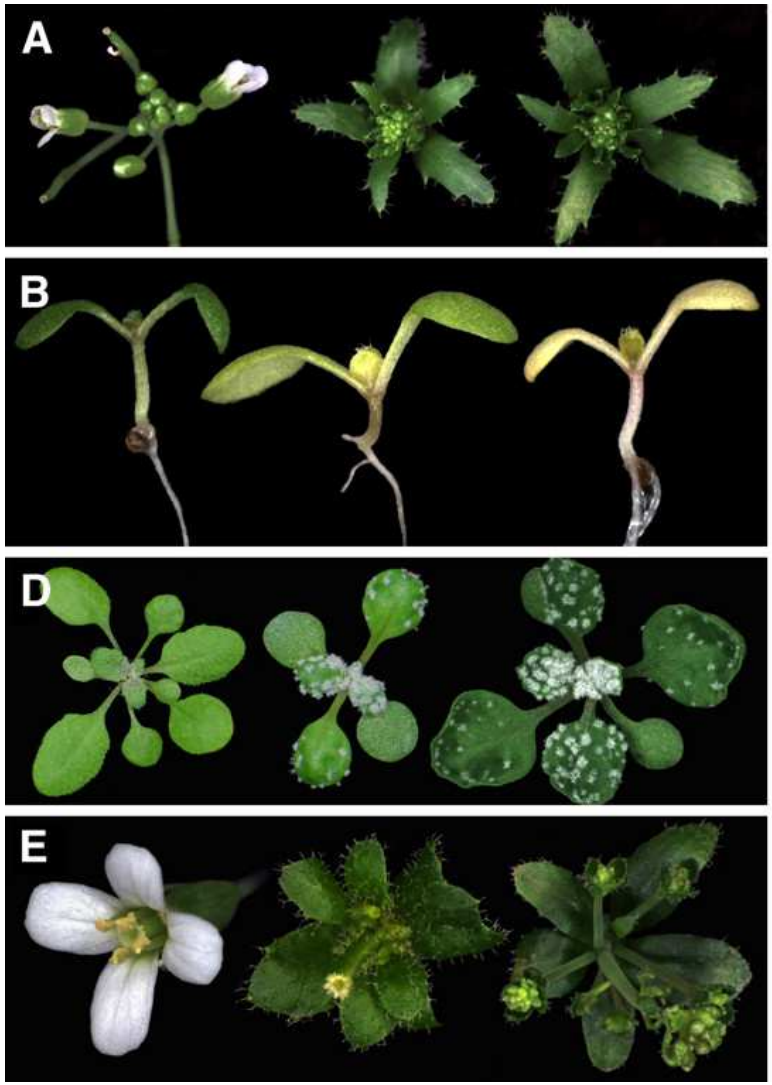
Compared with conventional RNAi, amiRNAs offer several advantages:

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.

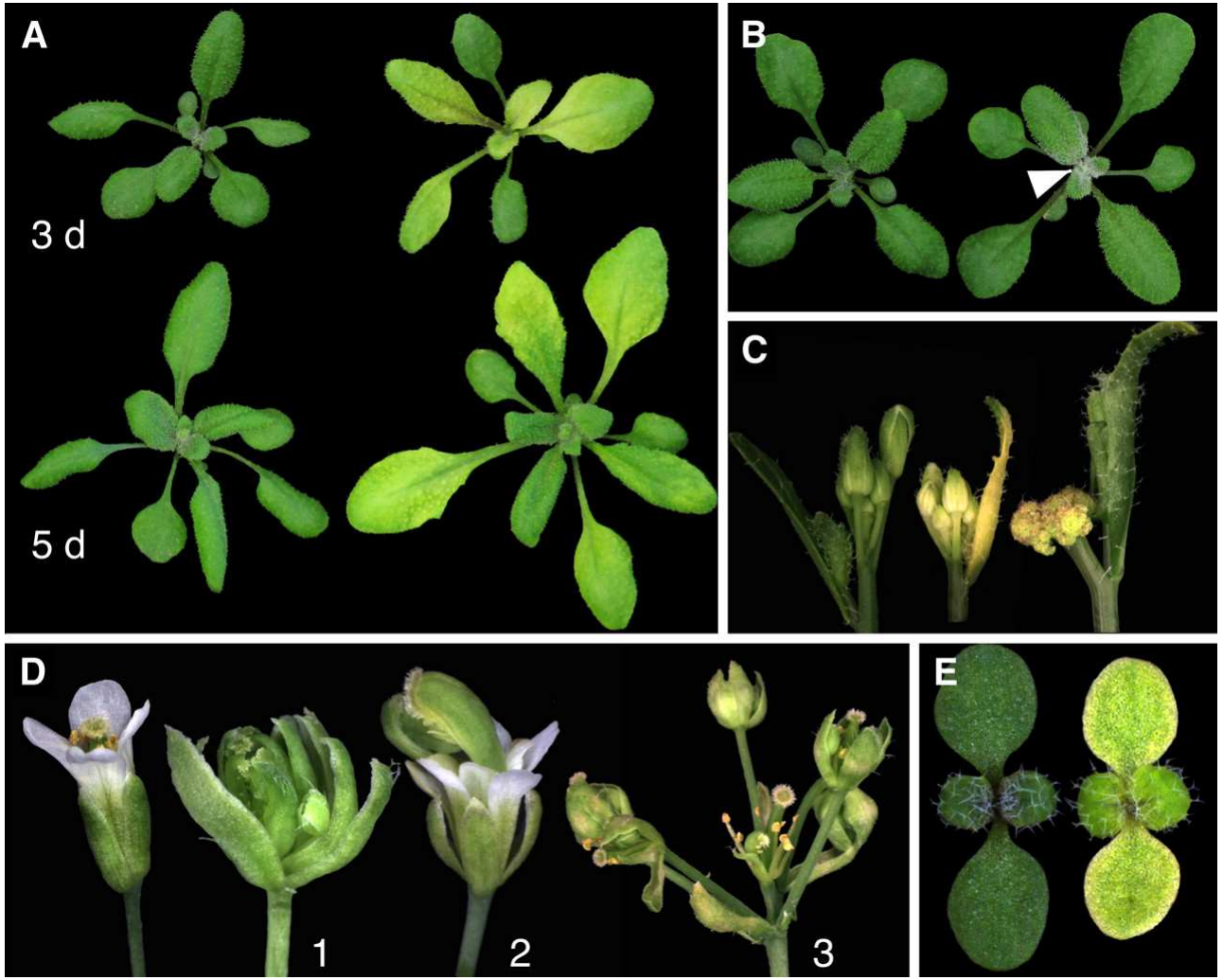
(B) Seedlings. From left to right: the wild type, gun4-1, and amiR-white-1 (MIR172a backbone) overexpresser. Bleaching of cotyledons is more pronounced in the amiR-white plants than in gun4-1, consistent with the more severe molecular profile of the amiR-white overexpressers.

(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



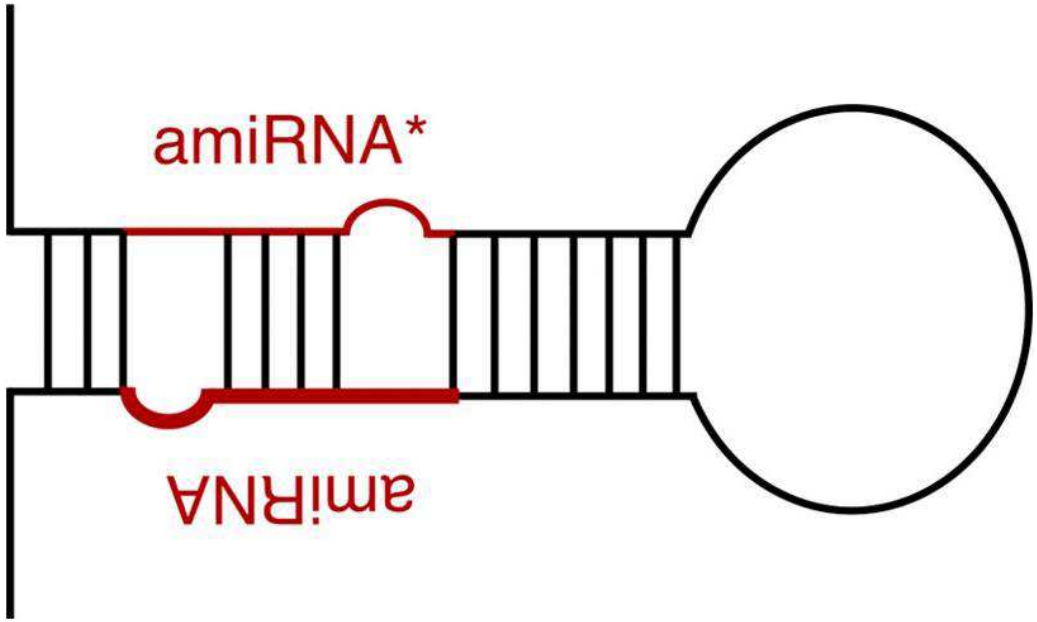
Schwab R. et.al. Plant Cell 2006:18:1121-1133

ENGINEERING OF AMIRNAs

miR319 (BACKBONE):

caaacacacgctcggacgcatattacacatgttcatacacttaatactcgctgttttgaatt
gatgttttaggaatatatatgt**agagagagcttccttgagtccattcacaggtcgtgatatgattaatta**
gcttccgactcattcatccaaataccgagtcgccaaaattcaaactagactcgttaaatgaatgaatgatgcg
gtagacaaattggatcattgattctctttgattggactgaagggagctccctctctcttttgtatccaatt
ttcttgattaatctttcctgcacaaaaacatgcttgatccactaagtgacatatatgctgcc
ttcgtatatatagttctggtaaaattaacattttgggtttatctttatttaaggcatcgcca
tg

miRNA319
miRNA319*



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

ENGINEERING OF AMIRNAS

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):

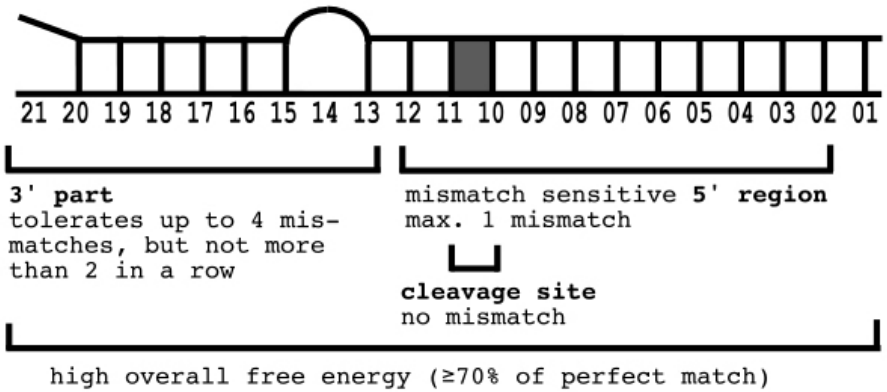
1. No mismatch between positions 2 and 12 of the amiRNA for all targets.
   Mismatches are not allowed for the target gene that is used as a template, but they might come up for additional intended targets since the target determinants allow for one mismatch.

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.

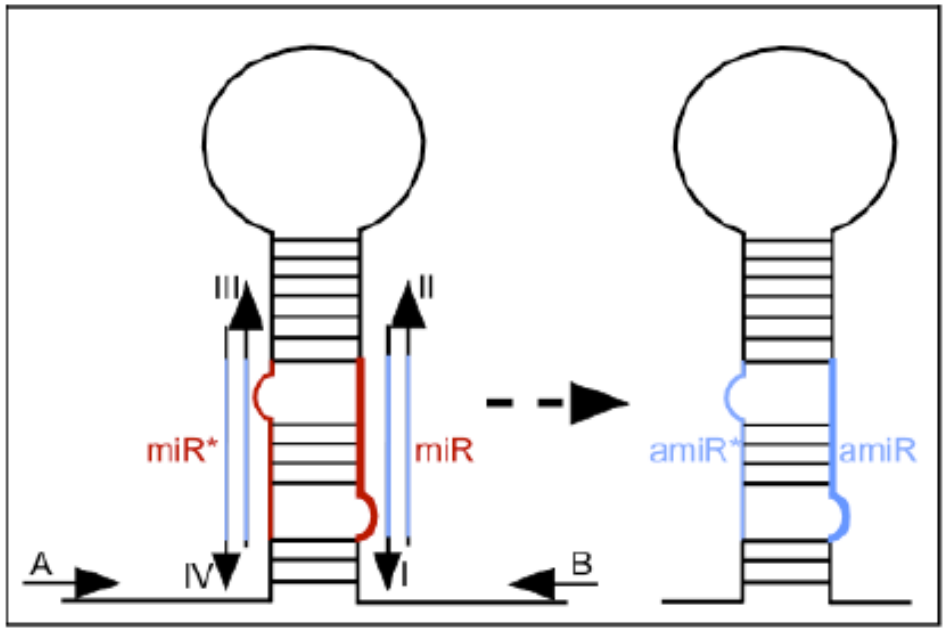
3. Similar mismatch pattern for all intended targets.
   There is no evidence that the pattern of mismatches matters, but similar patterns definitely don't hurt.

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

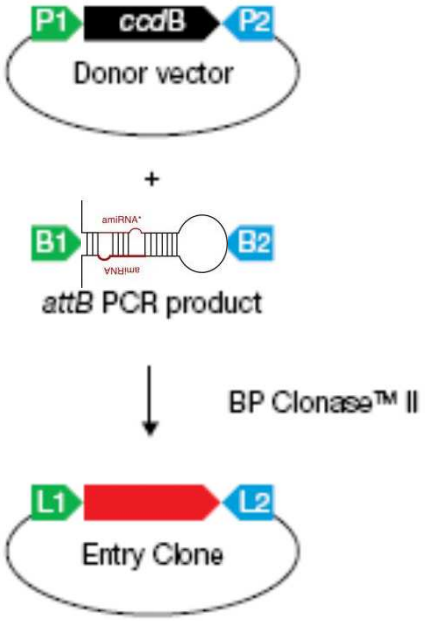
	forward oligo	reverse oligo	template
(a)	A	IV	pRS300
(b)	III	II	pRS300
(c)	I	B	pRS300
(d)	A	B	(a)+(b)+(c)

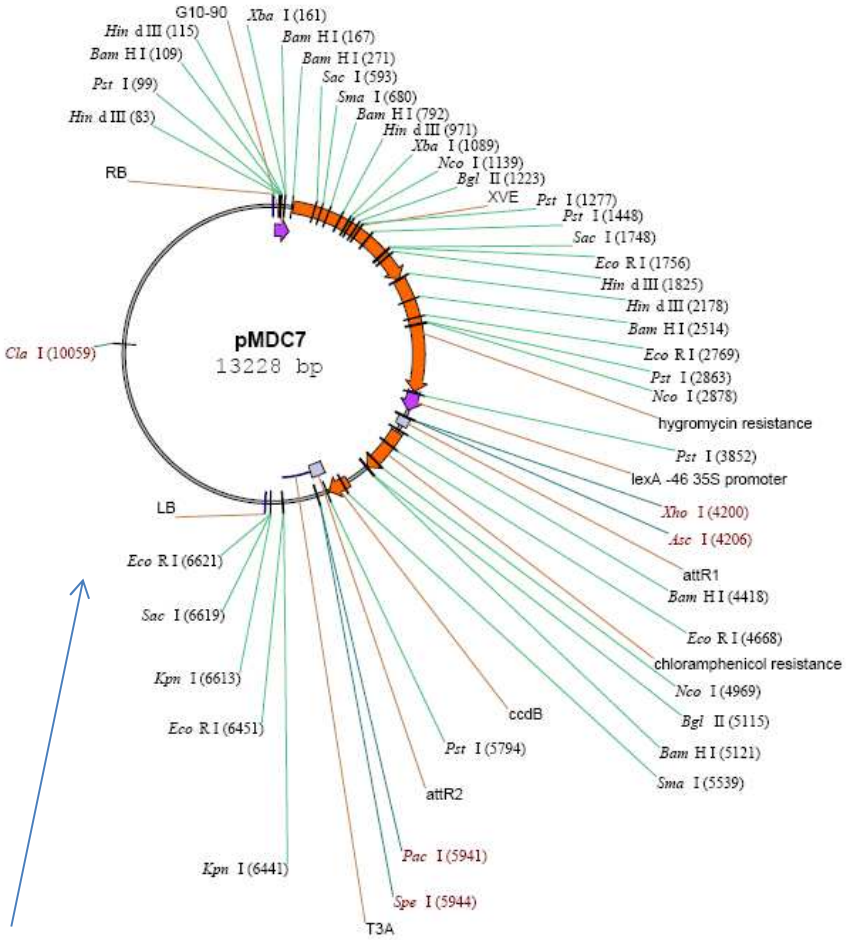
Schwab R. et.al. Plant Cell 2006:18:1121-1133

DNA CLONING USING IN VITRO SITE-SPECIFIC RECOMBINATION

The Gateway reactions:

1)





2)

The Plant Cell, Vol. 14, 1109–1120, May 2002, www.plantcell.org © 2002 American Society of Plant Biologists

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

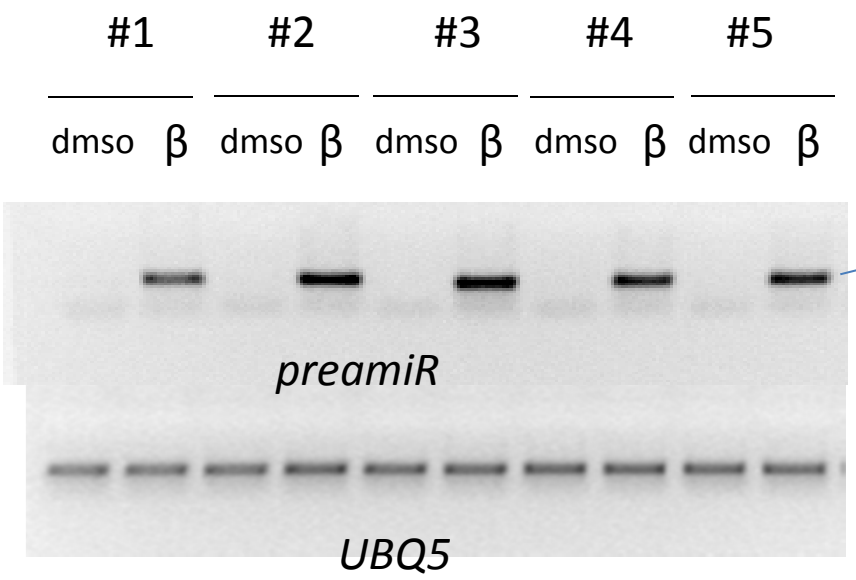
The results described above demonstrate that the *ANP* genes are involved in the control of cellular growth and division. Therefore, we tested the sensitivity of the *anp2 anp3* plants to the phytohormones abscisic acid, auxin, brassinosteroids, cytokinin, ethylene, and gibberellin using agar plate assays. 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.

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):

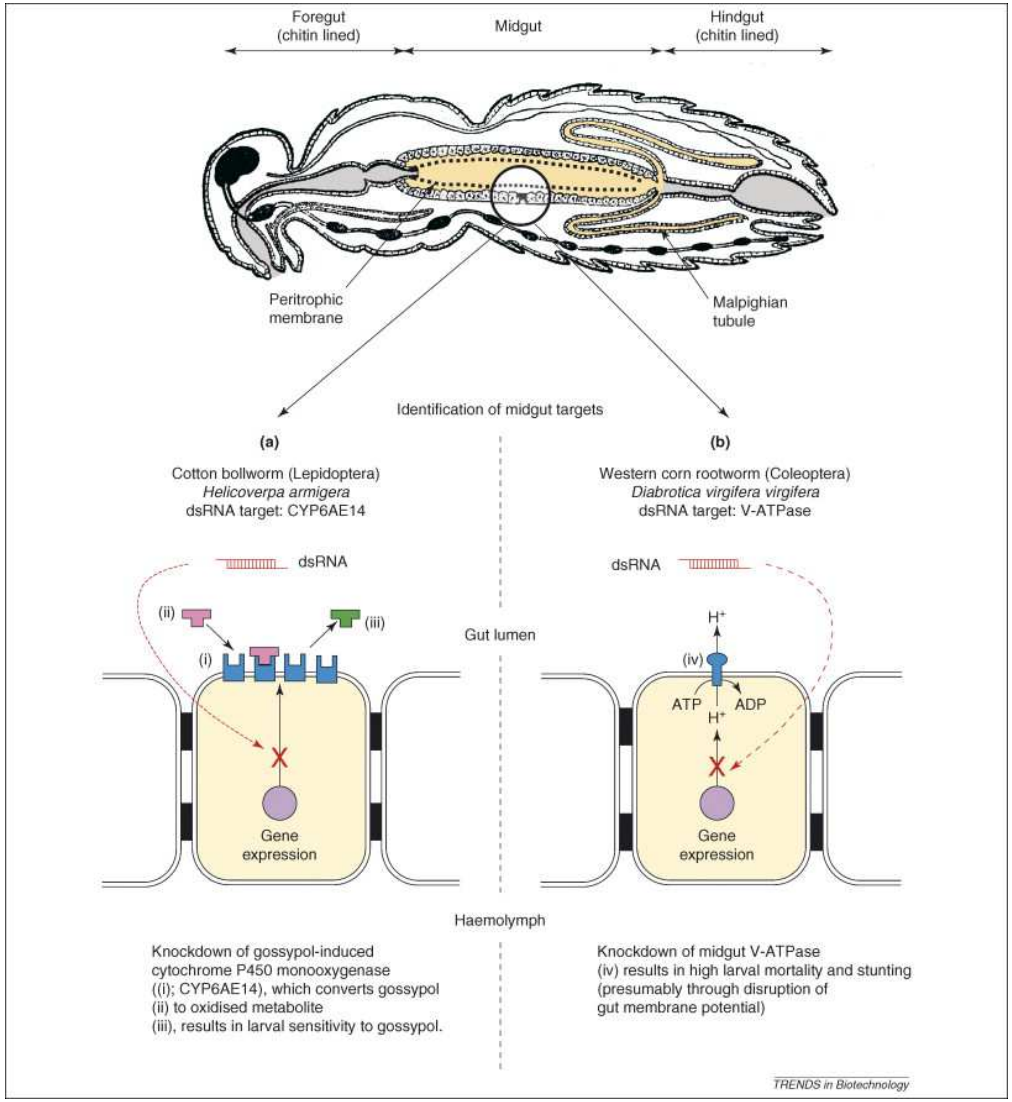
CAAACACACGCTCGGACGCATATTACACATGTTC
ATACACTTAATACTCGCTGTTTTGAATTGATGTTTT
AGGAATATATATGT**AGCAAGTAGTCGTGATTTGA**
ATTTCACAGGTCGTGATATGATTCAATTAGCTT
CCGACTCATTCATCCAAATACCGAGTCGCCAAA
ATTCAAACTAGACTCGTTAAATGAATGAATGAT
GCGGTAGACAAATTGGATCATTGATTCTCTTTG
ATATTCAATTCACGACTACCTGCTCTCTTTTGTA
TTCCAATTTTCTTGATTAATCTTTCCTGCACAAAAA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

amiR * - CAAGTAGTCGTGATTTGAATT
amiR – TATTCAATTCACGACTACCTG

INDUCIBILE PTGS

HOST-INDUCED GENE SILENCING (HIGS): UNA BIOTECNOLOGIA CONTRO LE MALATTIE DELLE PIANTE

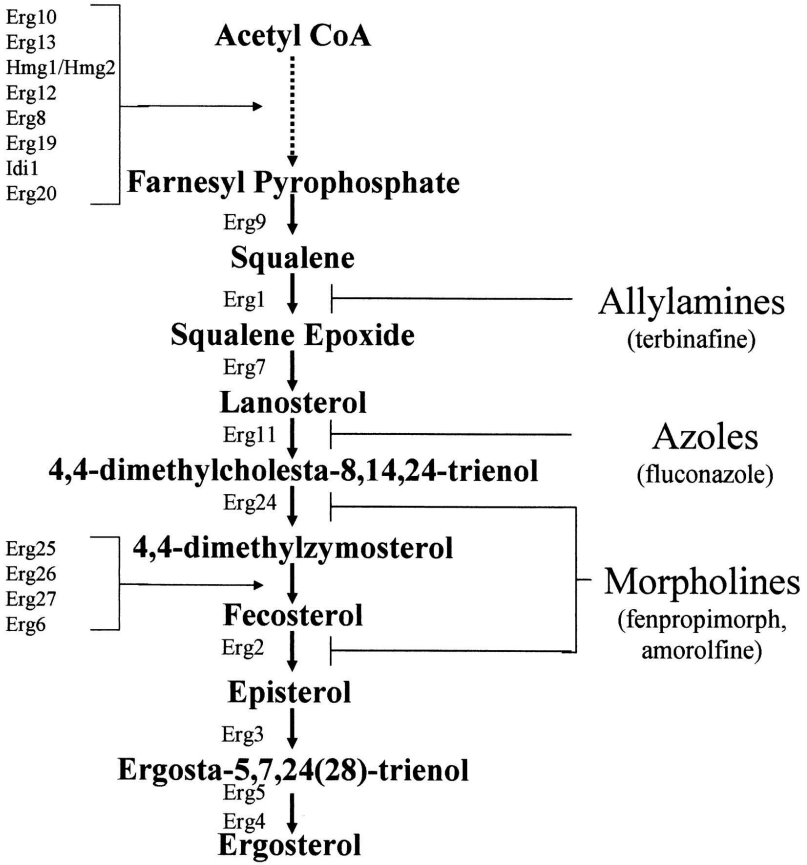
Espressione *in pianta* di costrutti per il silenziamento di geni di insetti fitofagi

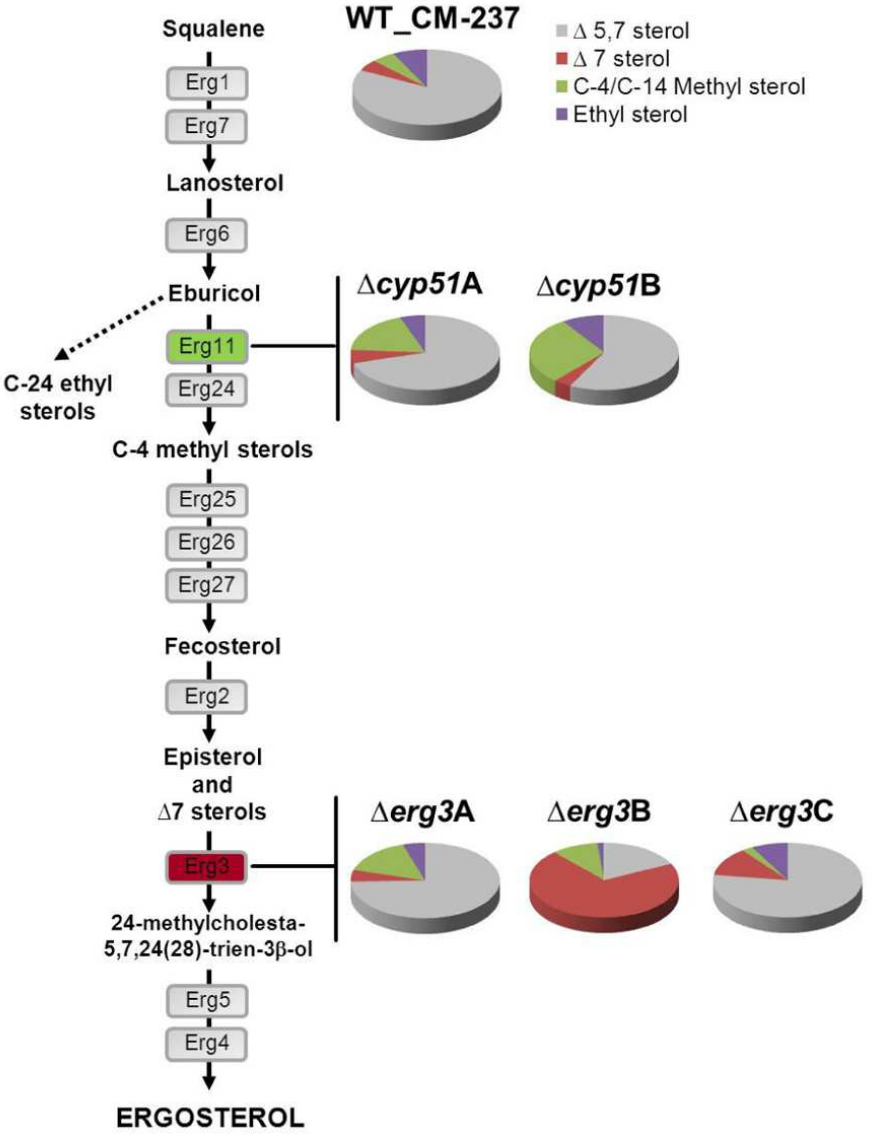


1. Cotton bollworm (lepidottero): CYP6AE14 conferisce resistenza all gossipolo. RNAi: sensibilità al gossipolo (Mao et al 2007 Nature Biotechnology)

2. Western cotton rootworm (coleottero): RNAi di una V-ATPasi espressa nell'intestino porta a mortalità larvale elevata (Baum et al 2017 Nature Biotechnology)

Biosintesi dell'ergosterolo nei funghi





Costrutti per il silenziamento di geni per la sintesi di ergosterolo in *Fusarium graminearum*

A)

Clone sequences of CYP51A (294nt)

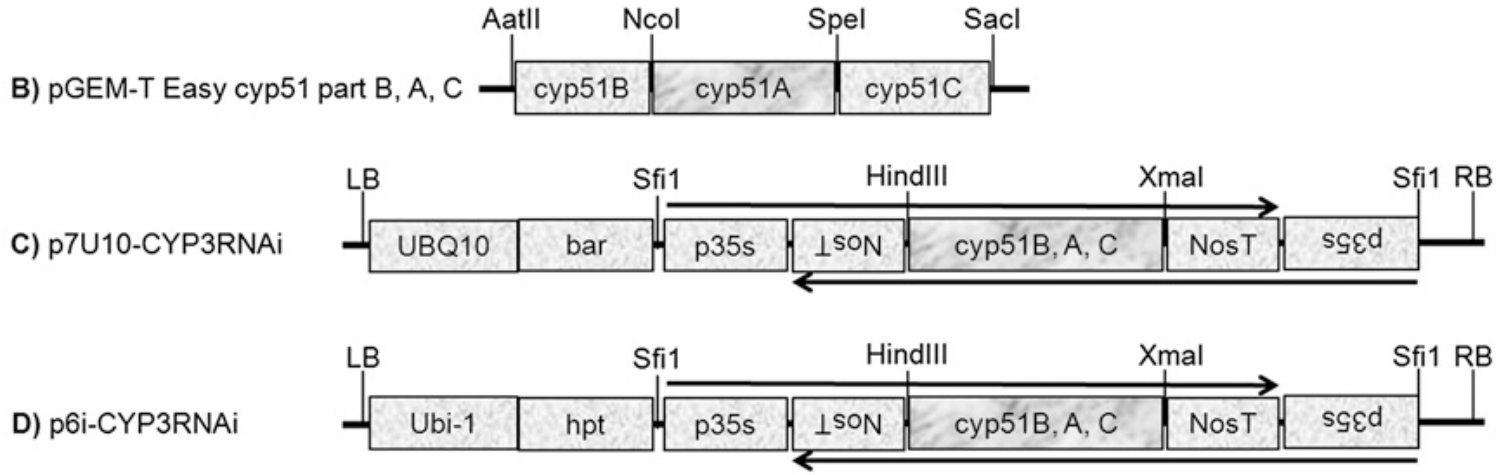
CGGTCCATTGACAATCCCCGTCTTTGGTAGCGATGTCGTATACGATTGTCCCAACTCGAAGCTCATGGAACAAAAGAAGT
TTGTCAAGTTTGGCCTTACGCAAAAAGCACTCGAGTCACACGTCCAGTTAATCGAGCGAGAGGTTCTTGACTACGTCGA
AACTGATCCATCCTTTTCTGGCAGAACTAGCACCATCGATGTCCCCAAGGCAATGGCTGAGATAACAATCTTTACTGCCT
CACGTTCTTTGCAGGGTGAGGAAGTTCGGAGAAAACTCACTGCCGAGTTTGCTGC

Clone sequences of CYP51B (220nt)

CAGCAAGTTTGACGAGTCCCTGGCCGCTCTCTACCACGACCTCGATATGGGCTTCACCCCCATCAACTTCATGCTTCAC
TGGGCCCCTCTCCCCTGGAACCGTAAGCGCGACCACGCCCAGCGCACTGTTGCCAAGATCTACATGGACACTATCAAG
GAGCGCCGCGCCAAGGGCAACAACGAATCCGAGCATGACATGATGAAGCACCTTATGAACTCT

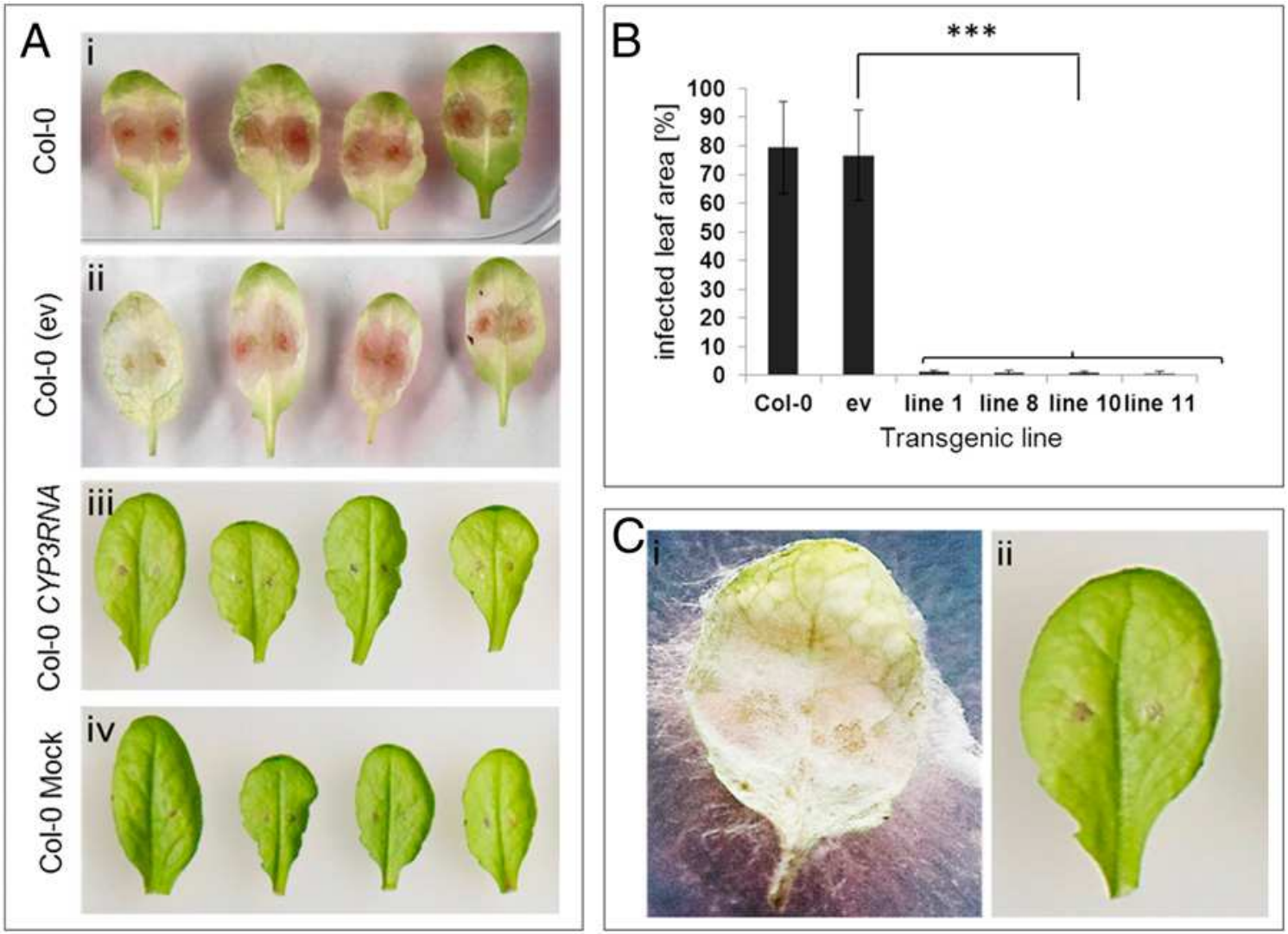
Clone sequences of CYP51C (238nt)

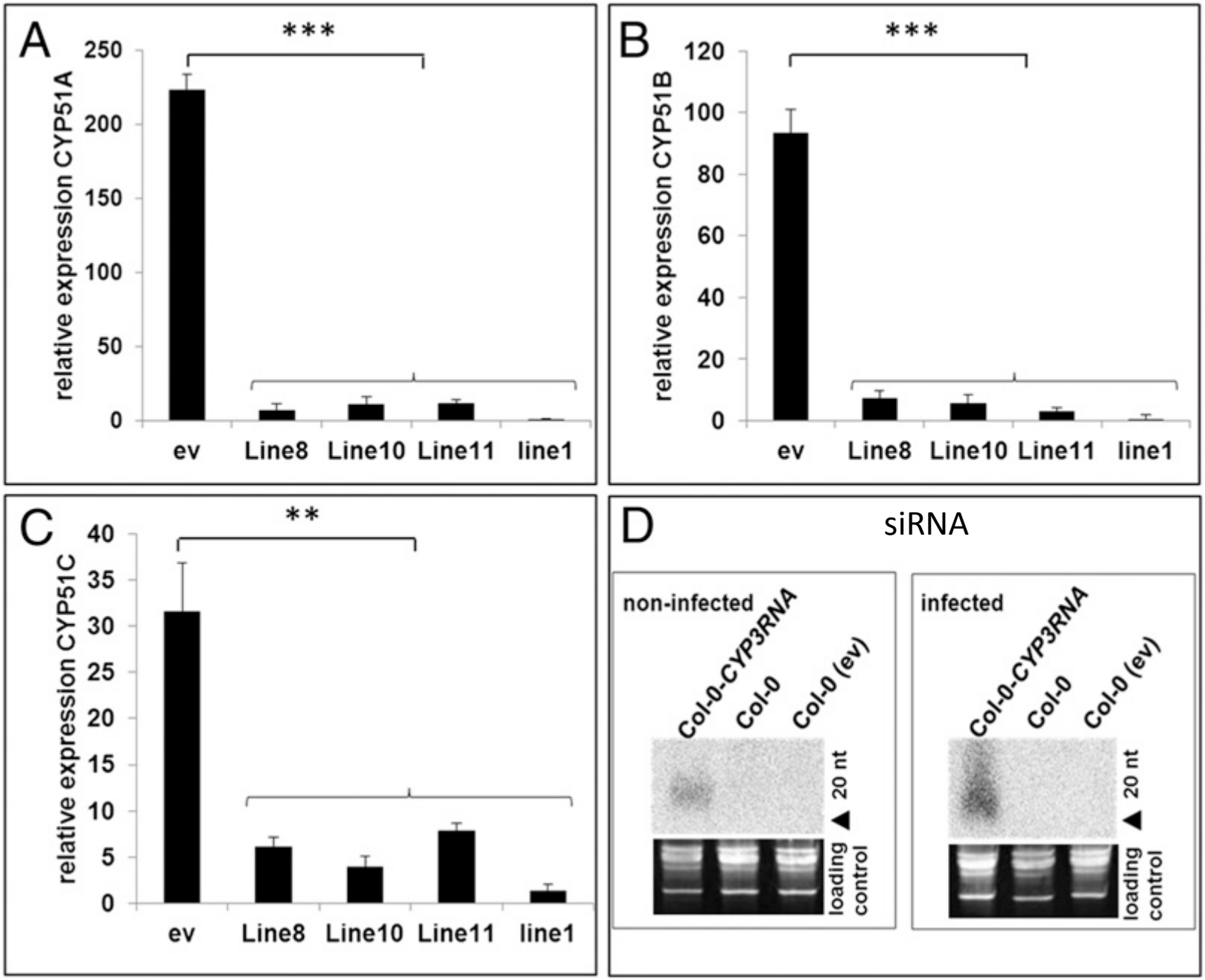
ATTGGAAGCACCGTACAATATGGCATCGACCCGTACGCTTTTTTCTTCGACTGCAGAGATAAATACGGCGACTGCTTTAC
CTTTATTCTCCTTGGCAAATCAACGACTGTCTTTCTTGGTCCCAAGGGCAATGACTTTATCCTCAACGGCAAACACGCCG
ATCTCAACGCCGAGGACGTTTATGGGAAACTTACCACGCCCGTGTTTGGTGAGGAGGTTGTTTATGACTGCTCCAATG



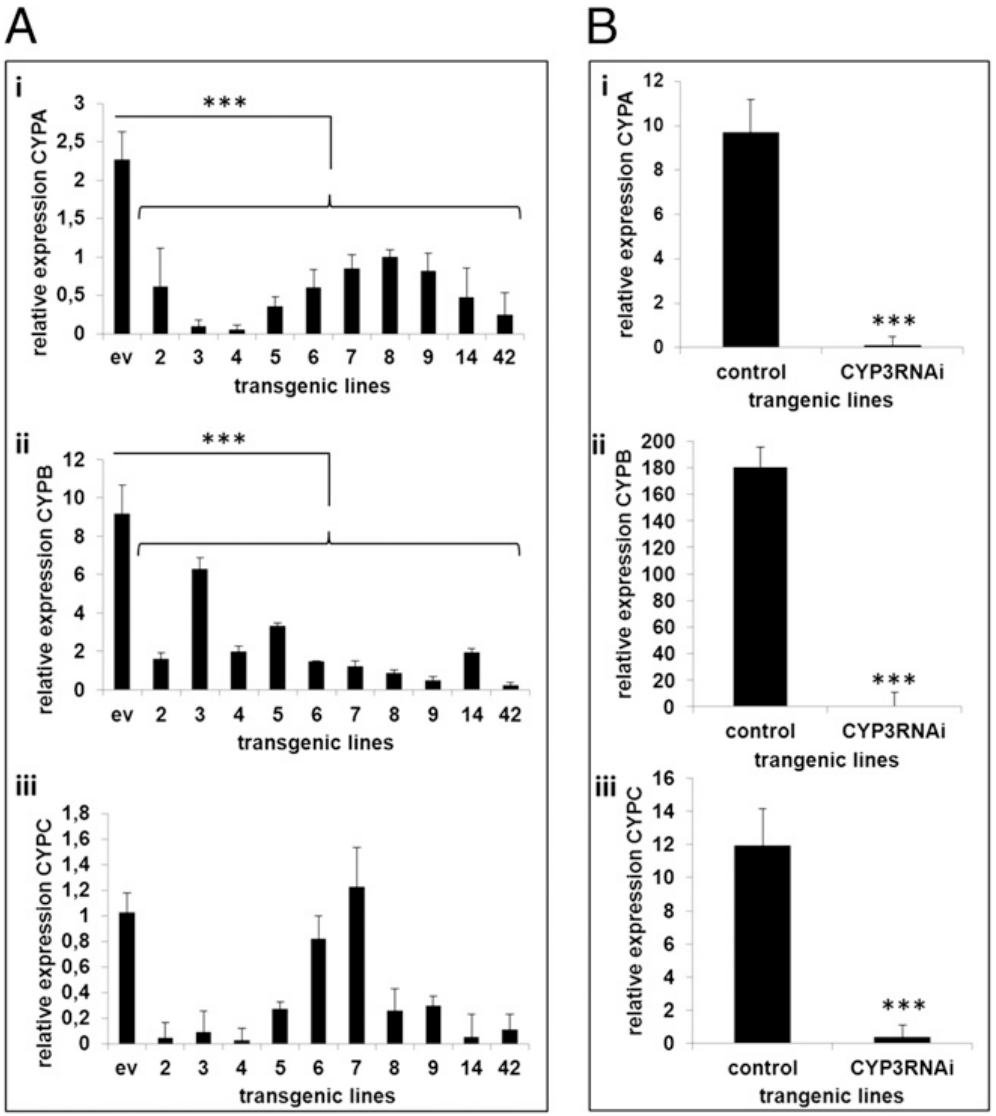
Koch et al 2013 PNAS

Piante di Arabidopsis che esprimono i costrutti per RNAi di CYP51A,B e C sono resistenti a *F. graminearum*

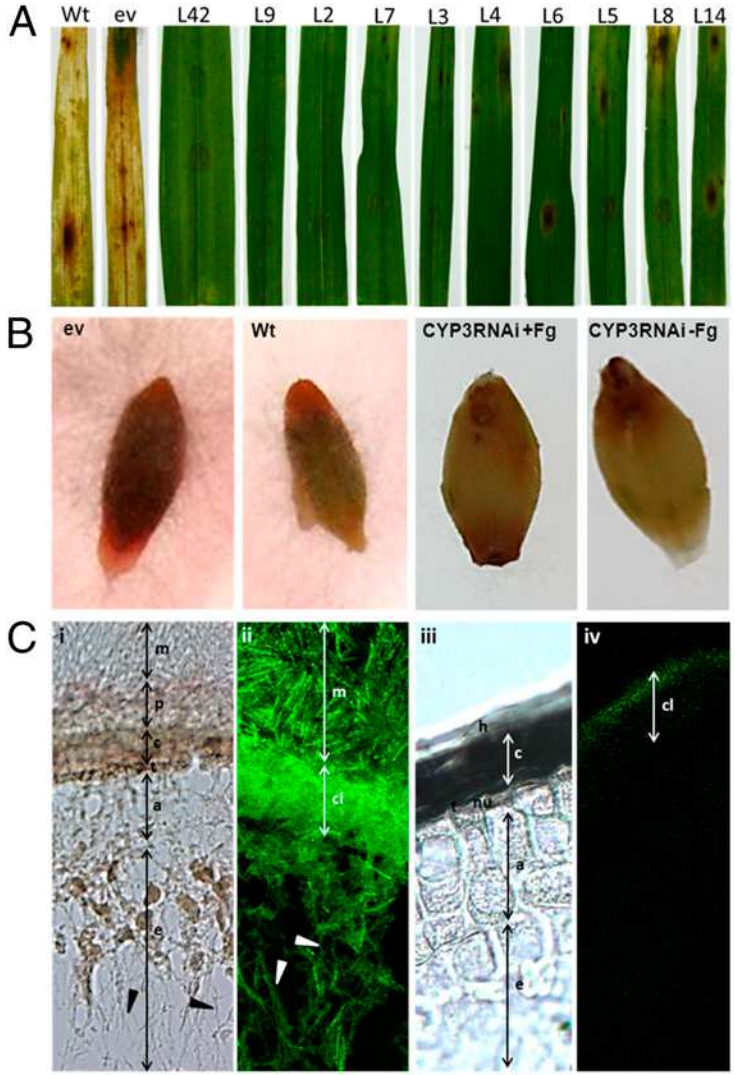



A
***
relative expression CYP51A
250
200
150
100
50
0
ev
Line8
Line10
Line11
line1
B
***
relative expression CYP51B
120
100
80
60
40
20
0
ev
Line8
Line10
Line11
line1
C
**
relative expression CYP51C
40
35
30
25
20
15
10
5
0
ev
Line8
Line10
Line11
line1
D
siRNA
non-infected
Col-0-CYP3RNA
Col-0
Col-0 (ev)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

I costrutti per RNAi riducono drasticamente l'espressione di CYP51A,B e C



I costrutti per RNAi contro FgCYP51A,B e C sono efficaci anche quando espressi in orzo



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]*

Foglie spruzzate con dsRNA silenziano un gene *GFP* espresso in *F. graminearum*

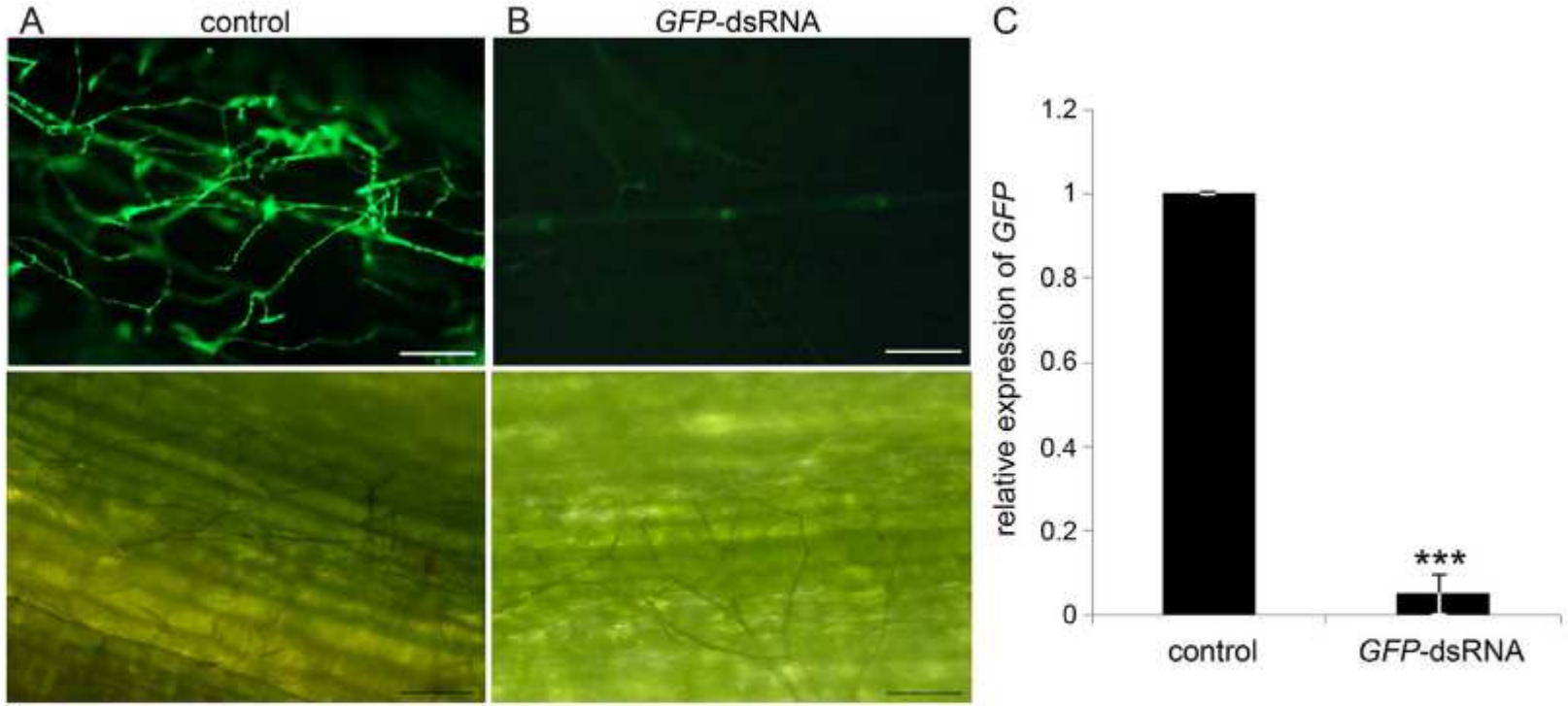


Fig 1. (A-C) Spray-induced gene silencing (SIGS) of *GFP* expression in *Fusarium graminearum* strain Fg-IFA65$_{GFP}$. Detached second leaves of three-week-old barley plants were locally sprayed with Tris-EDTA (TE, **A**, control) or *GFP*-dsRNA **(B)**. Forty-eight hours after spraying, distal, non-sprayed 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.

Foglie spruzzate con dsRNA contro CYP51A,B,C di *F. graminearum* mostrano minori sintomi

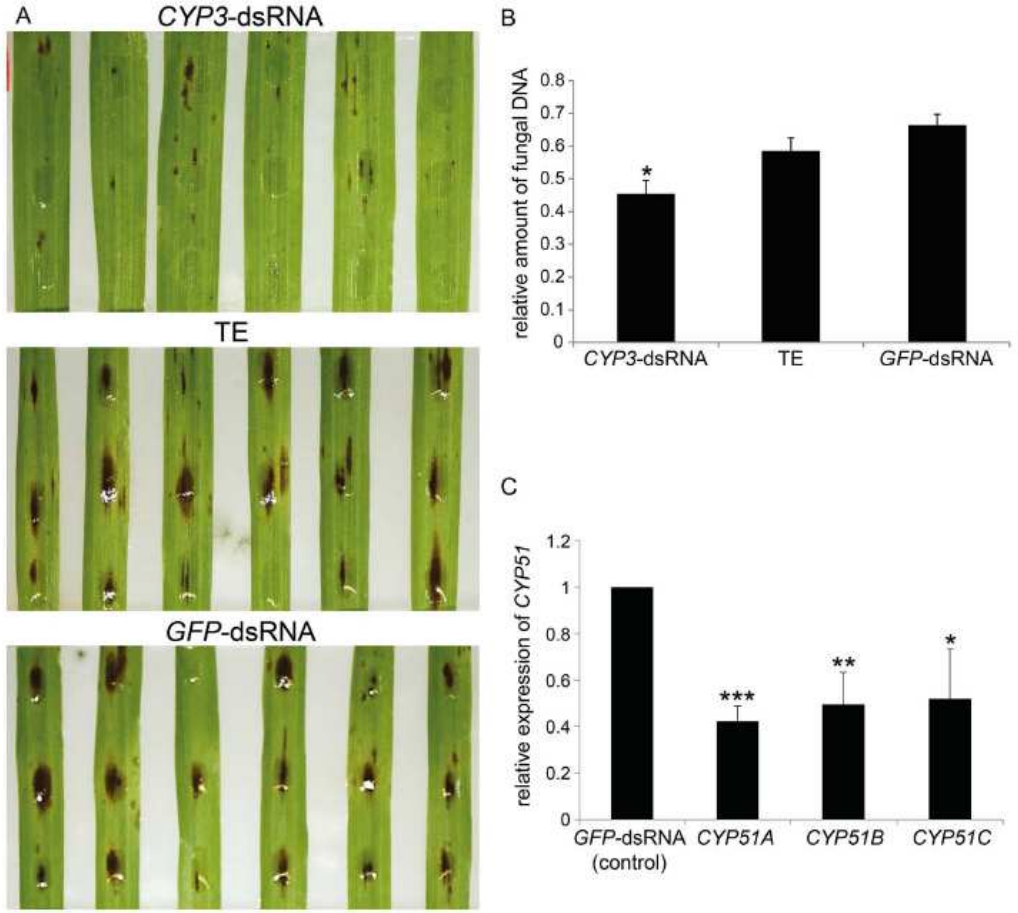
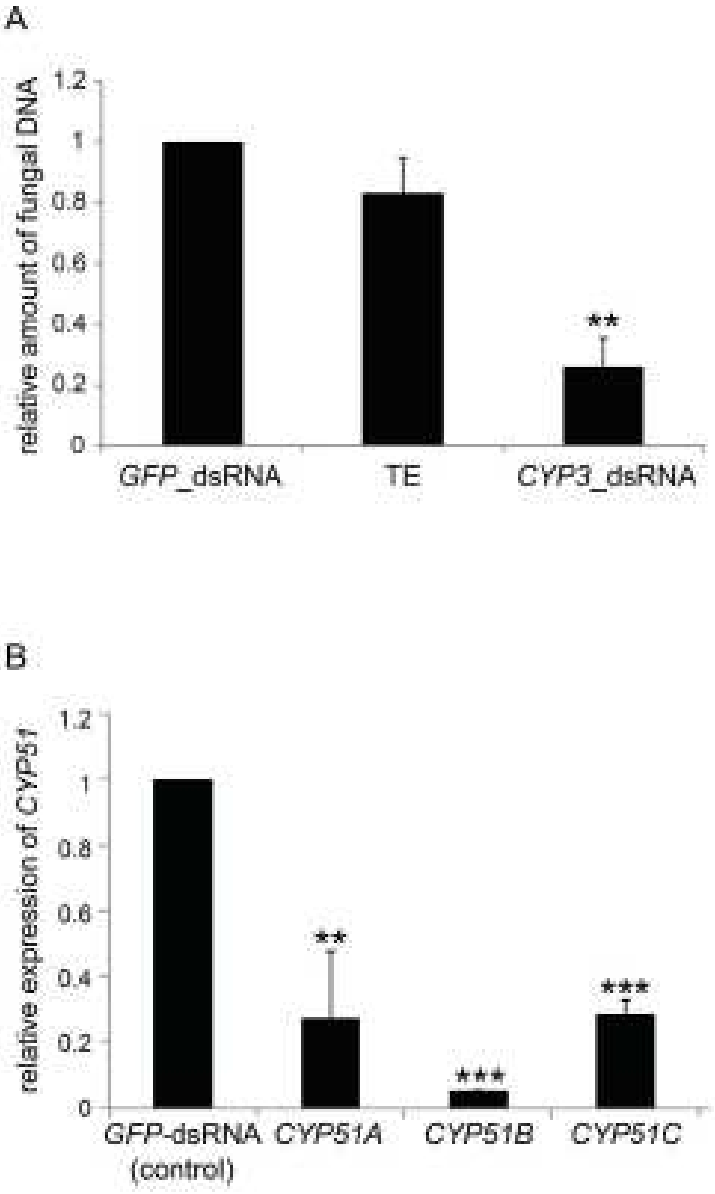


Fig 2. (A-C) SIGS-mediated control of *F. graminearum* on leaves sprayed with *CYP3*-dsRNA. (A) Detached second leaves of three-week-old barley were sprayed evenly with *CYP3*-dsRNA, TE (mock control), and *GFP*-dsRNA (negative control), respectively. After 48 hours, leaves were drop-inoculated with 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).


A
relative amount of fungal DNA
1.2
1
0.8
0.6
0.4
0.2
0
**
GFP_dsRNA
TE
CYP3_dsRNA
B
relative expression of CYP51
1.2
1
0.8
0.6
0.4
0.2
0
**
***
***
GFP-dsRNA (control)
CYP51A
CYP51B
CYP51C

Accumulo semi-sistemico di siRNA in piante spruzzate con dsRNA

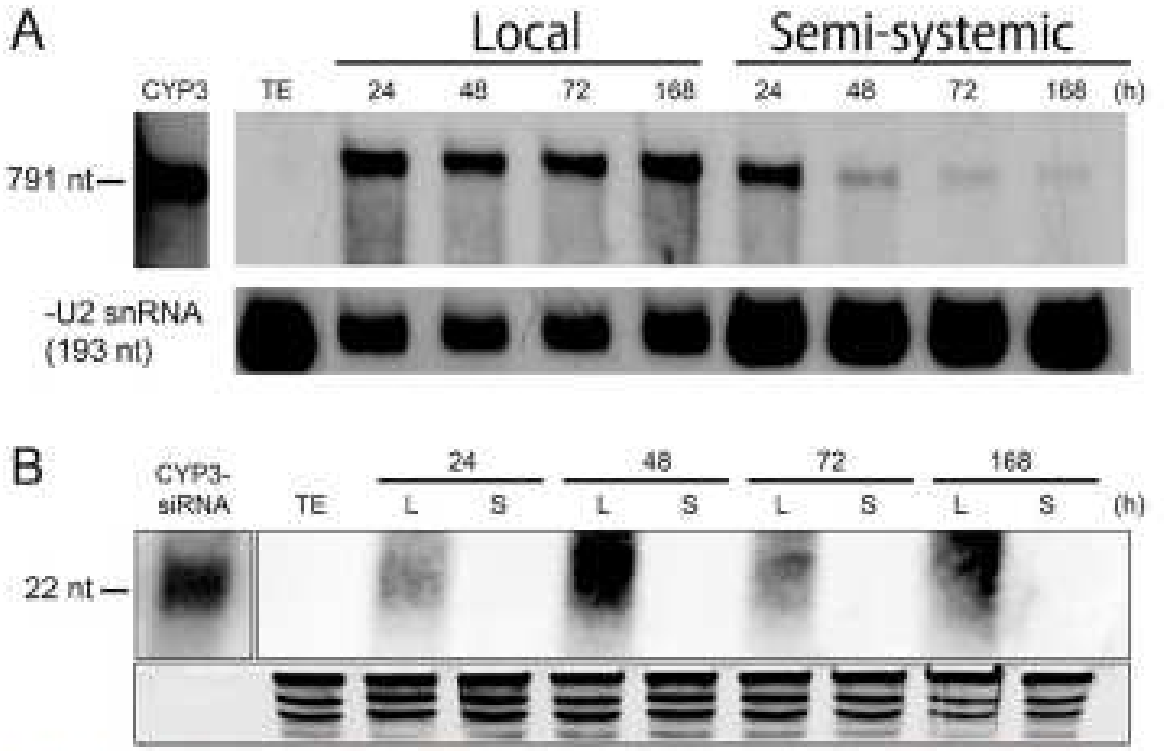


Fig 4. (A,B) Northern gel blot analysis of *CYP3*-dsRNA and *CYP3*-dsRNA-derived siRNA accumulation in local and distal (semi-systemic) barley leaf areas. (**A**) Detection of 791 nt long *CYP3*-dsRNA precursor in pooled leaf tissue from non-infected leaves using [α-32P]-dCTP labeled *CYP3*-dsRNA as 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.

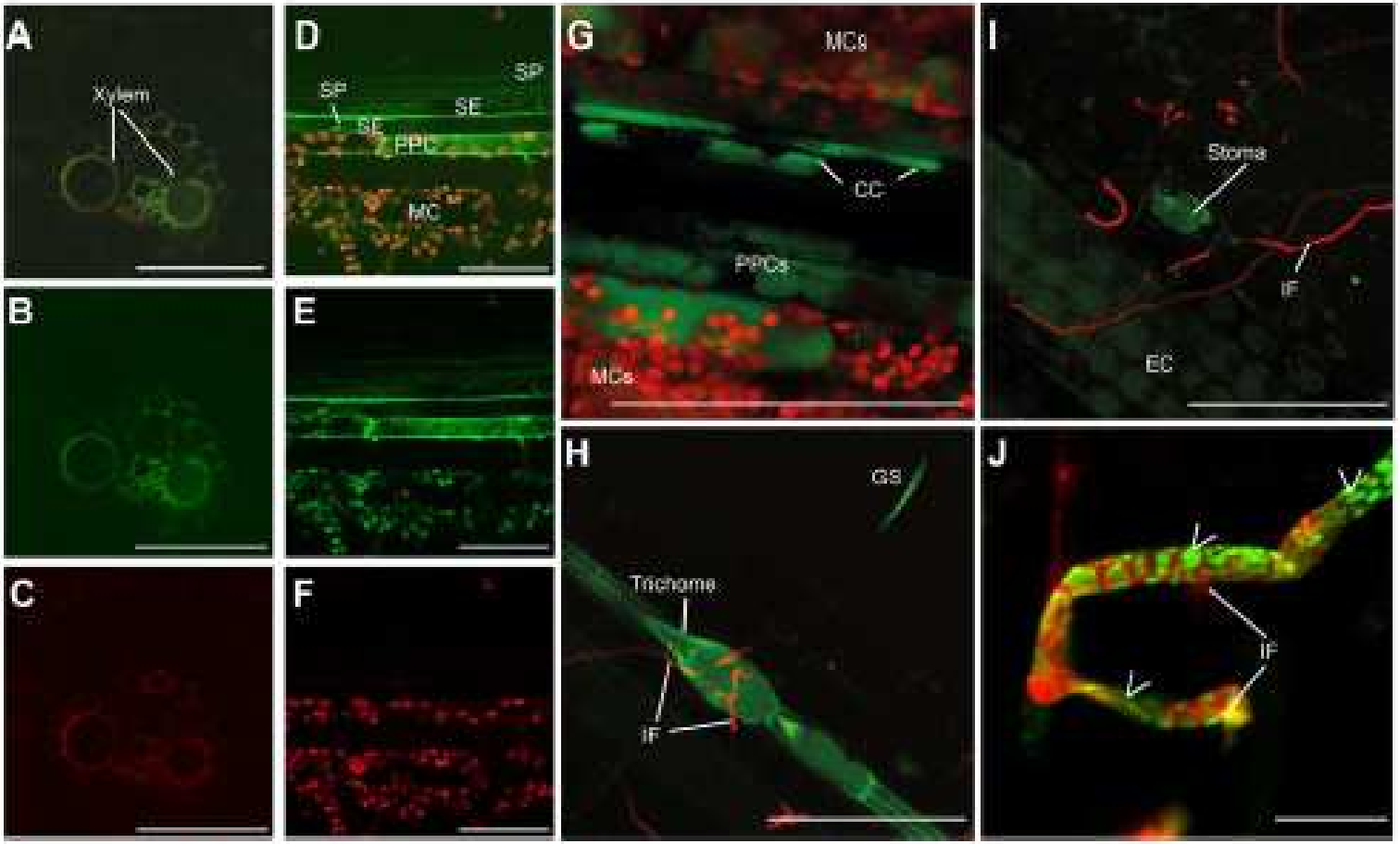
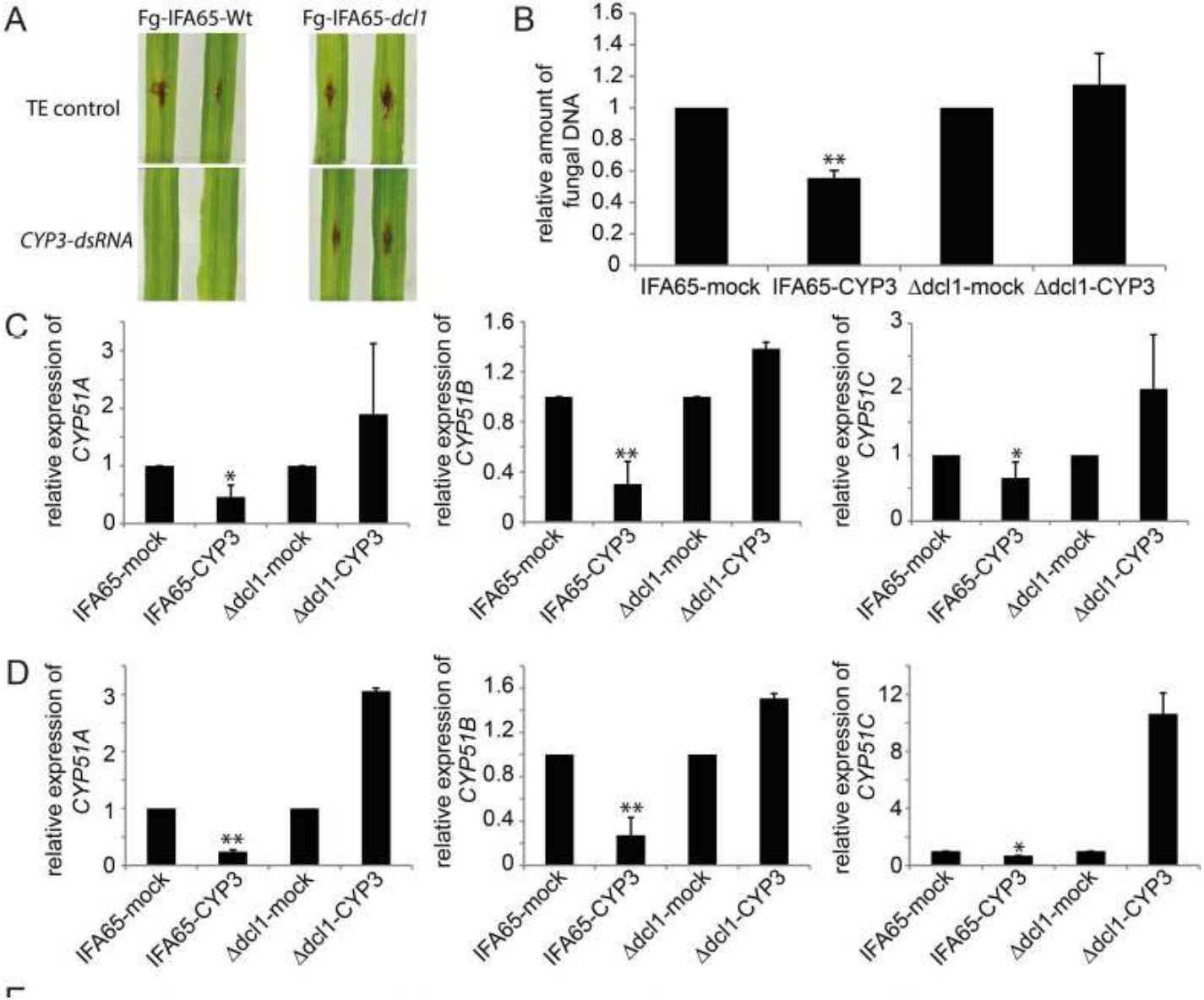


Fig 5. (A-J) Confocal laser scanning microscopy of ATTO 488-labeled *CYP3*-dsRNA$_{A488}$ in locally sprayed barley leaves. (A-C) Detection of *CYP3*-dsRNA$_{A488}$ (green) in xylem vessels of vascular bundles 24 h after spraying. **(D-G)** Longitudinal sections reveal uptake of *CYP3*-dsRNA$_{A488}$ by cells of the phloem tissue at 24 h after spraying. SE, sieve element; CC, companion cell; SP, sieve plate; PPC, phloem parenchyma cell; MC, mesophyll cell. The red cells result from the autofluorescence of chloroplasts (F,G). **(H-J)** Leaf hair cells (trichome), stomata, germinating spores (GS) and fungal hyphae strongly accumulated *CYP3*-dsRNA$_{A488}$. Fungal hyphae (IF) are stained with chitin-specific dye WGA-Alexa Fluor 594 (red) 24 h after inoculation. EC, epidermal cells. RNA signals in germinated conidia are marked by arrow heads. Scale bars 100 μm (A-H), 20 μm (F), and 10 μm (J).

DCL1 è necessario per la HIGS indotta da dsRNA spruzzato sulle foglie



PICCOLI RNA FUNGINI SILENZIANO GENI NELL'OSPITE

Fungal Small RNAs Suppress Plant Immunity by Hijacking Host RNA Interference Pathways

Arne Weiberg,[1,2,3]* Ming Wang,[1,2,3]* Feng-Mao Lin,[4] Hongwei Zhao,[1,2,3]† Zhihong Zhang,[1,2,3,5] Isgouhi Kaloshian,[2,3,6] Hsien-Da Huang,[4,7] Hailing Jin[1,2,3]‡

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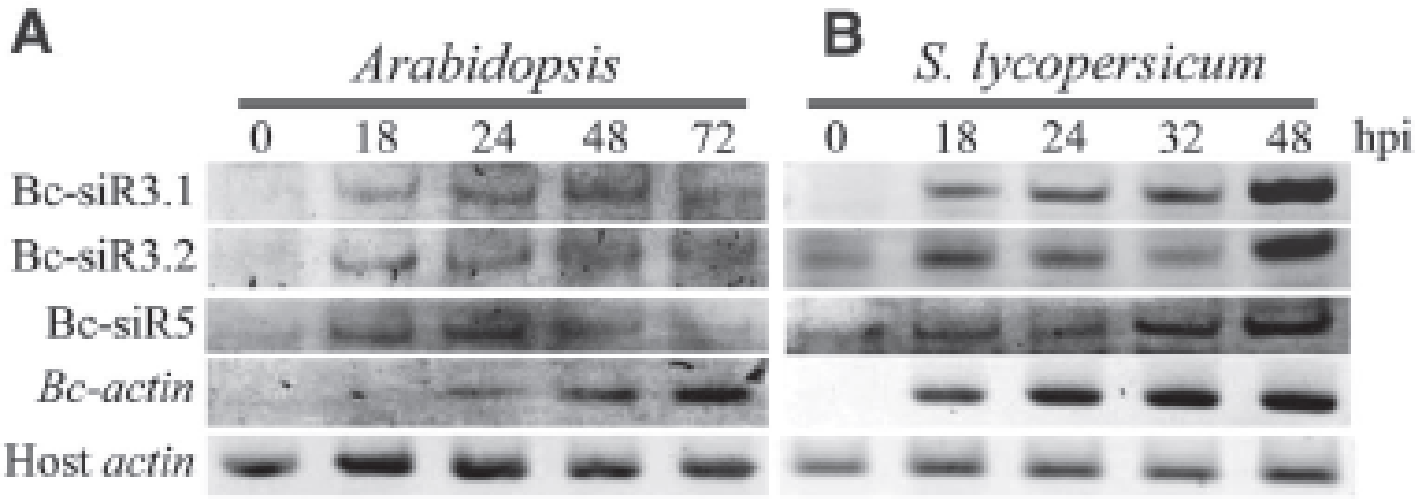
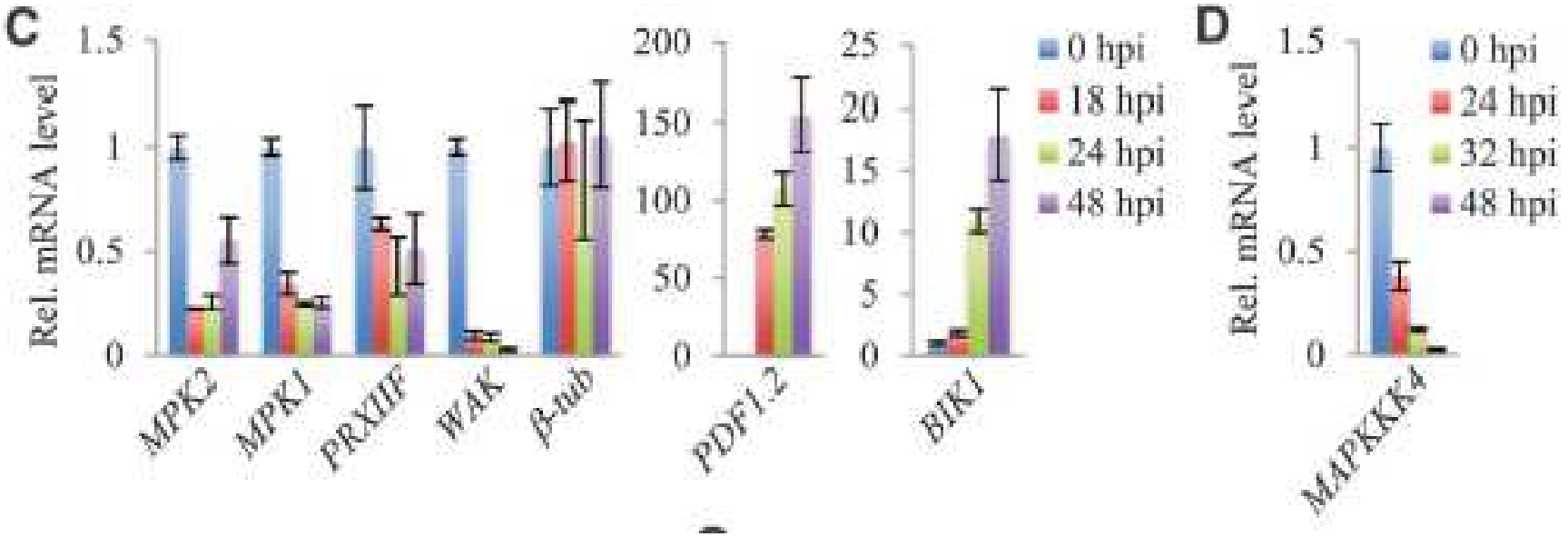
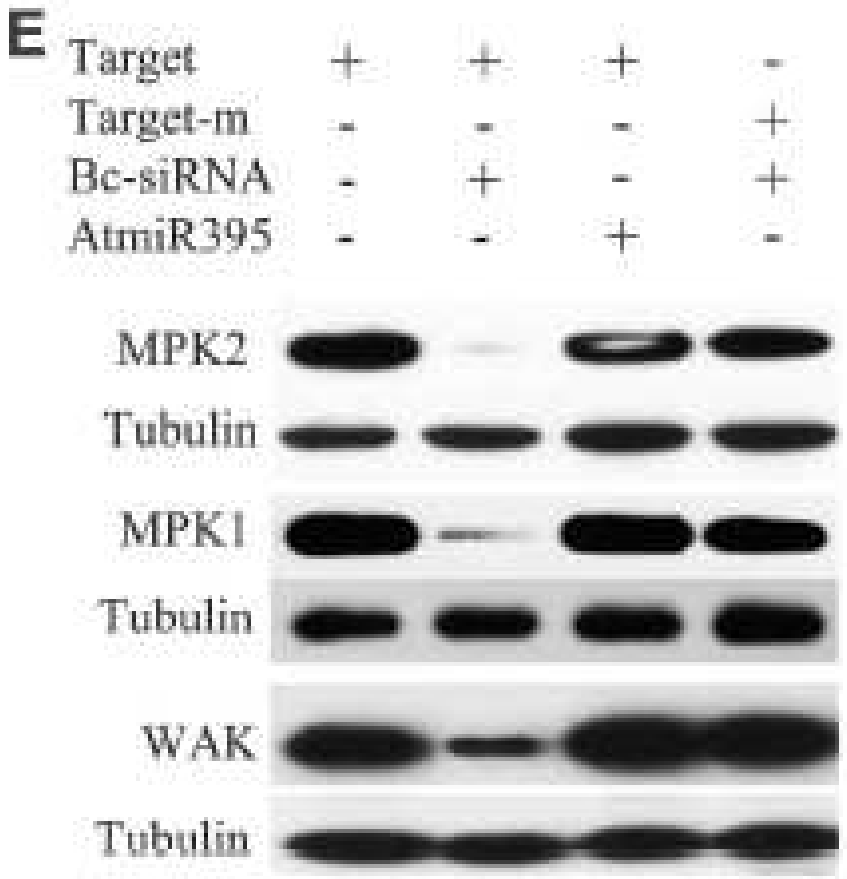
Botrytis cinerea produce siRNA durante l'infezione di Arabidopsis e pomodoro



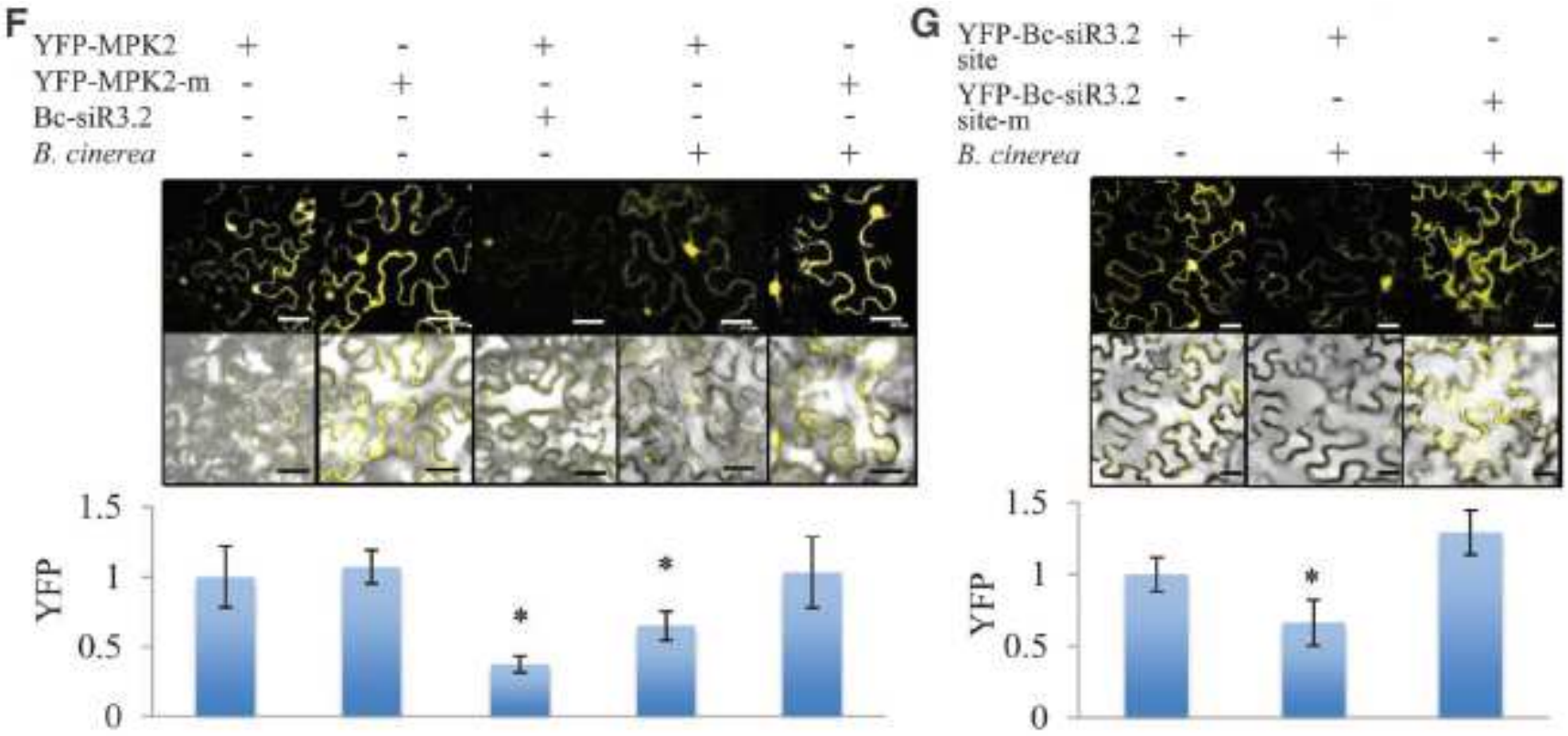
Geni target vegetali degli siRNA di *B. cinerea*



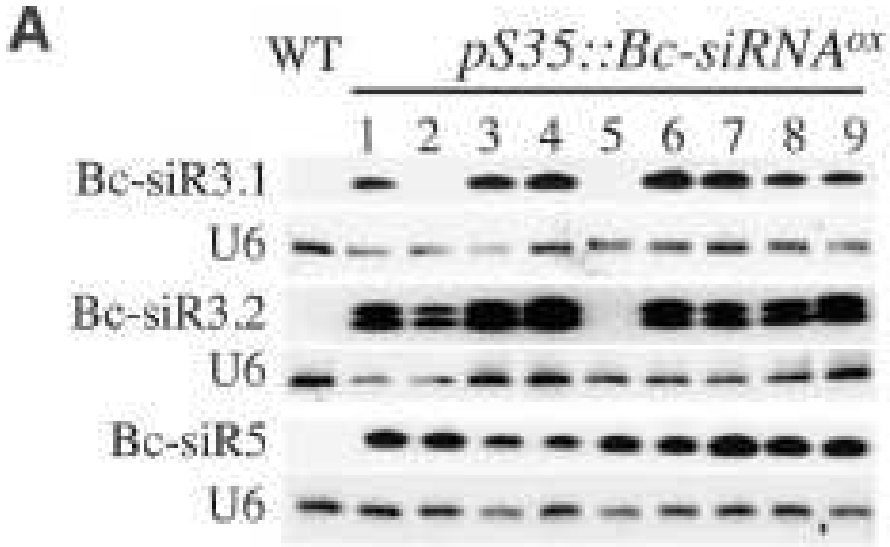
Bc-siR3.2 silenzia una MAP chinasi di pianta

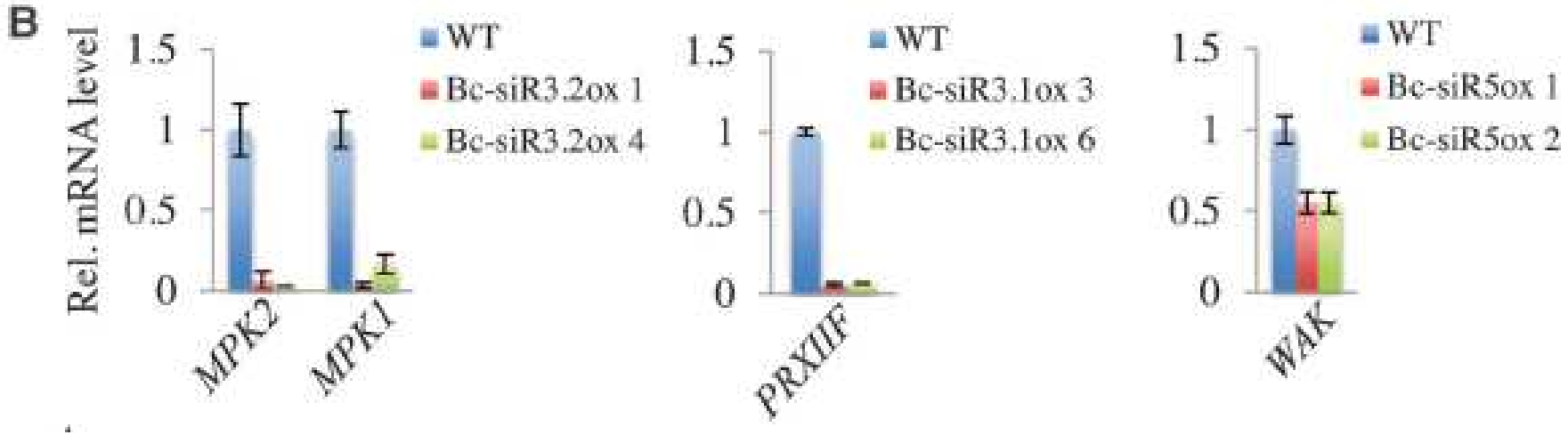


Bc-siR3.2 silenzia una MAP chinasi di pianta

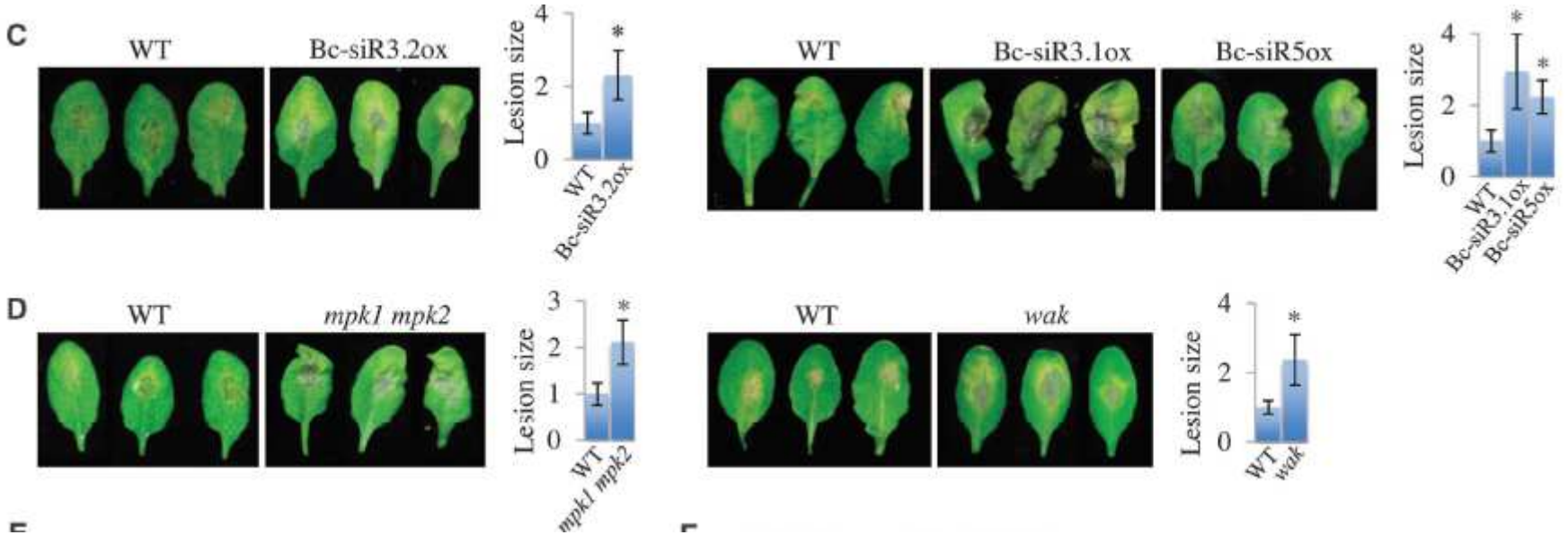


Bc-siR3.2 silenzia una MAP chinasi di pianta

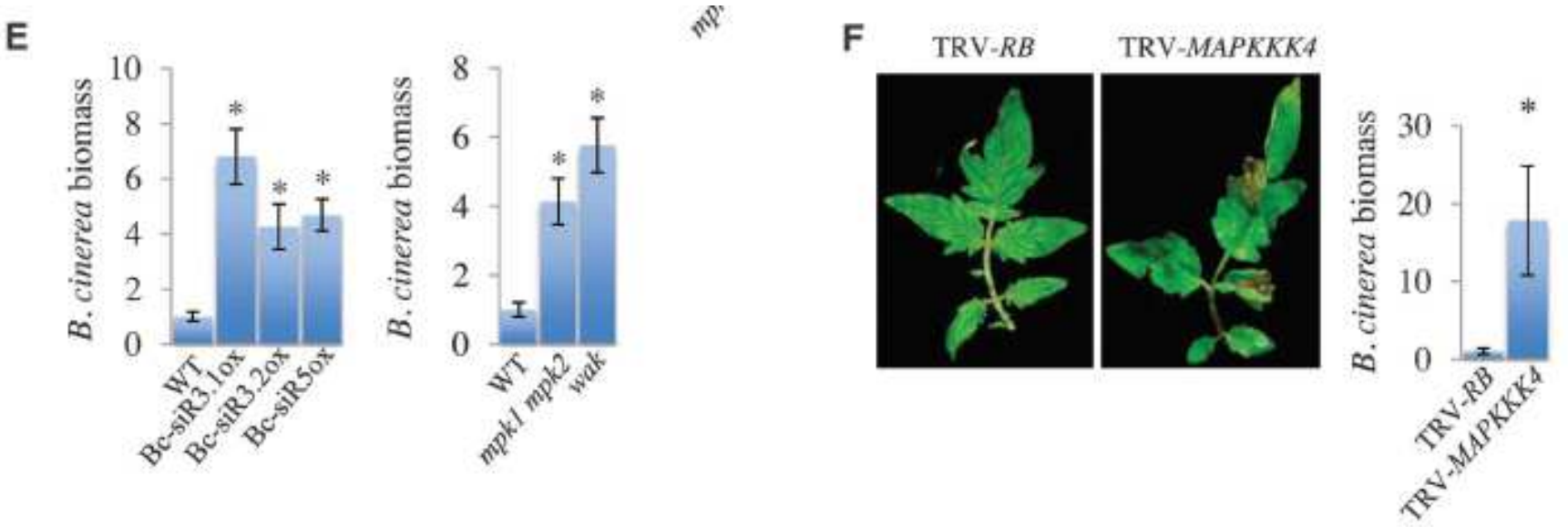




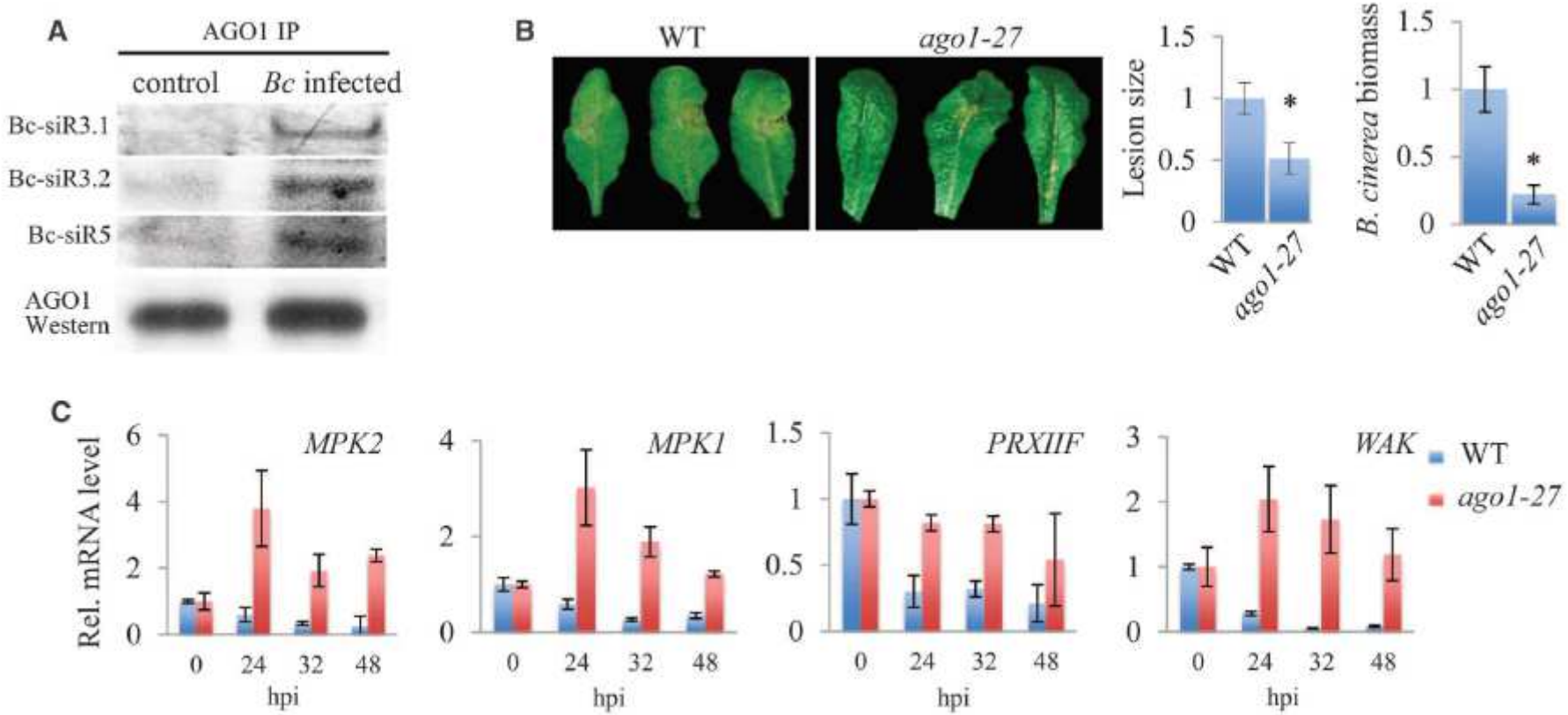
La sovraespressione di Bc-siR3.2 in Arabidopsis aumenta la suscettibilità a *B. cinerea*



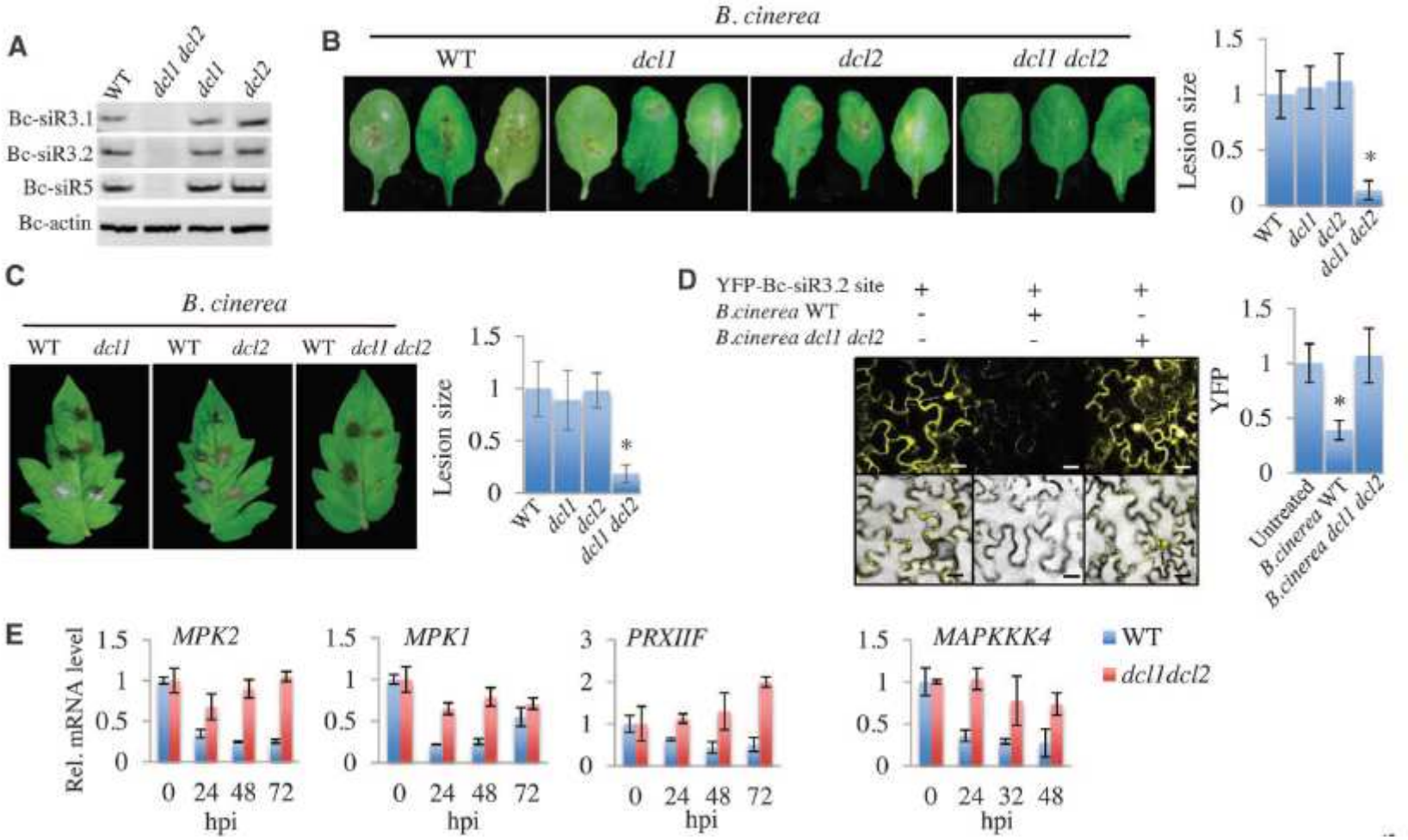
Bc-siR3.2 silenzia una MAP chinasi tripla di pomodoro



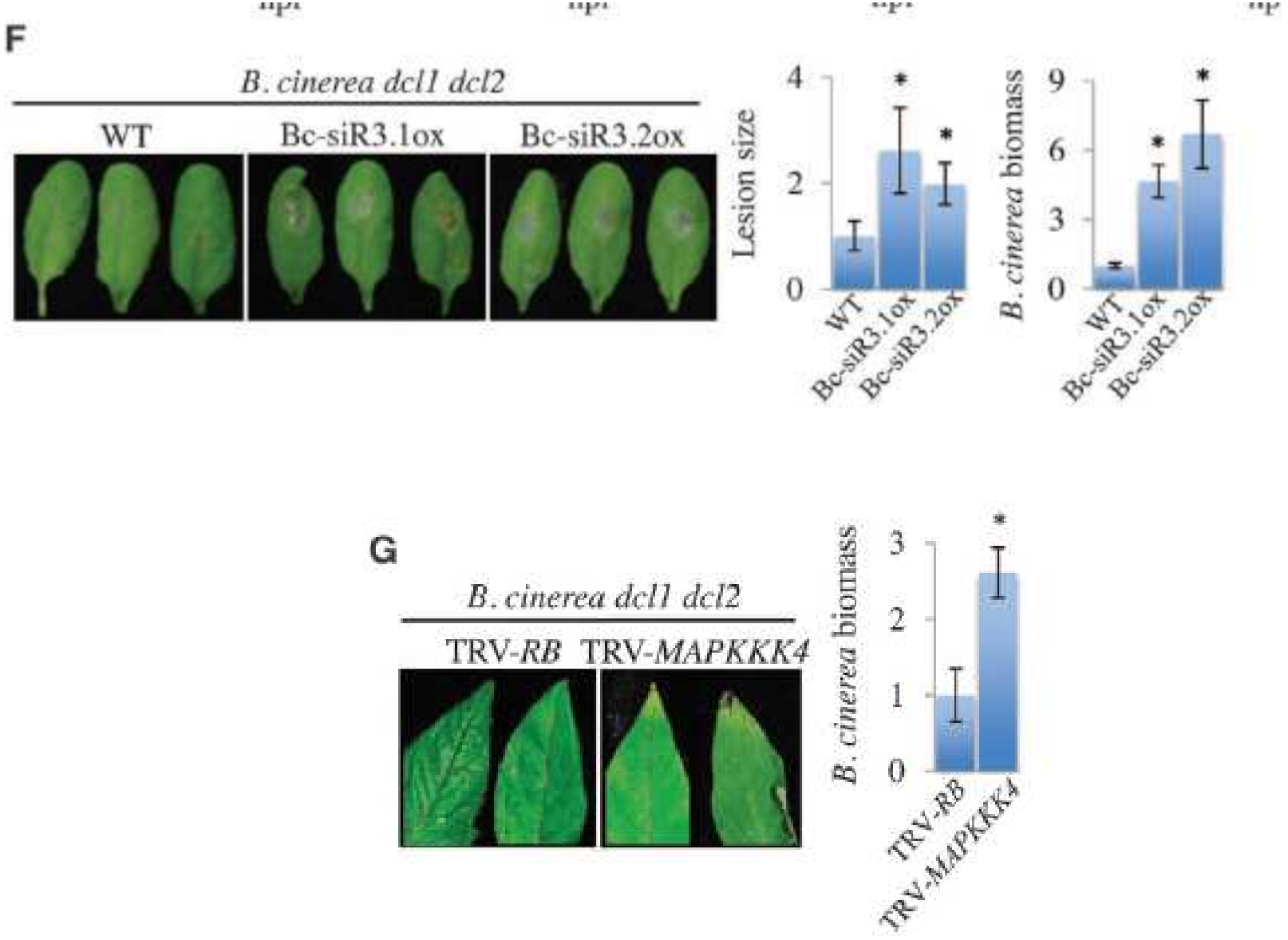
I siRNA di B. cinera richiedono AGO1 dell'ospite per ridurre la suscettibilità dell'ospite

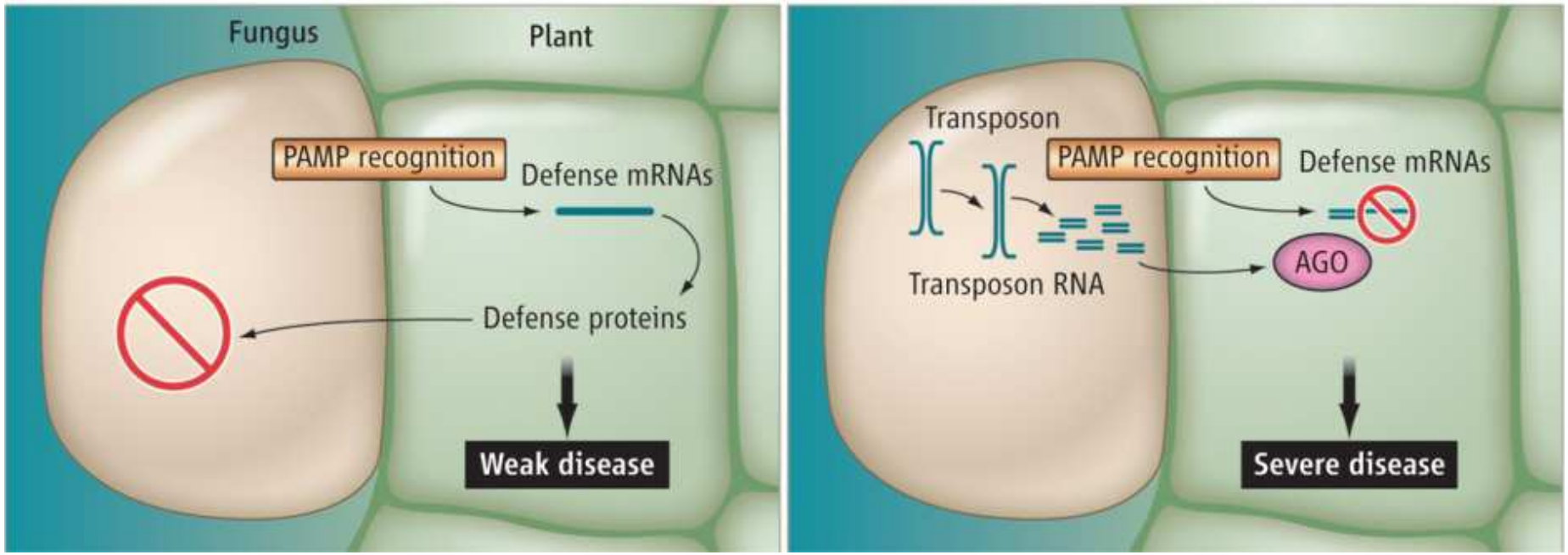


I siRNA di B. cinera richiedono DCL1 e 2 dell'ospite per ridurre la suscettibilità dell'ospite



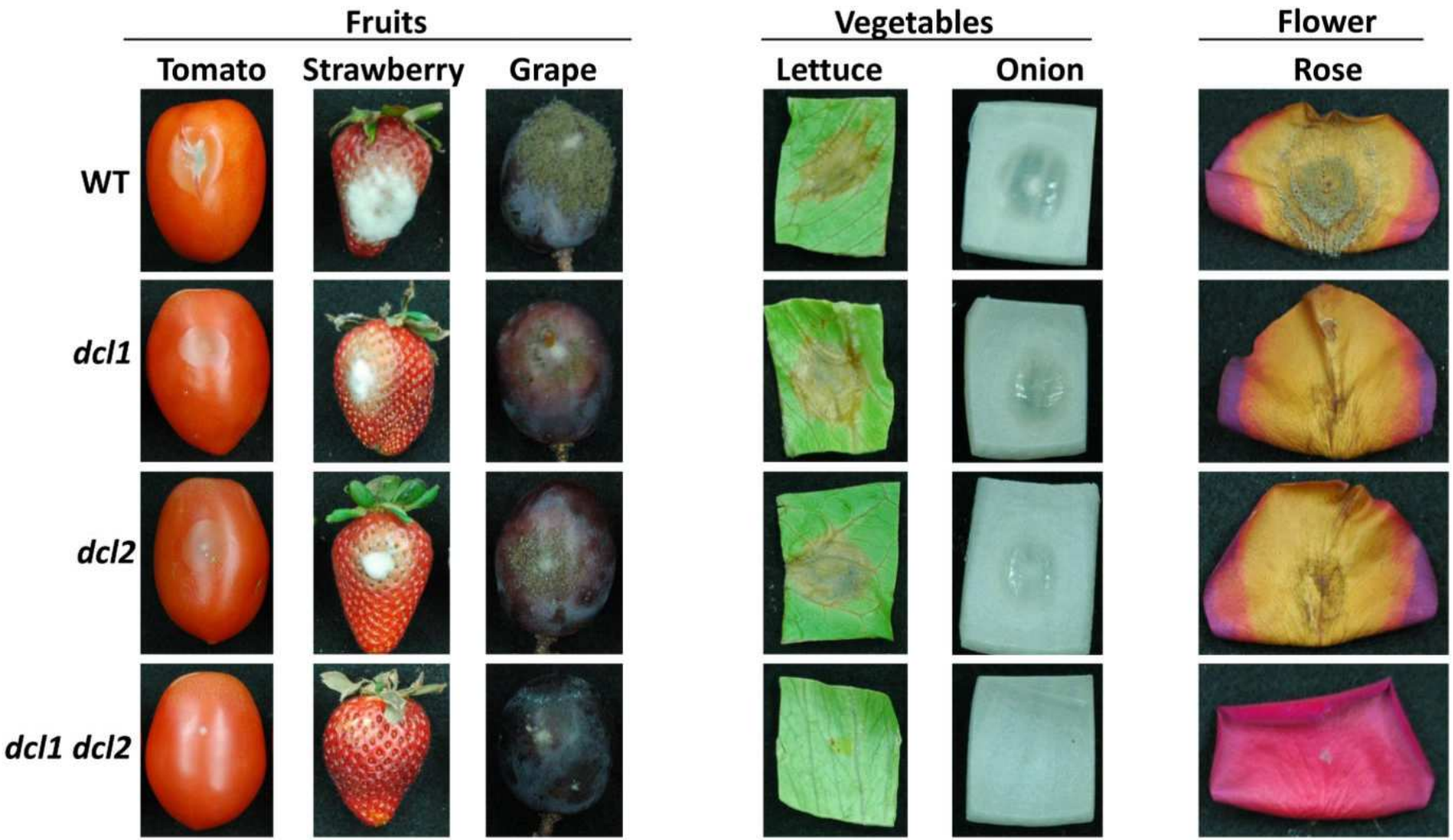
I siRNA di B. cinera richiedono DCL1 e 2 dell'ospite per ridurre la suscettibilità dell'ospite



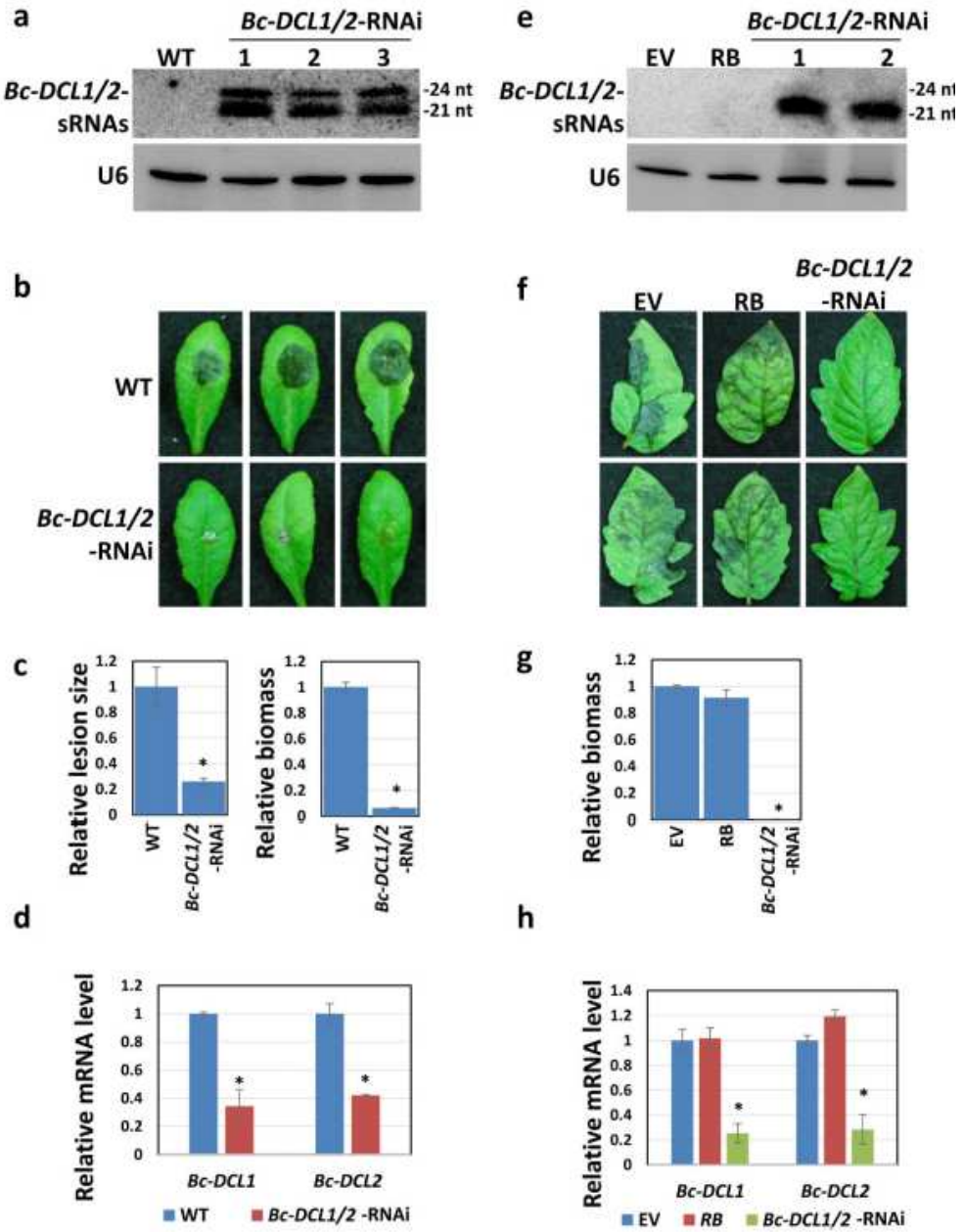

Fungus
Plant
PAMP recognition
Defense mRNAs
Defense proteins
Weak disease
Transposon
PAMP recognition
Defense mRNAs
AGO
Transposon RNA
Severe disease

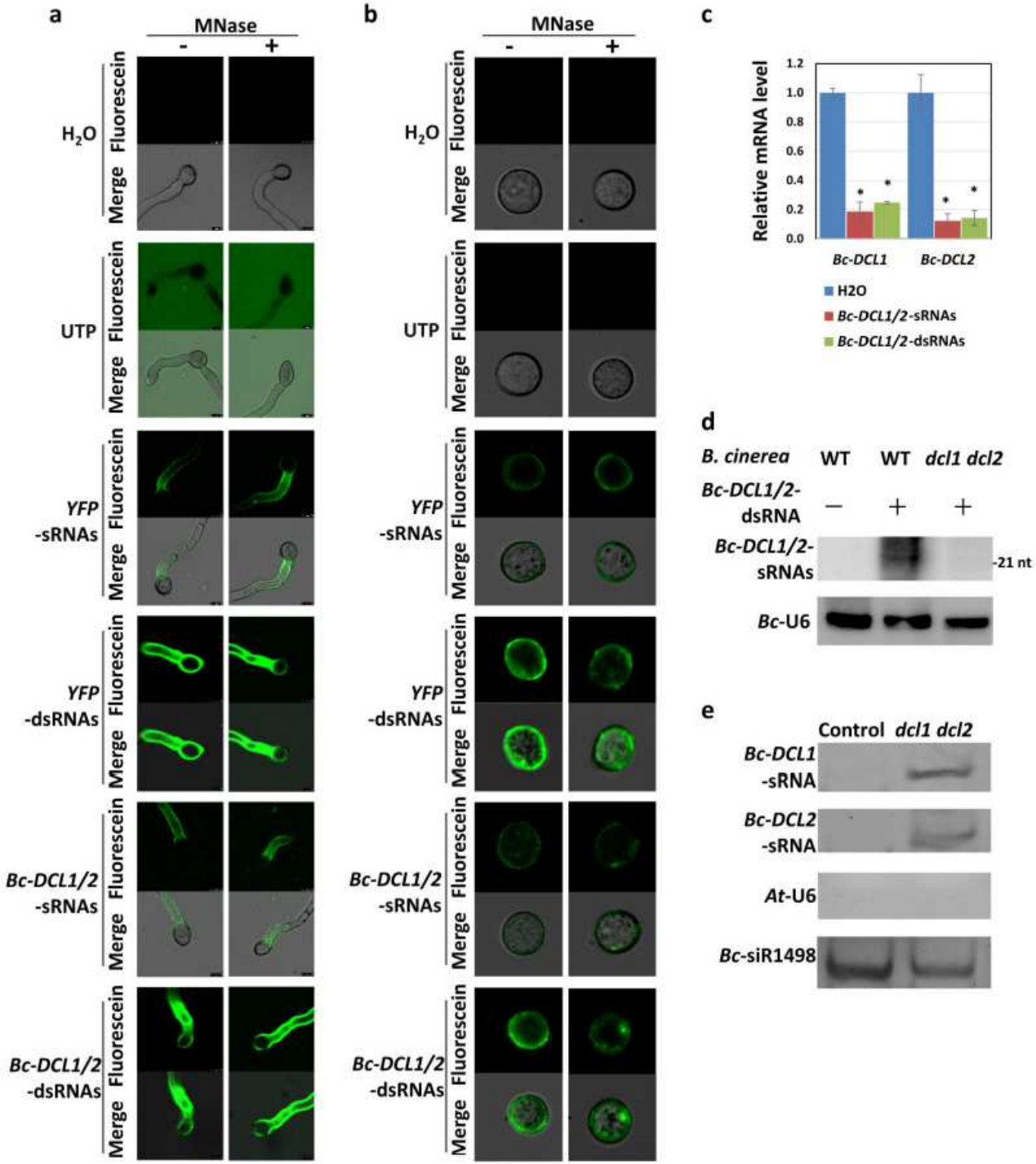
TRAFFICKING BIDIREZIONALE INTER-REGNO DI PICCOLI RNA

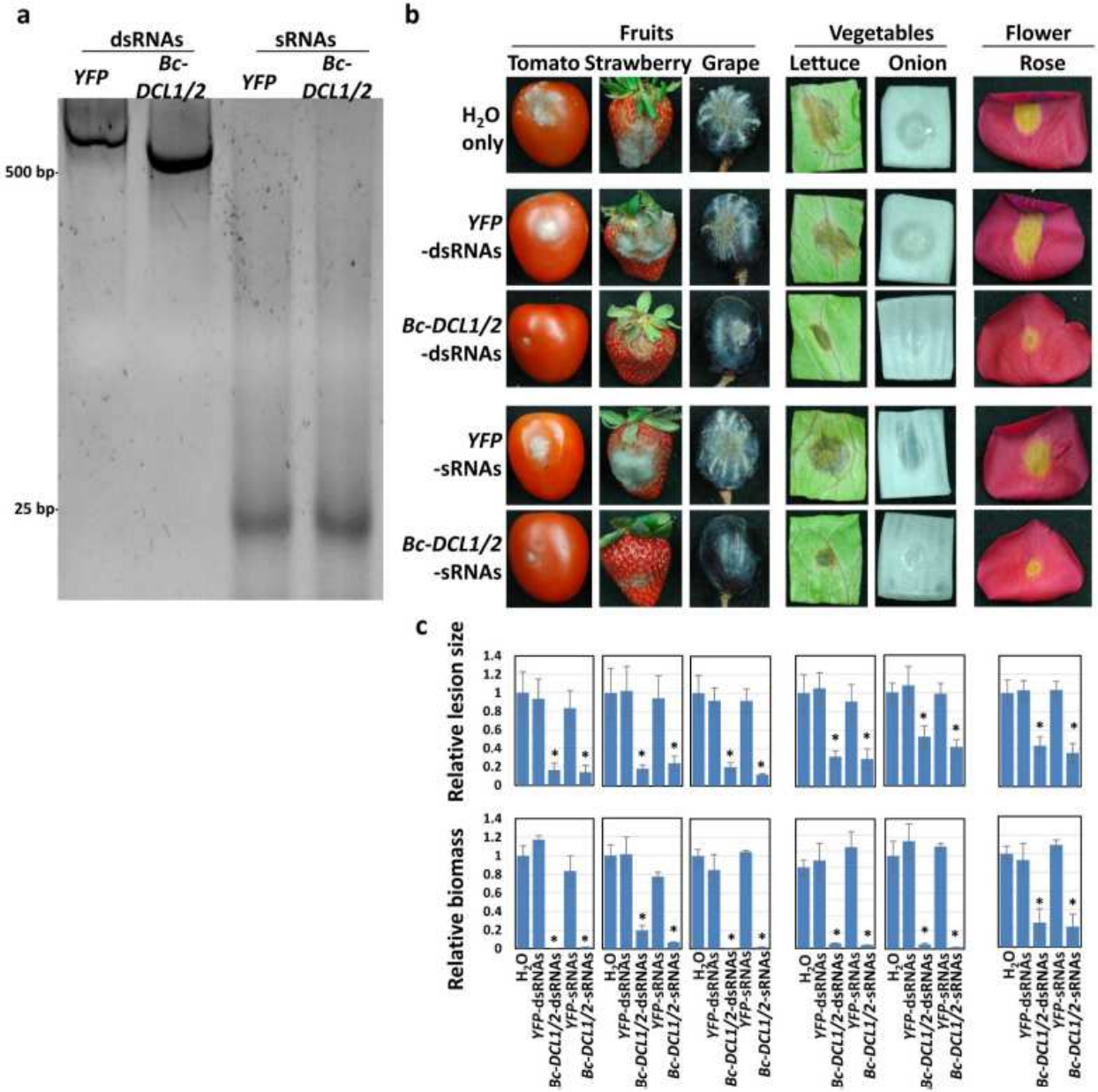
a


Fruits
Vegetables
Flower
Tomato
Strawberry
Grape
Lettuce
Onion
Rose
WT
dcl1
dcl2
dcl1 dcl2

b
Relative lesion size
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
dcl1
dcl2
dcl1 dcl2
*
c
Relative biomass
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
dcl1
dcl2
dcl1 dcl2
*


a
Bc-DCL1/2-RNAi
WT
1
2
3
Bc-DCL1/2-sRNAs
-24 nt
-21 nt
U6
e
Bc-DCL1/2-RNAi
EV
RB
1
2
Bc-DCL1/2-sRNAs
-24 nt
-21 nt
U6
b
WT
Bc-DCL1/2 -RNAi
f
EV
RB
Bc-DCL1/2 -RNAi
c
Relative lesion size
1.2
1
0.8
0.6
0.4
0.2
0
*
WT
Bc-DCL1/2 -RNAi
Relative biomass
1.2
1
0.8
0.6
0.4
0.2
0
*
WT
Bc-DCL1/2 -RNAi
g
Relative biomass
1.2
1
0.8
0.6
0.4
0.2
0
*
EV
RB
Bc-DCL1/2 -RNAi
d
Relative mRNA level
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Bc-DCL1
Bc-DCL2
WT
Bc-DCL1/2 -RNAi
h
Relative mRNA level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Bc-DCL1
Bc-DCL2
EV
RB
Bc-DCL1/2 -RNAi


a
MNase
-
+
H2O
UTP
YFP -sRNAs
YFP -dsRNAs
Bc-DCL1/2 -sRNAs
Bc-DCL1/2 -dsRNAs
Fluorescein
Merge
b
MNase
-
+
H2O
UTP
YFP -sRNAs
YFP -dsRNAs
Bc-DCL1/2 -sRNAs
Bc-DCL1/2 -dsRNAs
Fluorescein
Merge
c
Relative mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Bc-DCL1
Bc-DCL2
H2O
Bc-DCL1/2-sRNAs
Bc-DCL1/2-dsRNAs
d
B. cinerea
WT
WT
dcl1 dcl2
Bc-DCL1/2-dsRNA
−
+
+
Bc-DCL1/2-sRNAs
21 nt
Bc-U6
e
Control
dcl1 dcl2
Bc-DCL1 -sRNA
Bc-DCL2 -sRNA
At-U6
Bc-siR1498


a
dsRNAs
sRNAs
YFP
Bc-DCL1/2
YFP
Bc-DCL1/2
500 bp
25 bp
b
Fruits
Vegetables
Flower
Tomato
Strawberry
Grape
Lettuce
Onion
Rose
H2O only
YFP -dsRNAs
Bc-DCL1/2 -dsRNAs
YFP -sRNAs
Bc-DCL1/2 -sRNAs
c
Relative lesion size
Relative biomass
0
0.2
0.4
0.6
0.8
1
1.2
1.4
H2O
YFP-dsRNAs
Bc-DCL1/2-dsRNAs
YFP-sRNAs
Bc-DCL1/2-sRNAs