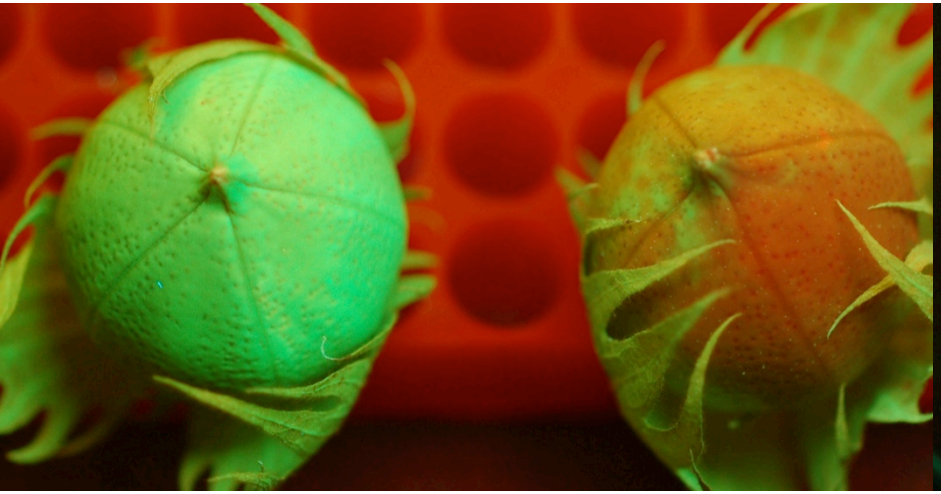
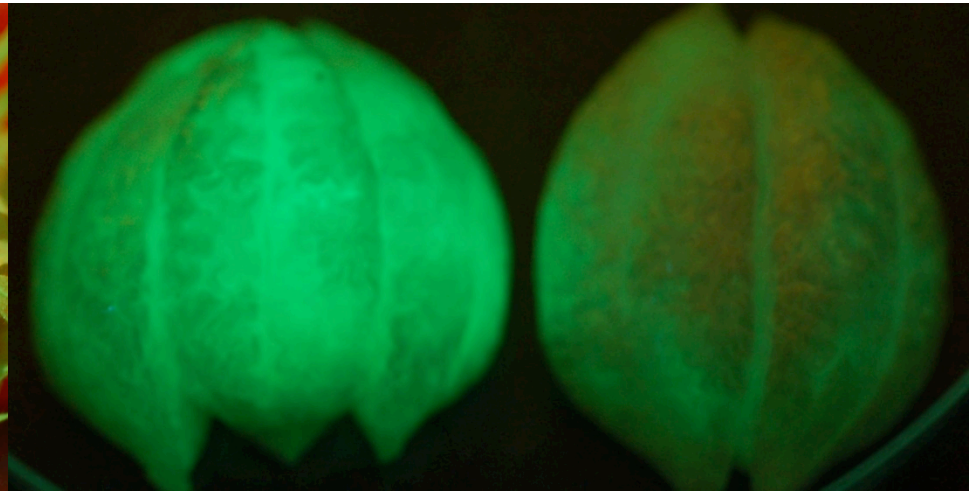
The Dynamics of Virus-induced Gene Silencing in Cotton Fiber

J. Rich Tuttle, Candace H. Haigler, Dominique Robertson





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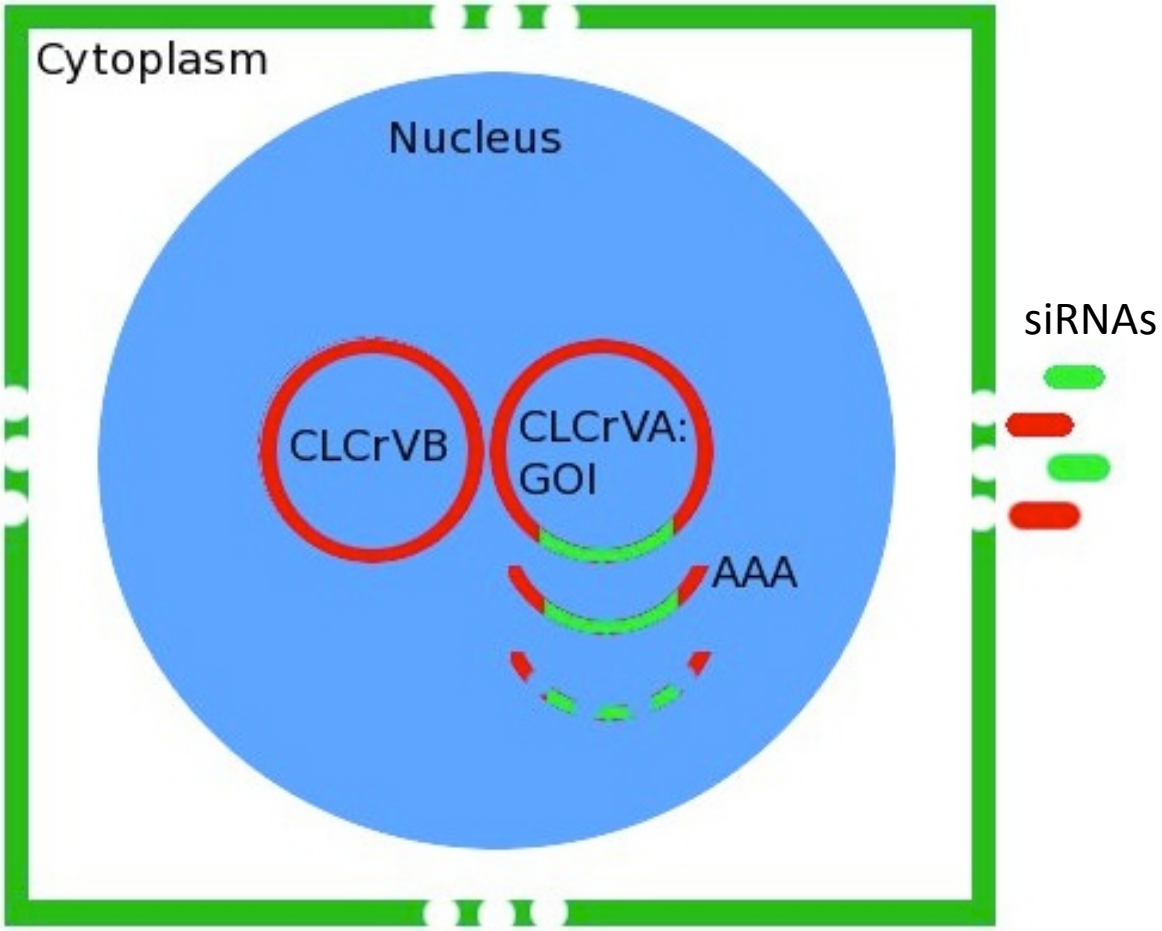
2012 ICGI
October 10th

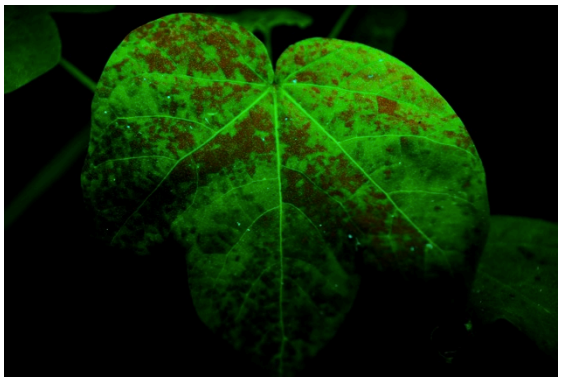
Outline

- Background
  - Virus-Induced Gene Silencing (aka VIGS)
  - CLCrV-based VIGS in cotton
- VIGS of 35S:GFP in cotton fiber
  - Reductions in total fluorescence
  - Down-regulation of transcript
  - As a marker for locating silenced tissues
- Differences between endogenous and transgene silencing
- Summary/Conclusions

What is Virus-Induced Gene Silencing (VIGS)?



CLCrVA:smRSGFP



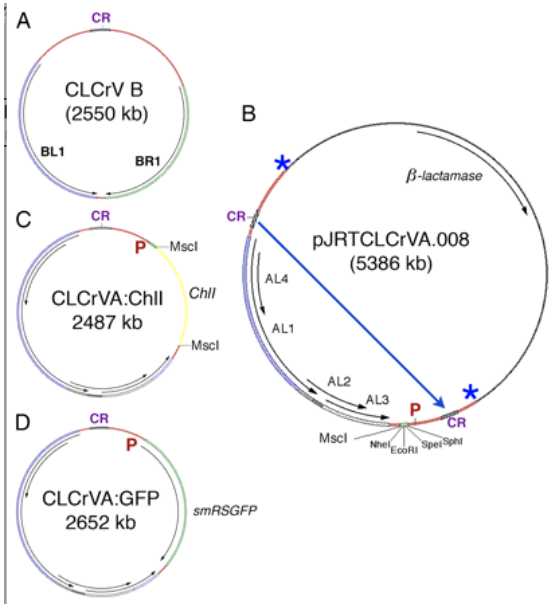
Plant Physiology 148:41–50 (2008)

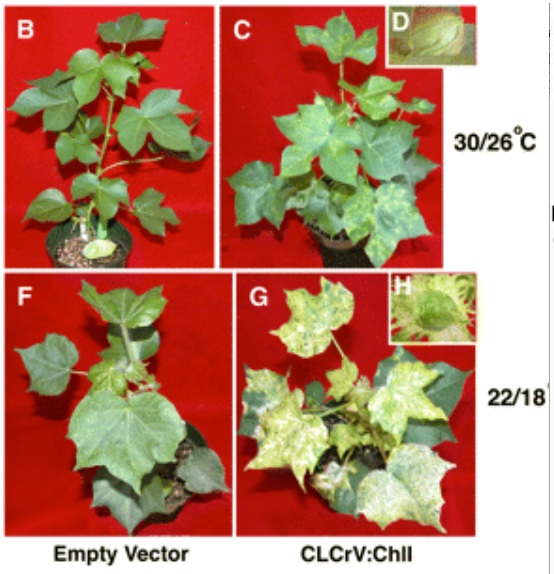
OPEN ACCESS ARTICLE

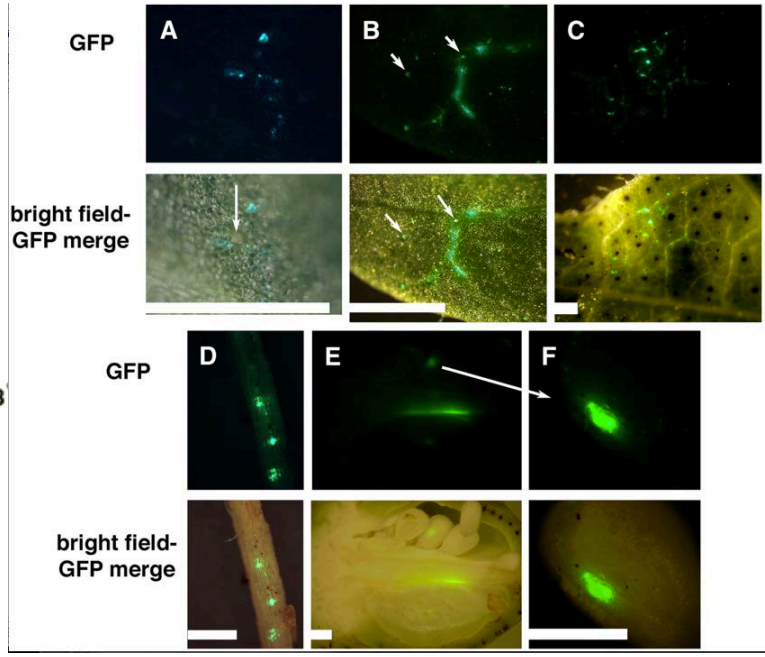
BREAKTHROUGH TECHNOLOGIES

Geminivirus-Mediated Gene Silencing from *Cotton Leaf Crumple Virus* Is Enhanced by Low Temperature in Cotton[1,[C],[OA]]

John R. Tuttle, A.M. Idris, Judith K. Brown, Candace H. Haigler and Dominique Robertson*

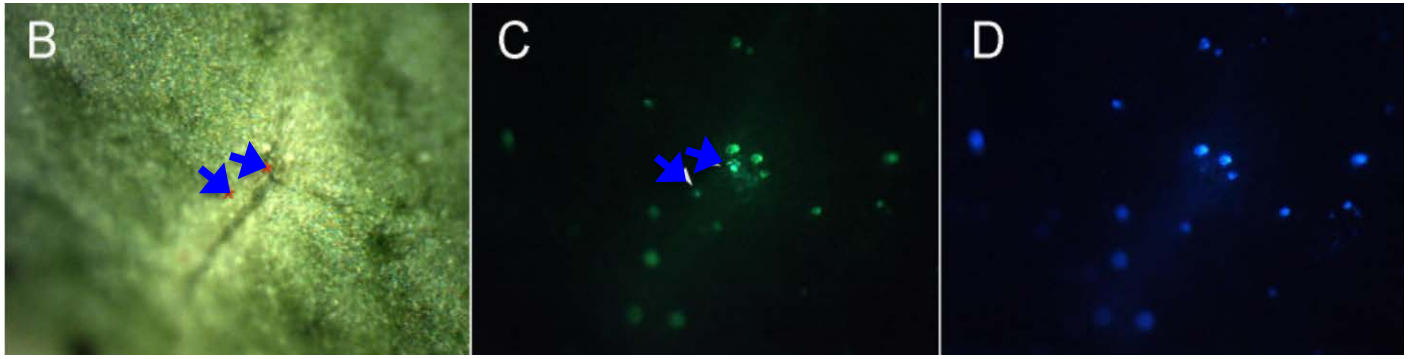


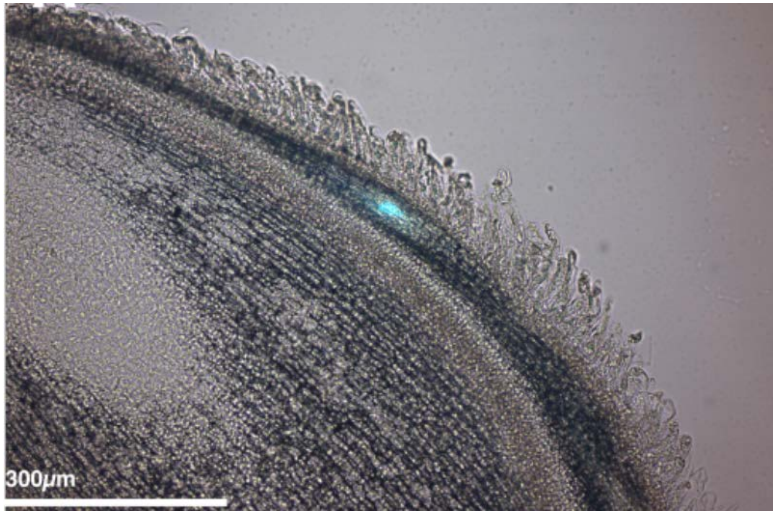




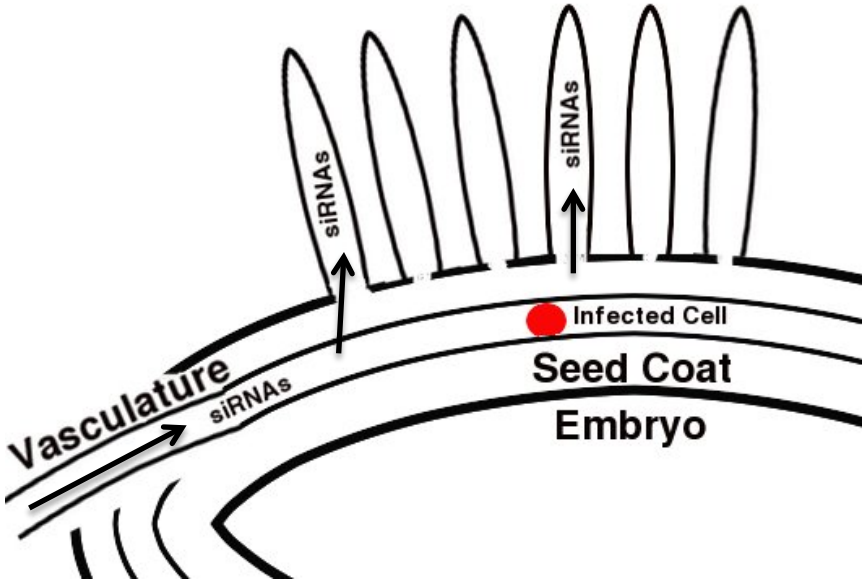
Silencing Spreads Beyond Infected Cells

CLCrVA:GFP + CLCrVB:ChlI (10 DPI seedling, cv DP5415)





CLCrVA:GFP in 3 DPA ovule (cv DP5415)

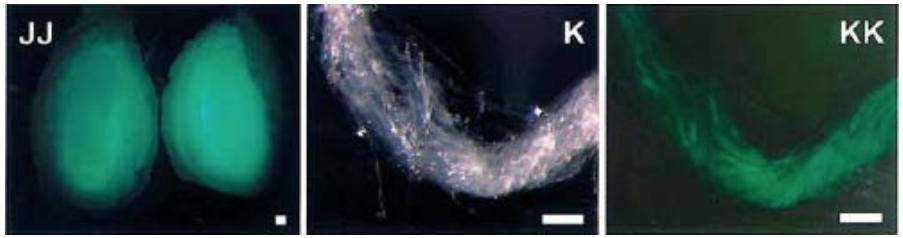


Using GFP as a Marker for Gene Silencing in Cotton Fiber

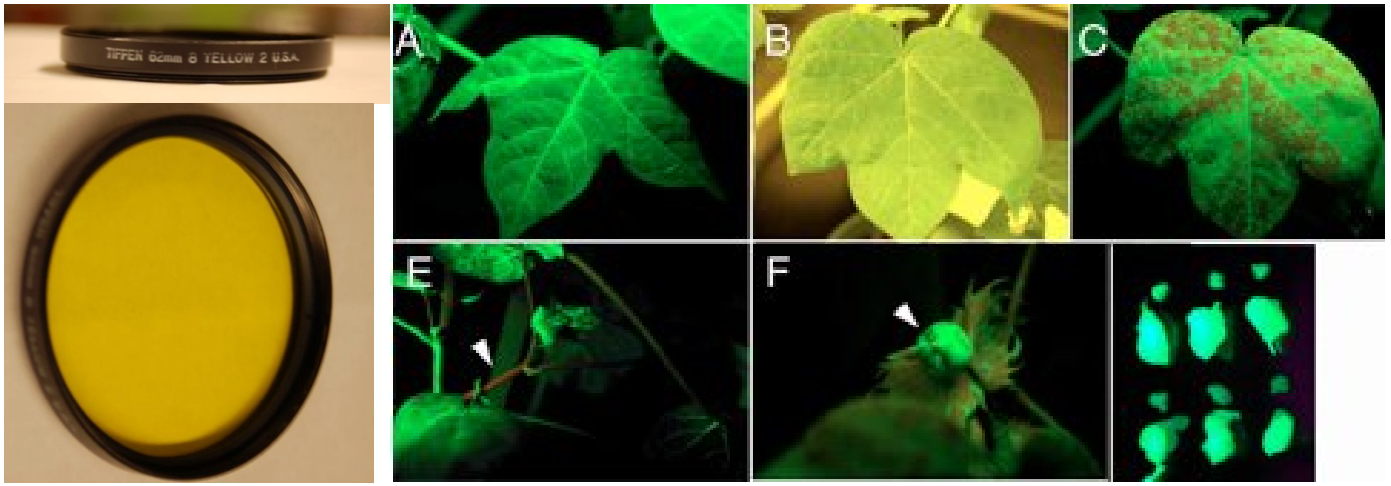
Plant Molecular Biology **50:** 463–474, 2002.

Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP

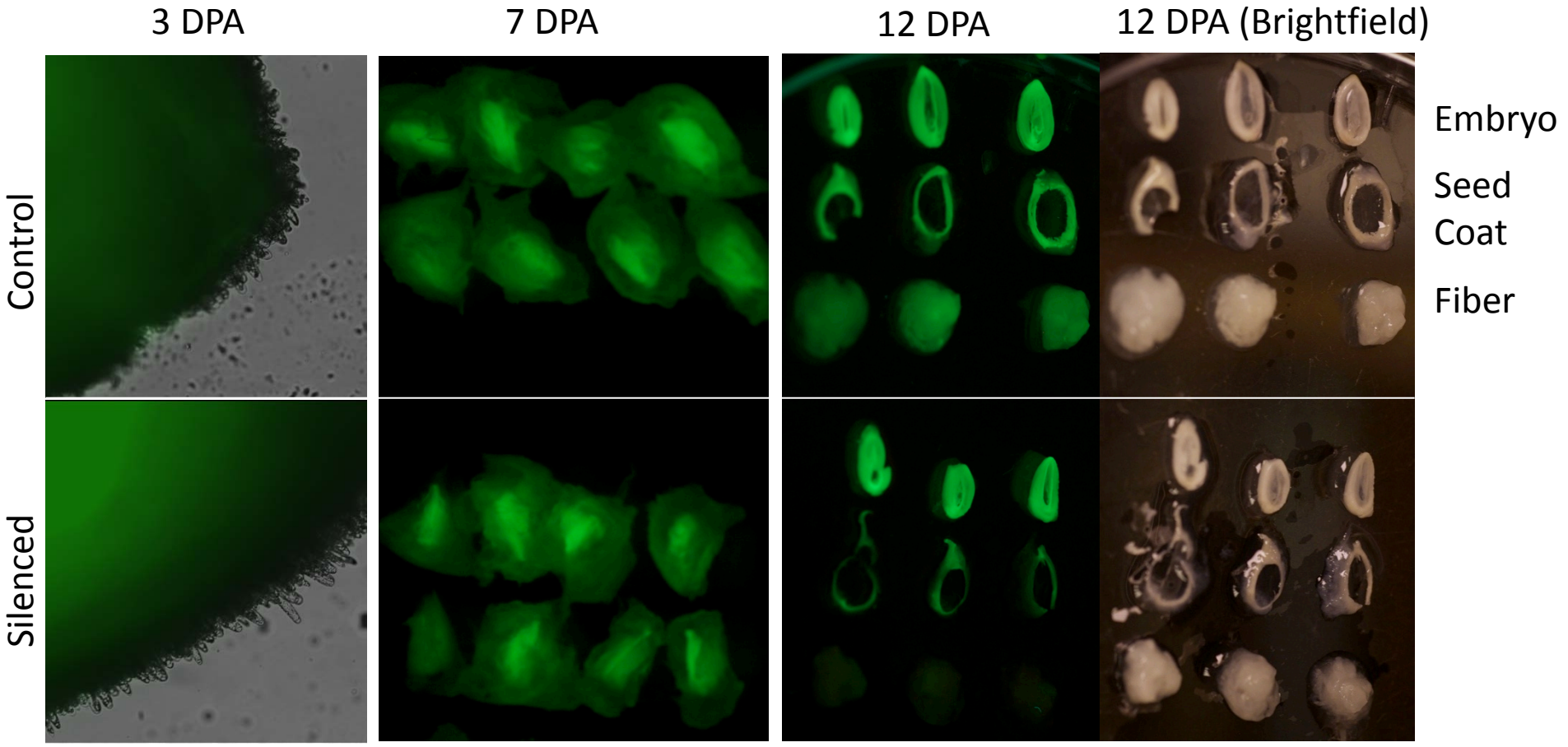
Ganesan Sunilkumar, LeAnne Mohr, Emily Lopata-Finch, Chandrakanth Emani and Keerti S. Rathore*



Photographed with a 365nm hand-held UV light and a yellow lens filter



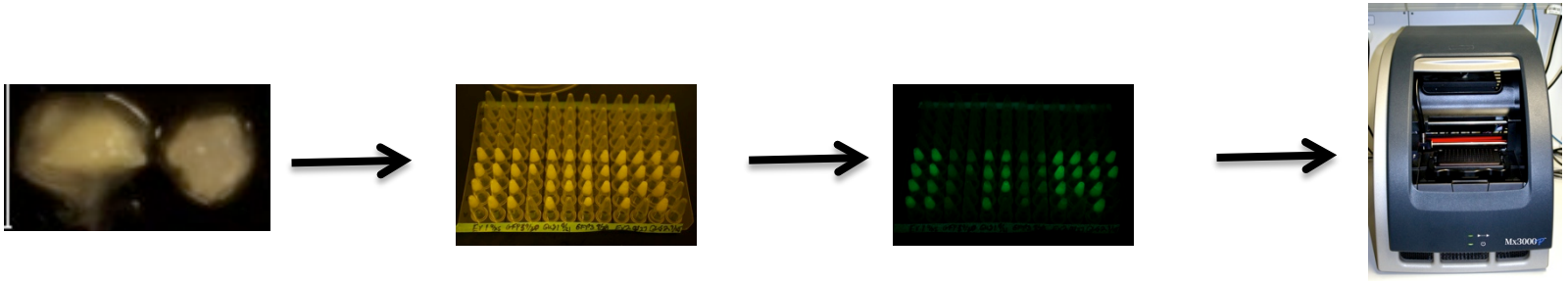
GFP Silencing in the Fiber is Masked by Fluorescence in Adjacent Tissues

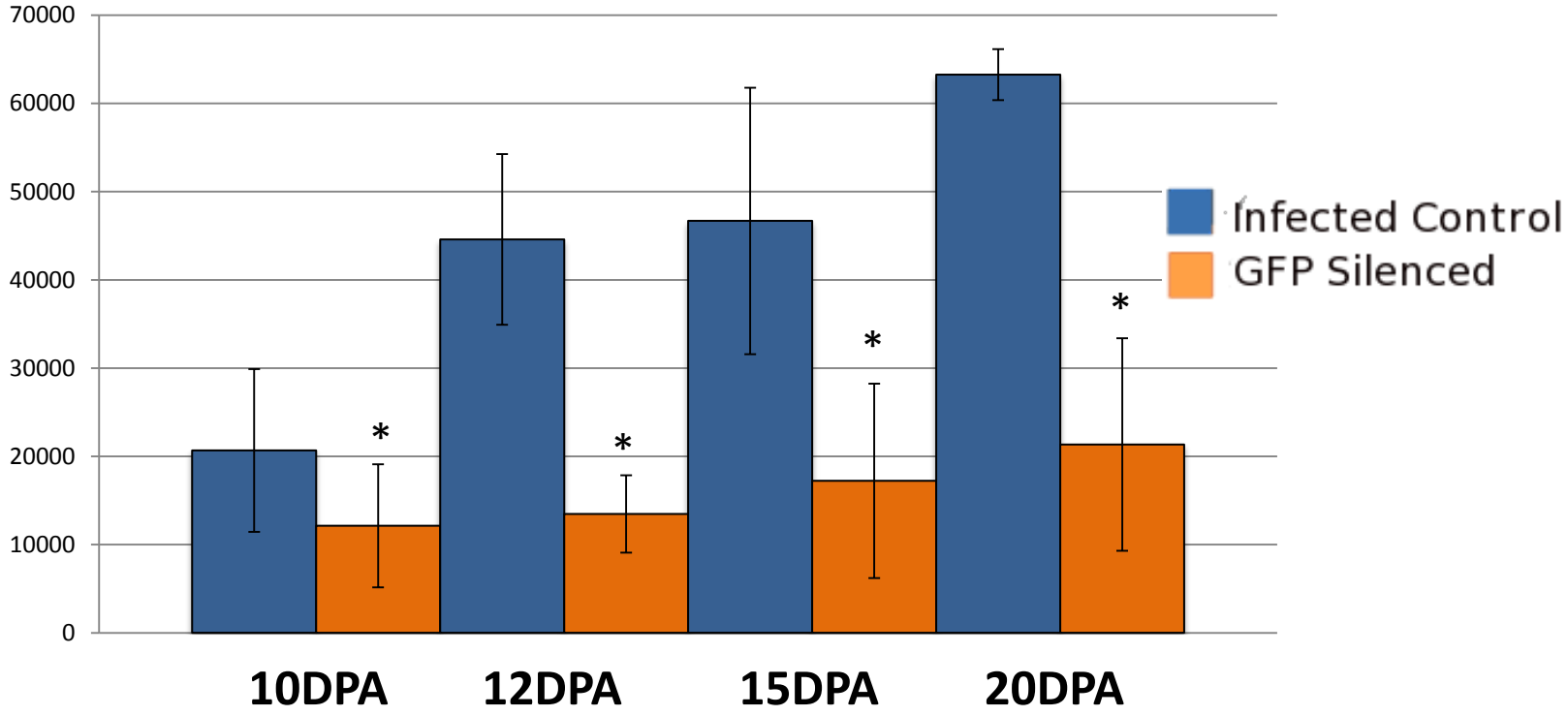


Nikon IX81

365nm UV, camera w/ yellow lens filter

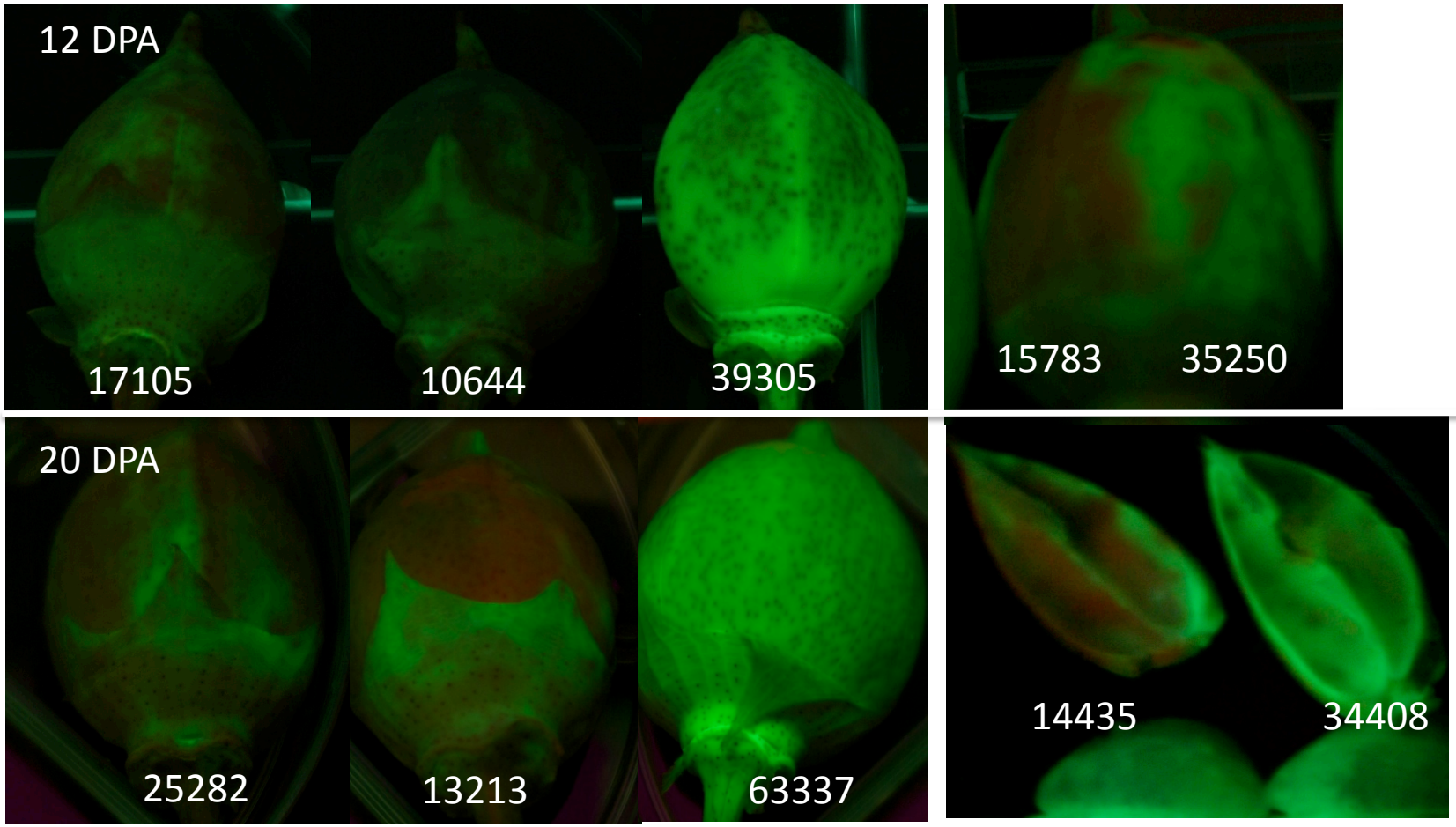
GFP Fluorescence is Reduced from 10 to 20 DPA





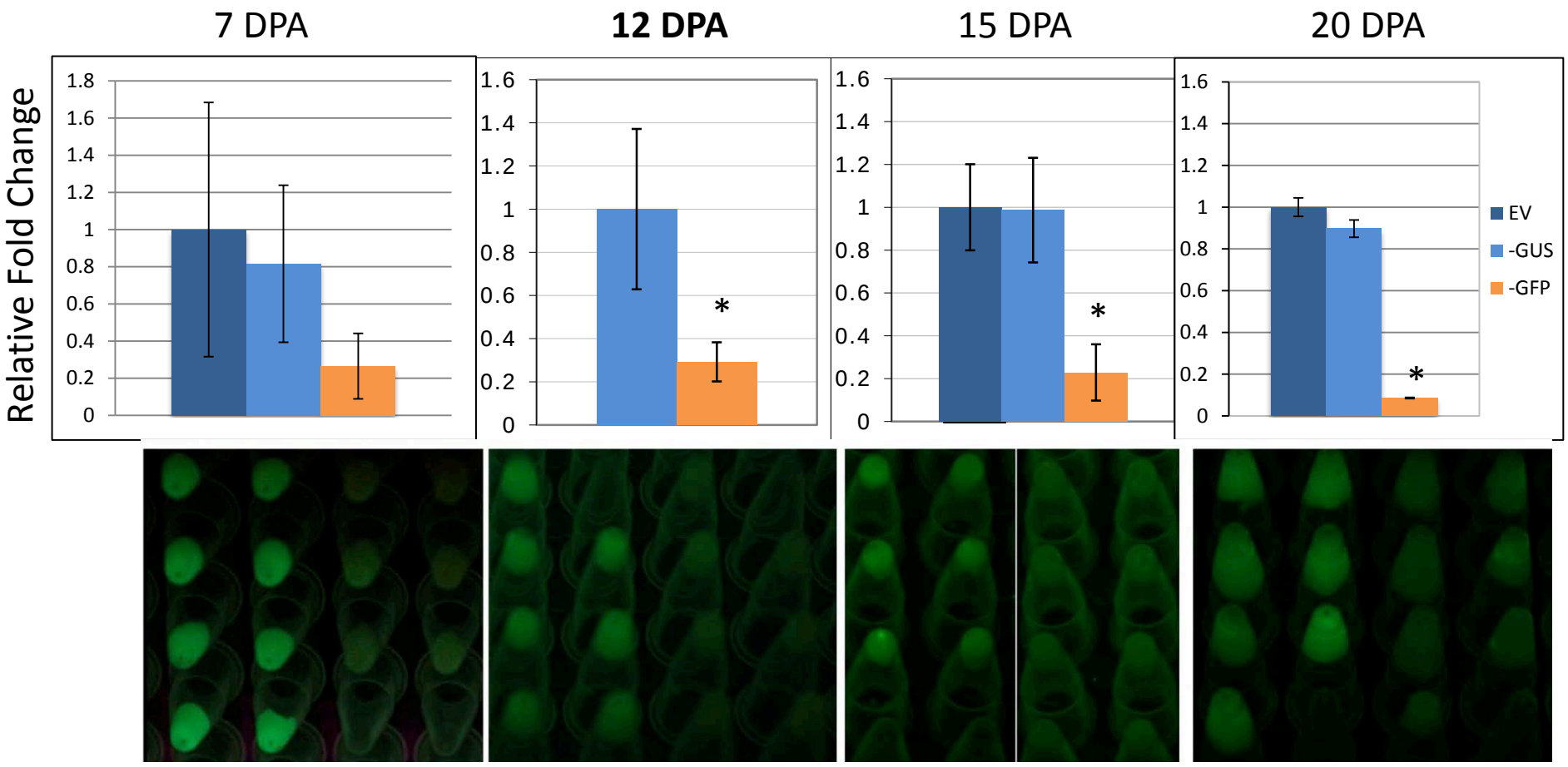
Error Bars are Standard Deviation, Asterisks = Significant Reductions
(Student's t-test p value ≤ 0.05)

Silencing in the Boll Wall Corresponds to Reduced GFP Fluorescence in Fiber



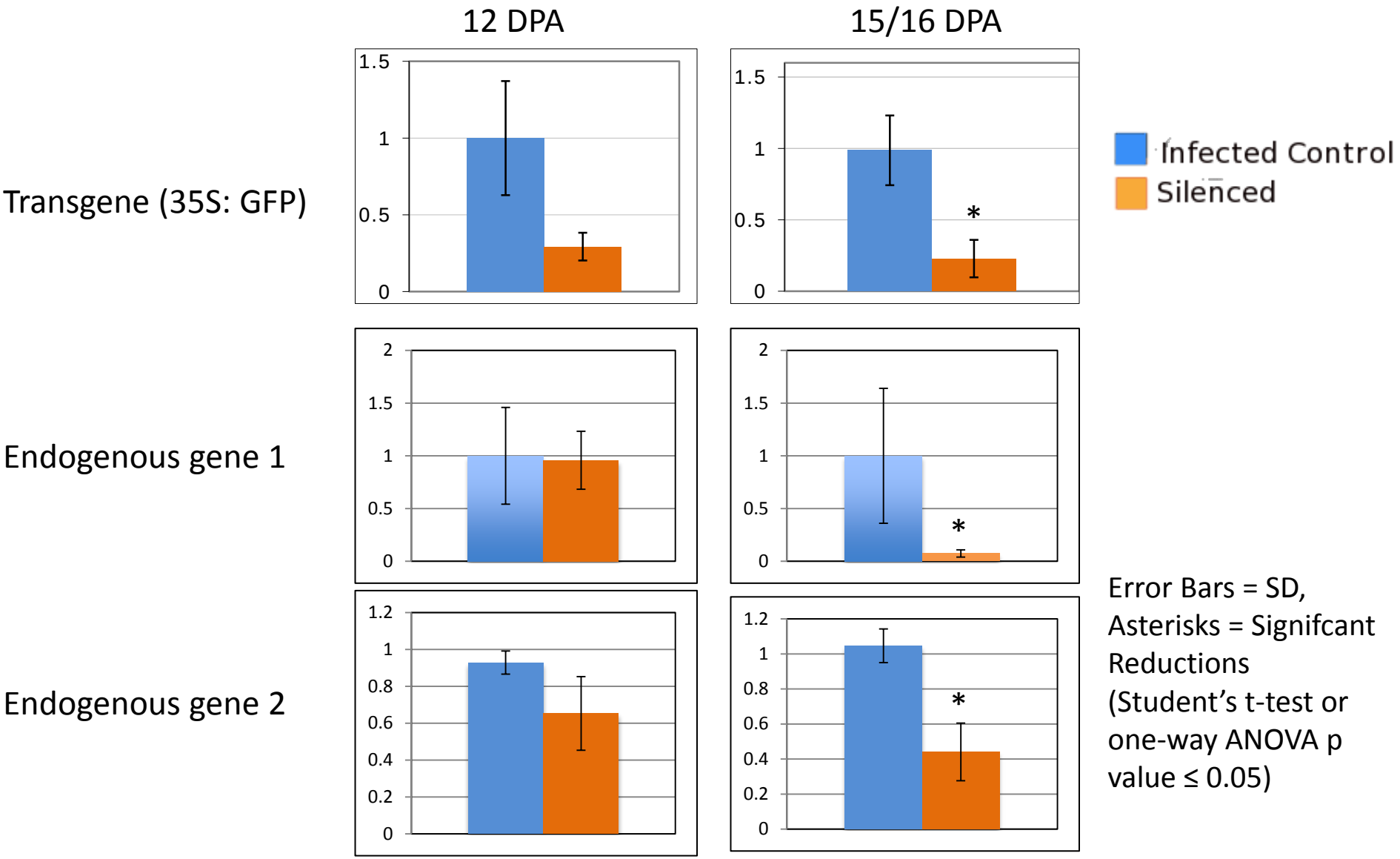
Values shown are average fluorescence of fibers beneath the imaged boll section

qRT-PCR Data Validates Silencing at 12-20 DPA

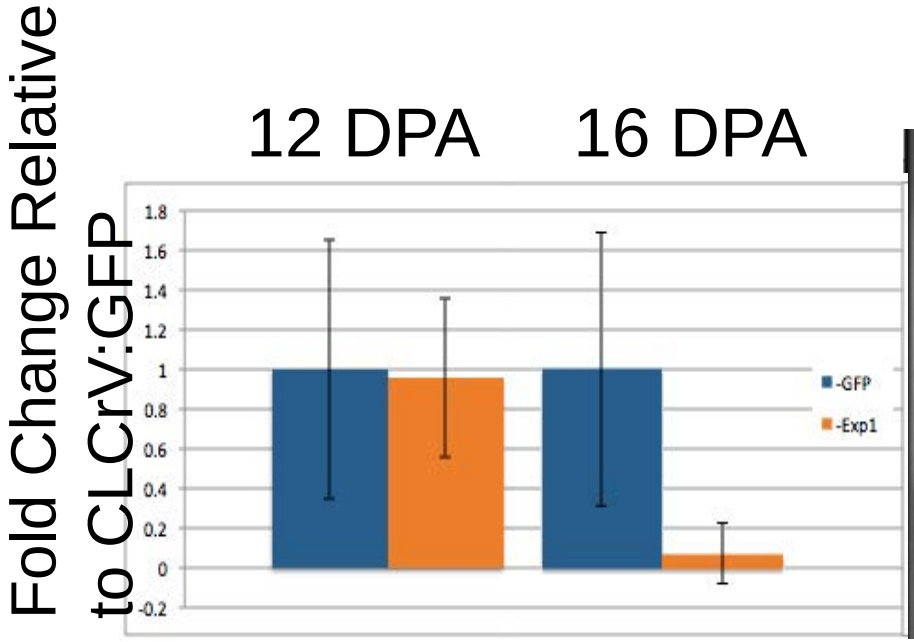


qRT-PCR suggests silencing is effective from at least 12 DPA to 20 DPA. Controls are shown in green, silenced in red, error bars are SD, asterisks indicate significant reductions.

Endogenous gene silencing is not effective at 12 DPA



Silencing of alpha Expansin1 (Exp1) results in short fiber phenotype



~14 fold reduction at 16 DPA

Error bars are SD.

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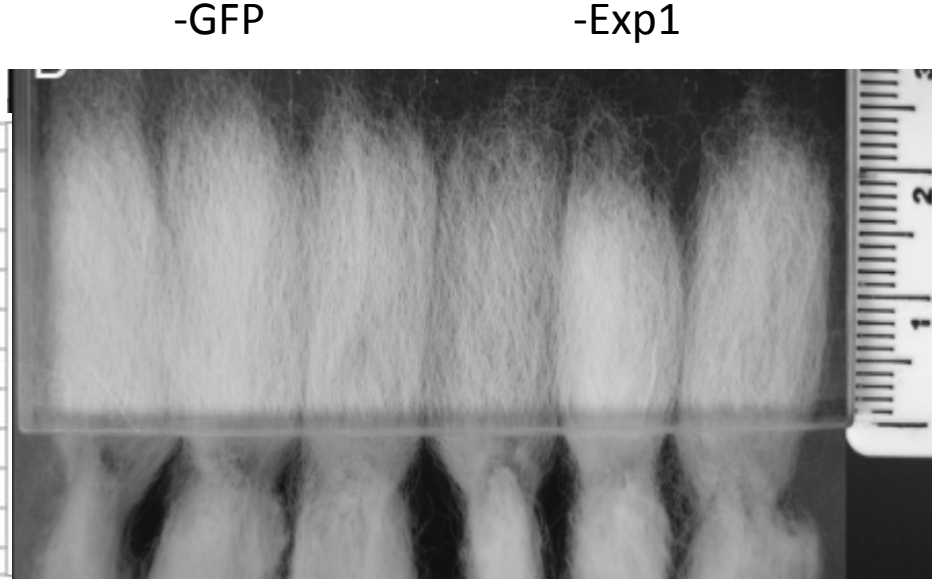


Mature fiber length is reduced in *Exp1*-silenced fibers

$\mu_{\text{-GFP}}$ = 25±0.3 mm (n=290).

$\mu_{\text{-Exp1}}$ = 22.8±0.1 mm (n=278)

p= 6.3E-46 (two-tailed non-parametric T-test)

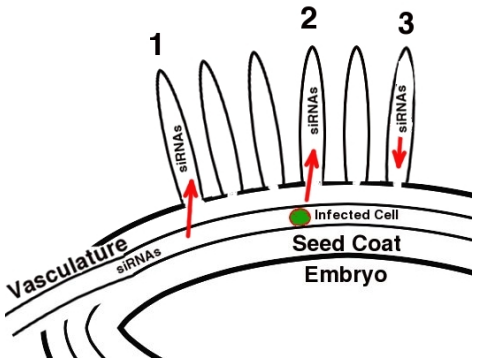
Silencing in fiber may be impacted by symplastic isolation.

The Control of Single-Celled Cotton Fiber Elongation by Developmentally Reversible Gating of Plasmodesmata and Coordinated Expression of Sucrose and K^+ Transporters and Expansin

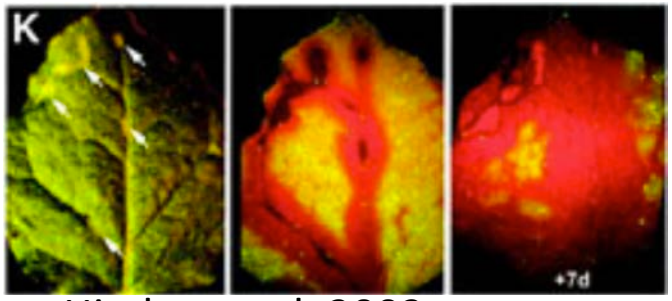
Yong-Ling Ruan[1], Danny J. Llewellyn and Robert T. Furbank

Confocal imaging of the membrane-impermeant fluorescent solute carboxyfluorescein (CF) revealed that the fiber plasmodesmata were initially permeable to CF (0 to 9 DAA), but closed at ~10 DAA and re-opened at 16 DAA. A

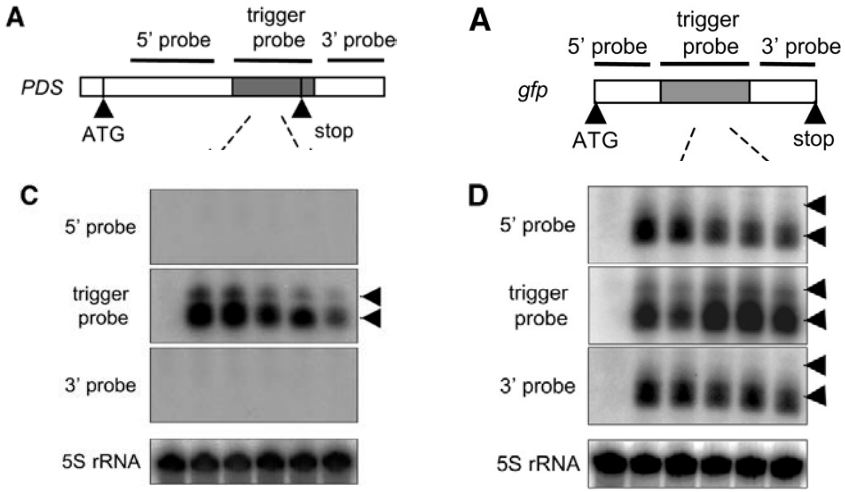
siRNAs may move into the fiber from other organs (1), from adjacent infected cells (2), or may be produced within the fiber (3)



Transgene silencing is 1) more extensive than endogenous gene and 2) more prone to transitivity (production of secondary siRNAs from target transcript).



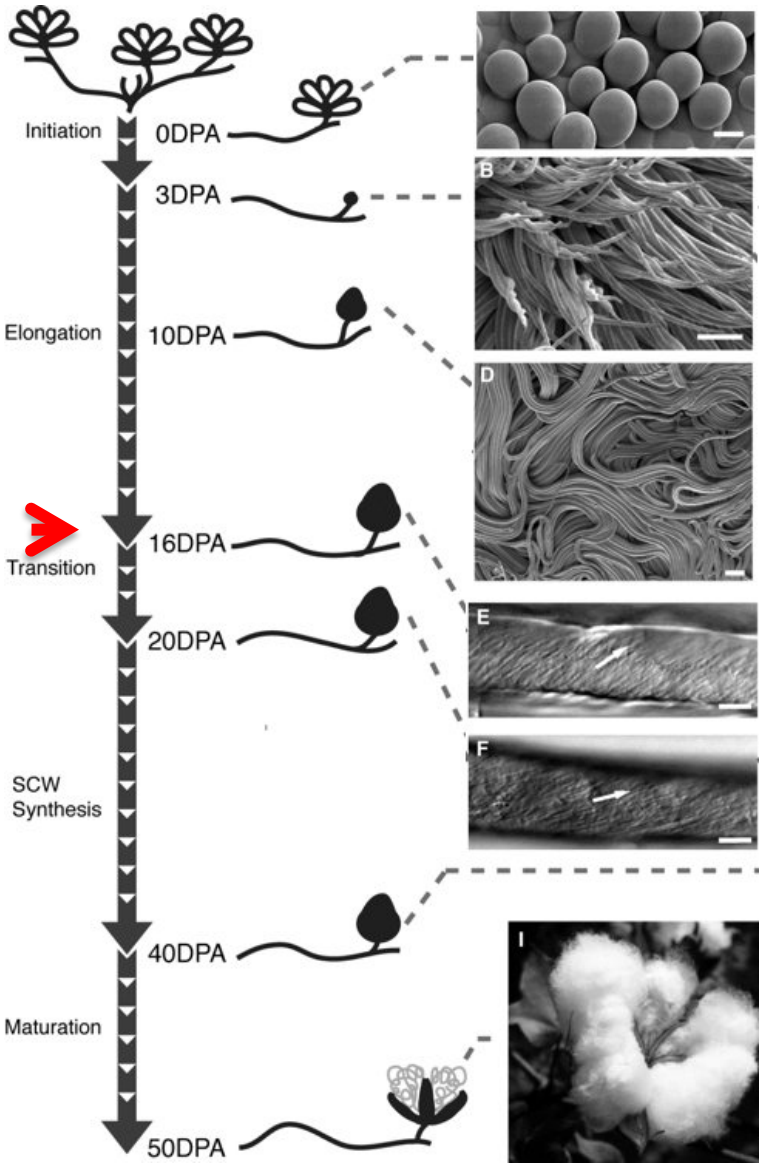
Himber et al. 2003



Miki D et al. Plant Physiol. 2005;138:1903-1913

Conclusions

- CLCrV-based VIGS may be used to silence genes in cotton fiber
  - Rapid functional analysis of genes in vegetative organs and fibers
- GFP silencing on the boll is associated with silencing in the fibers
  - Useful marker for selecting tissue for phenotyping
  - But less informative for accurately characterizing the precise timing of silencing
- Endogenous gene silencing is well-established for
  - Transition to Secondary Cell Wall Deposition and Onward



Courtesy M.R. Stiff

Thanks!...Questions?

Contributors
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Tallisin Cochran
Lillian Martin
Keerti Rathore

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Cotton Incorporated (Graduate Fellowship)
National Science Foundation Genome Grant

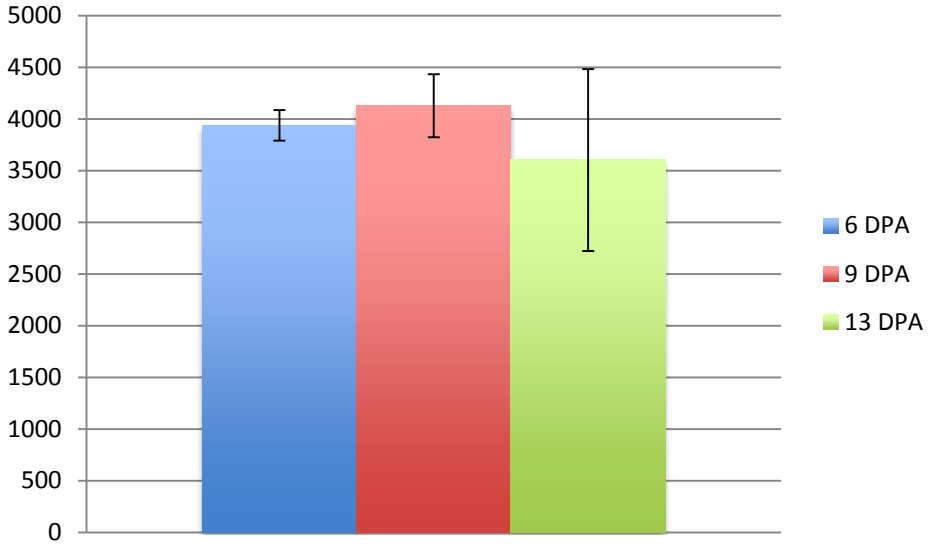
To request the biolistic vector: www.addgene.org

To request the agrobacterium vector or for additional information: jrtuttle@ncsu.edu

Supplementary Material

Does fluorescence increase in non-transgenic fibers?

Fiber Fluorescence of non-transgenic lines does not increase with development (preliminary data derived from cv. DP5415).

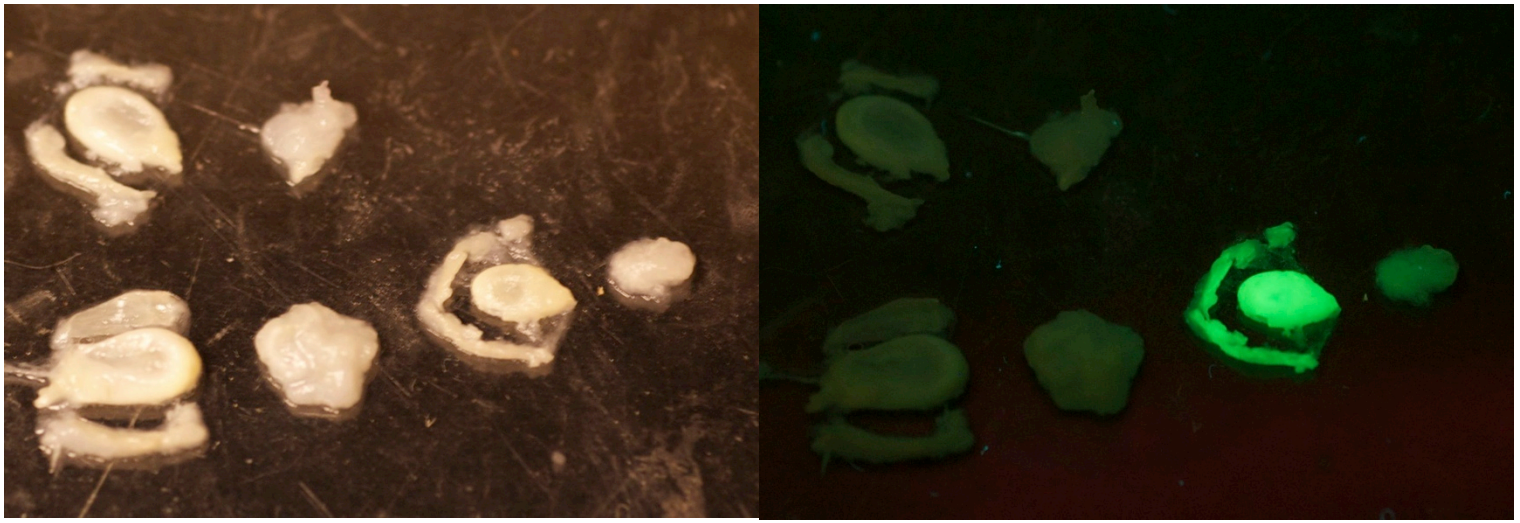


Maximum fluorescence is less than half of that seen for silenced transgenics.

What is the background fluorescence of a non-transgenic seed like?

Brightfield

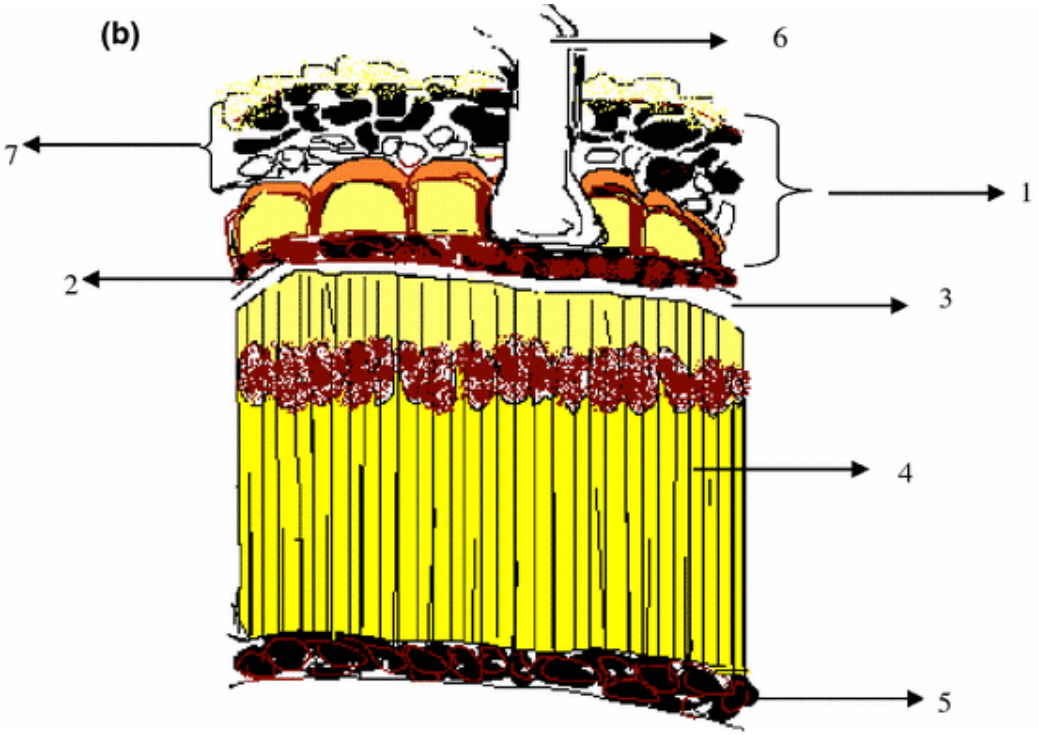
365nm UV



Left, DP5415 (non-transgenic)
Right, control infected 35S:GFP
Unknown DPA.

How did you dissect your seeds/separate embryo and seed coat?

Ovules were longitudinally hand-sectioned to ~1-2mm thick and seperated at the palisade cell layer under a disecting scope.



Schematic of a cross-section of cotton seed coat showing anatomical components. 1 epidermis layer, 2 outer pigment layer, 3 colorless layer, 4 palisade layer, 5 inner pigment layer, 6 cotton fiber, 7 cuticle (Yan et al. 2009)

Why does GFP fluorescence increase as the fiber develops?

Maybe due to:

1) Developmental regulation of the 35S promoter?
2) Conditions of the fiber cell? Kim and coworkers were unable to detect transient expression of 35S:GFP in bombarded 2 DPA ovules despite successful expression of 35S:GUS (2002).
3) Simply an accumulation of GFP protein over time?

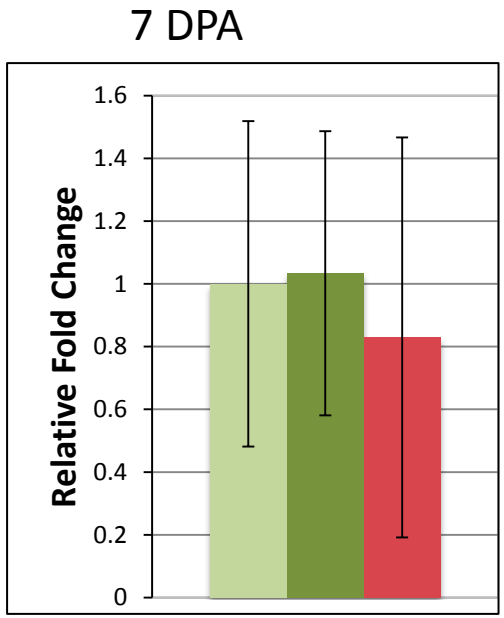
Why is silencing in the boll sectored?

van Iersel and coworkers (1995) used carboxyfluorescein as a fluorescent tracer for vascular tissue.

- "After application of CF tracer to the mid-vein of a sympodial leaf, the dye was not distributed equally throughout the petiole, peduncle, or subtended fruit…. This may have been the result of callose formation due to leaf injury while applying the dye, or **different parts of the leaf may supply specific parts of the fruit**."

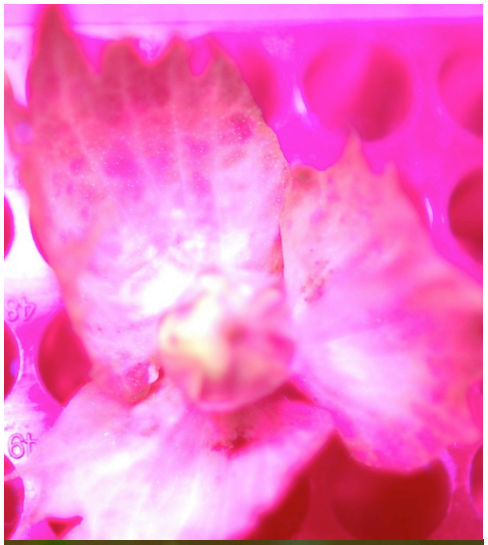
Both virus and siRNAs rely on phloem translocation for long distance movement. Both virus accumulation and silencing are sectored in the leaf.

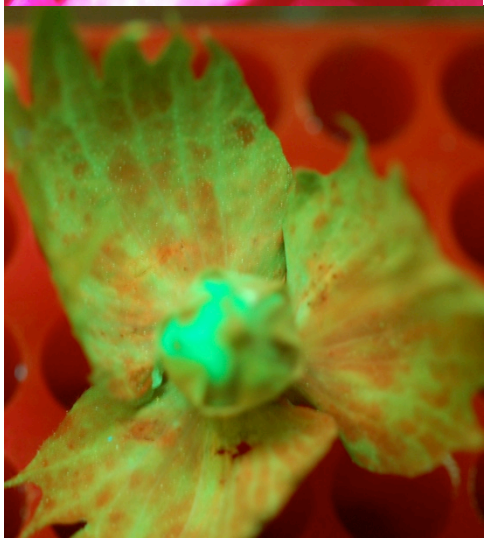
What happened with RT-PCR of 7 dpa?

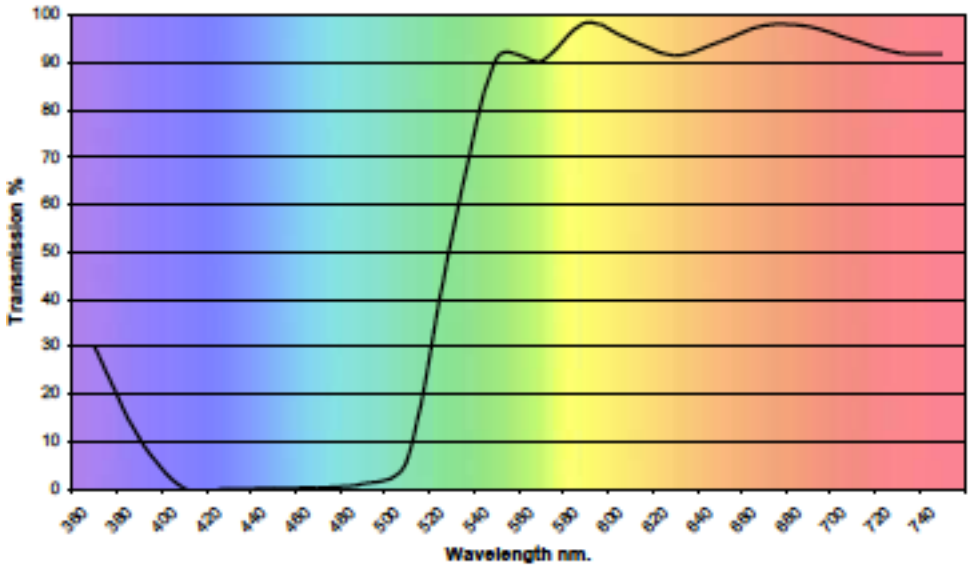


Young fibers were isolated by vortexing with 0.1mm glass beads in 5mL of LN2 for 30s and repeating 3X. I believe the glass beads are disrupting highly fluorescent adjacent tissues in addition to the young fibers resulting in mixed RNA preps.

Why use the yellow filter for GFP imaging?



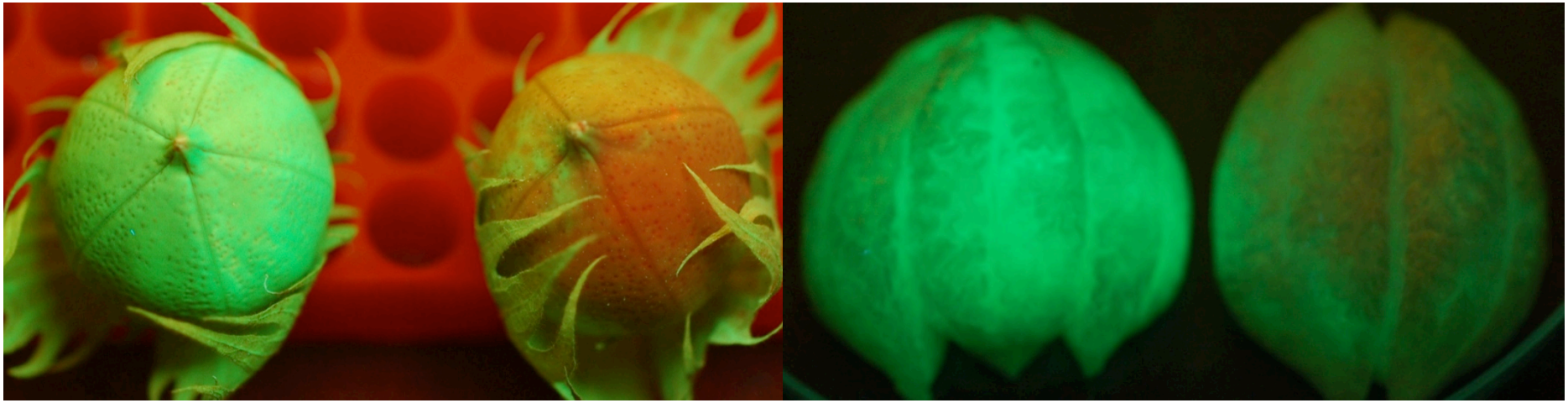




The filter cuts out reflected UV by blocking everything below ~500nm and improves contrast in the silenced tissues.

Do you ever see escapes?

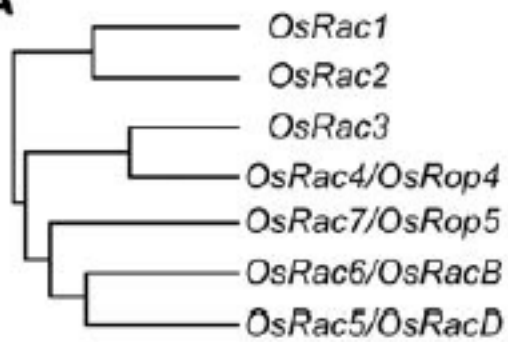
Yes, approximately 11% (4/35) of bolls from silenced plants escape silencing.



20DPA, from same GFP-silenced plant collected on the same day

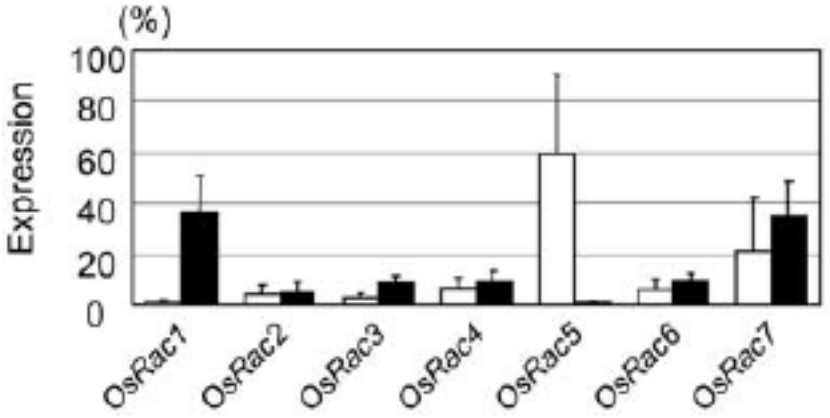
What are the requirements for silencing single genes vs multiple family members?

A



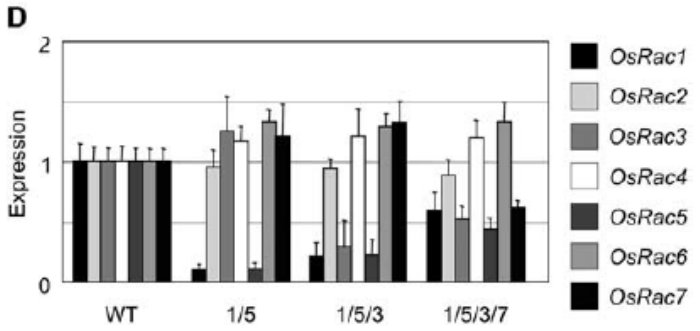
B

	OsRac1	perfect match	OsRac5	perfect match
OsRac1	100%		73%	24 nt
OsRac2	82%	49 nt	75%	21 nt
OsRac3	74%	21 nt	76%	34 nt
OsRac4	75%	21 nt	74%	22 nt
OsRac5	73%	24 nt	100%	
OsRac6	75%	27 nt	82%	47 nt
OsRac7	72%	23 nt	78%	21 nt



Using highly conserved 518-bp 5' inverted repeat of either OsRac1 (white) or OsRac5 (black)

D



Using Inverted repeats of 3' gene-specific regions

Miki et al. 2005

What is the affect of the virus on the plant?

- Reduced plant height
- 27% fewer bolls
- 24% less seed cotton
- Our vector is highly attenuated due to removal of the coat protein

Butler et al. 1986

www.plantwise.org

Other considerations

- Timing and level of target gene expression (higher expression more prone to errors thus more prone to RNA degradation).
- Number of family members
  - Sequence homology
  - Expression pattern
  - Possible compensatory affects.
- Region of gene used for targeting
- Targeting sequence length (longer = better)