Genetic Engineering of Plants for Enhanced Disease Resistance

We Were the First to Demonstrate Transgenic Plants with Enhanced Disease Resistance

(selected papers)

Cetiner S, JM Jaynes, B Blackmon. (1987) Effect of Novel Lytic Peptides on Plant Pathogenic Fungi. Hortscience 22(5): 1057.

Destefanobeltran L, JM Jaynes, C Clark. (1987) Effect of Novel Lytic Peptides on Plant Pathogenic Bacteria. Phytopathology 77(12):1768.

Jaynes JM and JH Dodds. (1987) Synthetic Genes Make Better Potatoes. New Scientist 1578: 62-64.

Jaynes JM, D Xanthopoulos, L Destefano-Beltran, and JH Dodds. (1987) Increasing Bacterial Disease Resistance in Plants Utilyzing Antibacterial Genes from Insects. BioEssays. 6(6): 263-270.

Destefano-Beltran L, PG Nagpala, M Selim Cetiner, JH Dodds, and JM Jaynes. (1990) Enhancing Bacterial and Fungal Disease Resistance in Plants: Application to Potato. Molecular Biology of the Potato. 205-221.

Nordeen RG, SL Sinden, JM Jaynes, and LD Owens. (1992) Activity of Cecropin SB37 Against Protoplasts from Several Plant Species and Their Bacterial Pathogens. Plant Science. 82:101-107.

Destefano-Beltran L, PG Nagpala, SM Cetiner, T Denny, and JM Jaynes. (1993) Using Genes Encoding Novel Peptides and Proteins to Enhance Disease Resistance in Plants. In Biotechnology in Plant Disease Control, 175-189. Ed. I. Chet.

Jaynes JM, P Nagpala, L Destefano-Beltran, JH Huang, JH Kim, T Denny and S Cetiner. (1993) Expression of a Cecropin B Lytic Peptide in Transgenic Tobacco Confers Enhanced Resistance to Bacterial Wilt Caused By Pseudomonas Solanacearum. Plant Science: 89:43-53.

Cary JW, Rajasekaran K, Jaynes JM, Cleveland, TE. (2000). Transgenic Expression of a Gene Encoding a Synthetic Antimicrobial Peptide Results in Inhibition of Fungal Growth In Vitro and In Planta. Plant Science. 154: 171-181.

Rajasekaran, K., Cary, J.W., Jaynes, J.M. and T.E. Cleveland. Disease Resistance Conferred by the Expression of a Gene Encoding a Synthetic Peptide in Transgenic Cotton (Gossypium hirsutum L.) Plants. (2005) Plant Biotechnology Journal 3(6) 545-554.

Rajasekaran, K., J.M. Jaynes and J. W. Cary. Transgenic Expression of Lytic Peptides in Food and Feed Crops to Control Phytopathogens and Preharvest Mycotoxin Contamination. (2009) In press.

First Attempts at Design

Cecropin B KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL

SB-37 MPKWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKAL

Shiva-1 MPRWRLFRRIDRVGKQIKQGILRAGPAIALVGDARAV

Amino acid differences between cecropin B and SB-37

Amino acid differences between SB-37 and Shiva-1

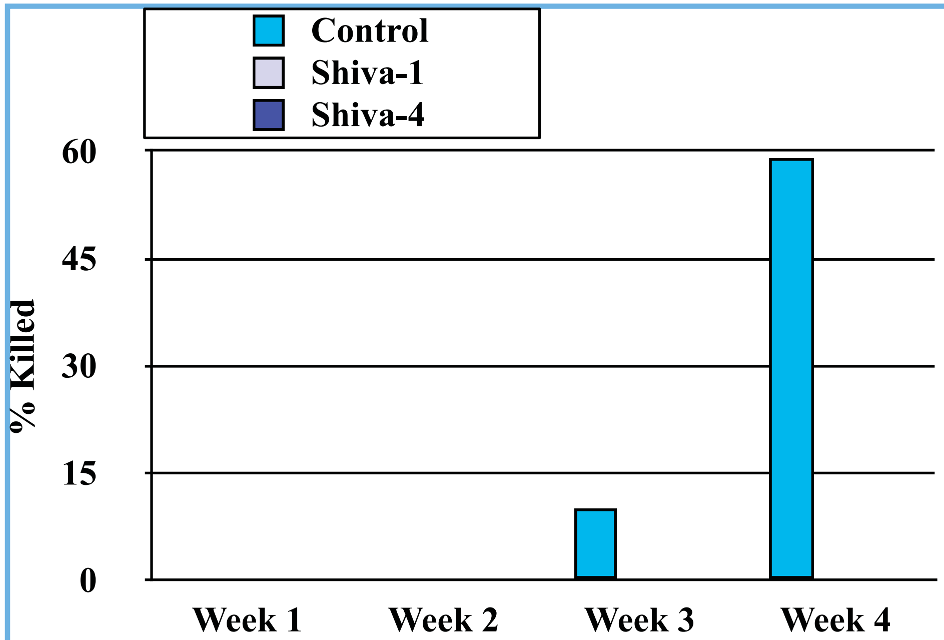
Sensitivity of Plant Pathogenic Bacteria to Lytic Peptides

Microorganism	SB-37	Shiva-1
C. michiganense spp. michiganense	3	1
E. carotovora spp. carotovora	2	0.5
P. solanacearum	64	40
P. syringae pv. tabaci	5	2
X. campestris pv. campestris	0.6	0.4

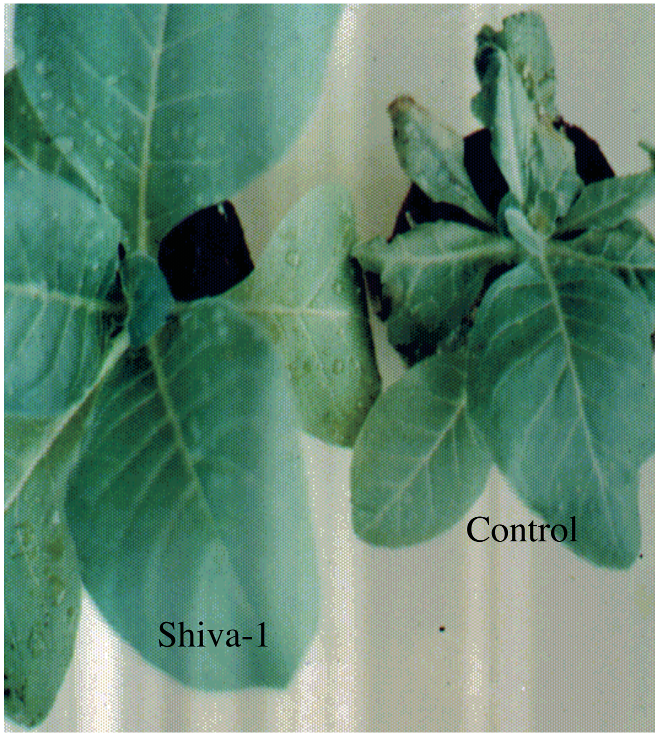
Numbers are concentration in μM of peptide necessary to kill 50% of the cells

First Improved Resistance

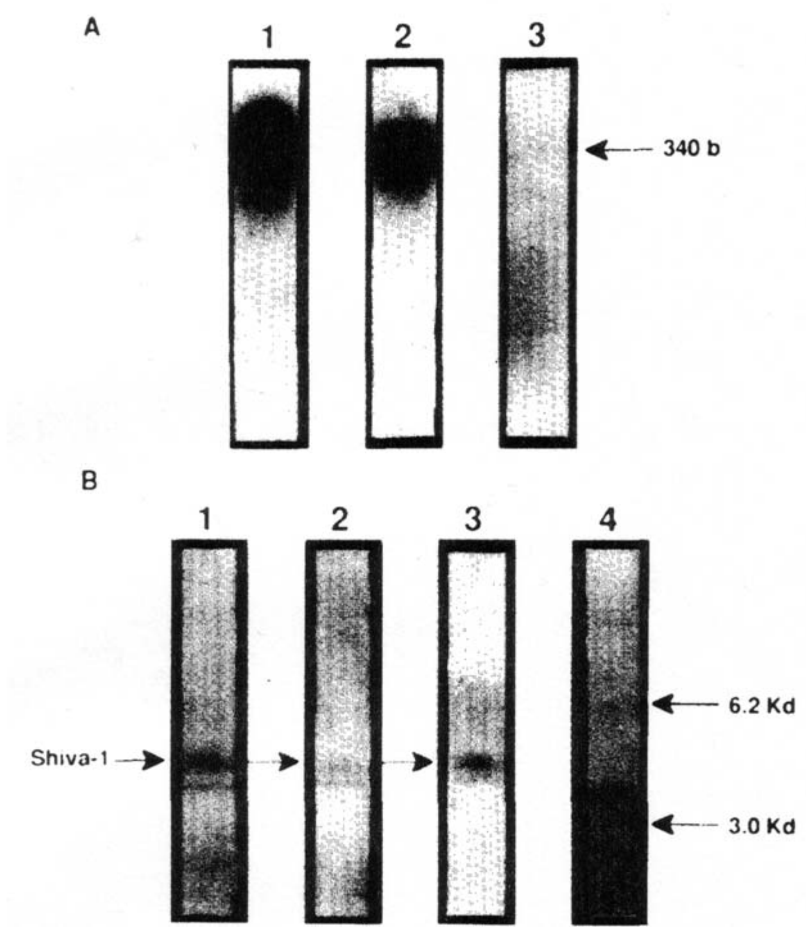
Jaynes JM, P Nagpala, L Destefano-Beltran, JH Huang, JH Kim, T Denny and S Cetiner. (1993) Expression of a Cecropin B Lytic Peptide in Transgenic Tobacco Confers Enhanced Resistance to Bacterial Wilt Caused By Pseudomonas solanacearum. *Plant Science*: 89:43-53



- Shiva-1 conferred enhanced resistance to bacterial wilt (*Pseudomonas solanacearum*) in tobacco
- SB-37 showed no protection



Presence of Shiva-1 in Transgenic Plants



A--The expression of the Shiva-1 gene was tested at the RNA level by northern analysis. Lanes 1 and 2 were samples derived from Shiva-1 plants numbers 3 and 4, respectively. Lane 3 extract was derived from transgenic control plants.

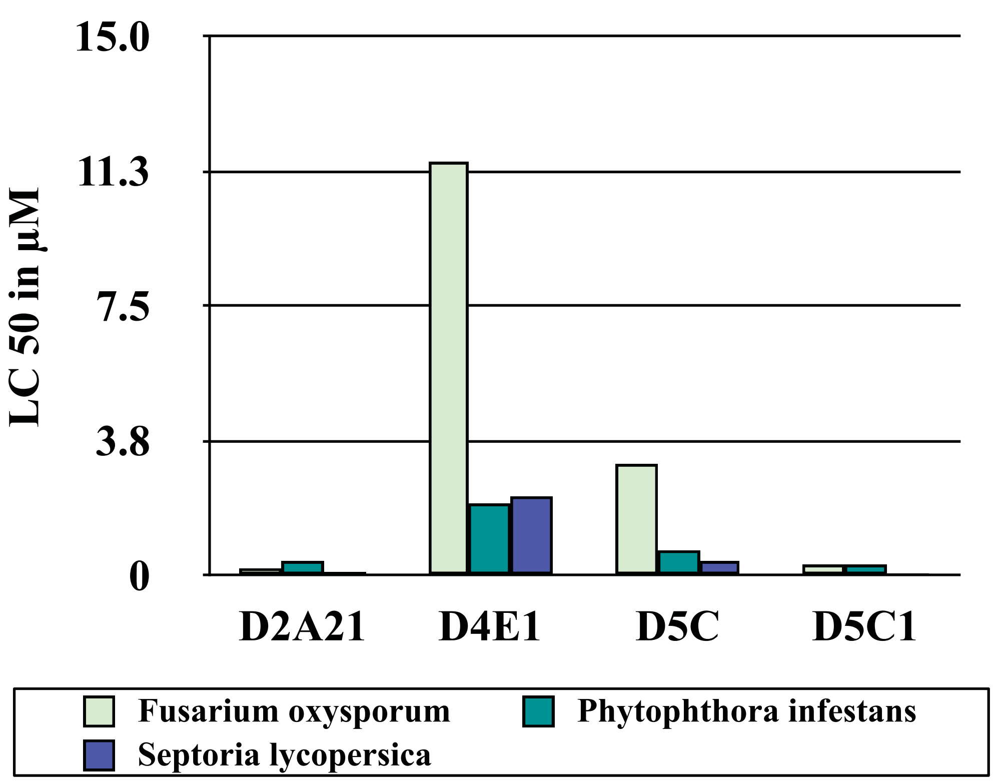
B--Western analysis was conducted on total protein extracted from tobacco leaf tissue. Lanes 1 and 2 were samples derived from Shiva-1 plants numbers 3 and 4, respectively. Lane 3 contained a few micrograms of Shiva-1 peptide which had been chemically synthesized. Lane 4 extract was derived from transgenic control plants.

Peptides Possess Broad Spectrum Antimicrobial Activity

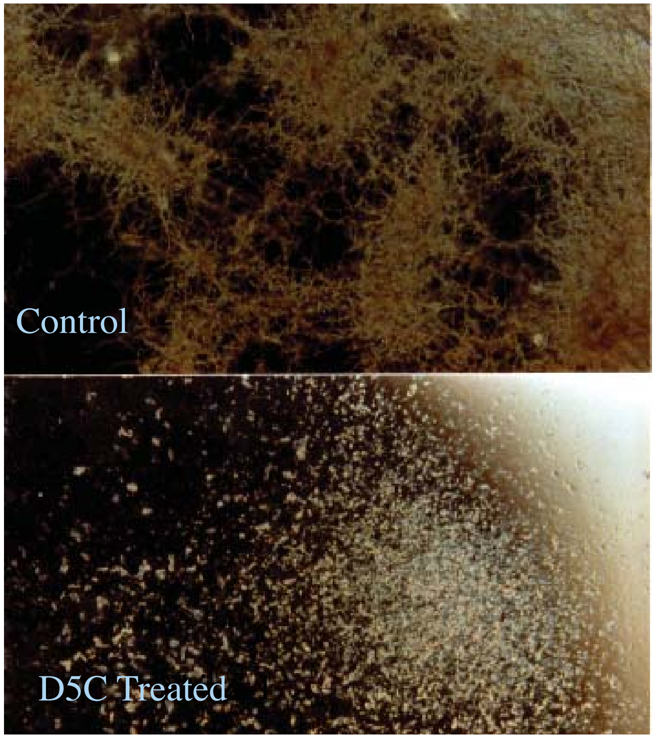
	IC50 in µM	MIC in µM
Alternaria alternata	12.39	>25.0
Aspergillus flavus	7.75	25.0
Aspergillus flavus 70-GFP	11.01	25.0
Cercospora kikuchii	8.67	>25.0
Colletotreichum destructivum	13.02	>25.0
Claviceps purpurea	1.60	20.0
Fusarium graminearum	2.10	25.0
Fusarium moniliforme	0.88	12.5
Fusarium oxysporum	2.05	12.5
Penicillium italicum	5.92	>25.0
Phytophthora cinnamomi	nd	4.7
Phytophthora parasitica	nd	4.7
Pseudomonas syringae pv. Tabaci	0.52	2.3
Pythium ultimum	nd	13.3
Rhizoctonia solani	nd	26.7
Thielaviopsis basicola	0.52	6.0
Verticillium dahliae	0.60	5.3
Xanthomonas campestris pv. Malvacearum	0.19	1.3

nd = not determined

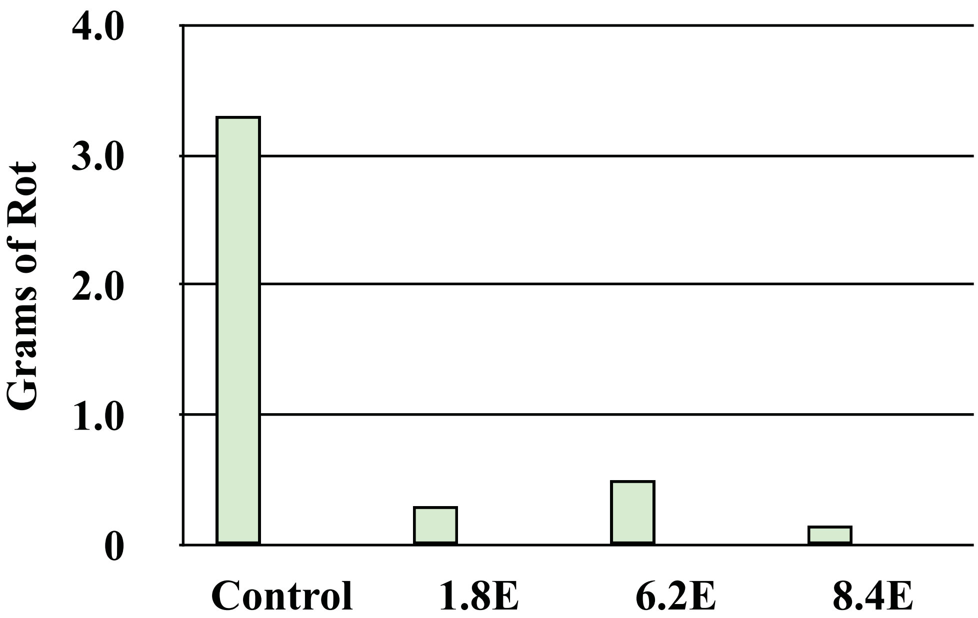
New Peptides Are More Active



Aspergillus flavus



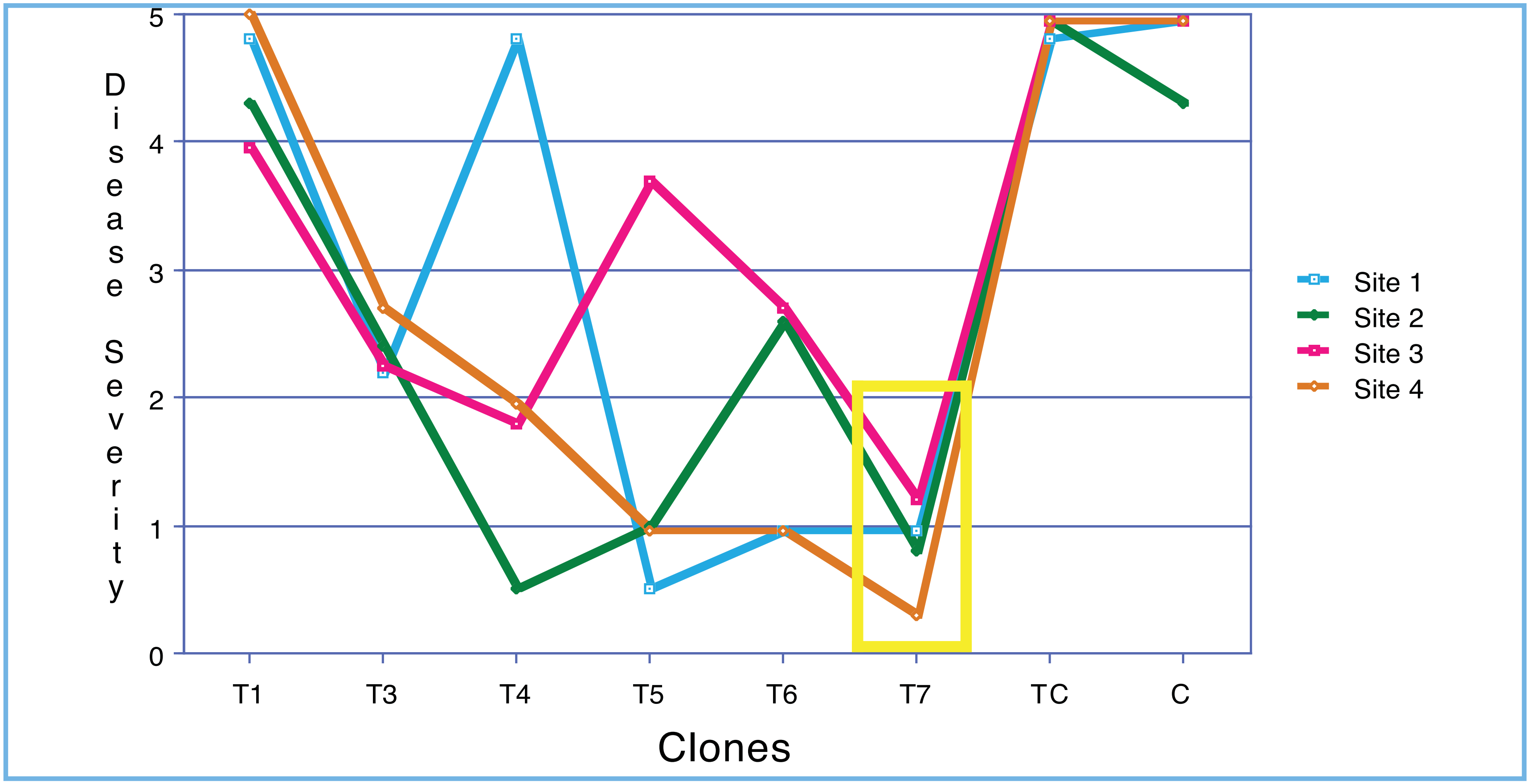
Resistance to Bacterial Diseases



*Tubers derived from potato plants transformed with D4E1

Erwinia carotovora Infection

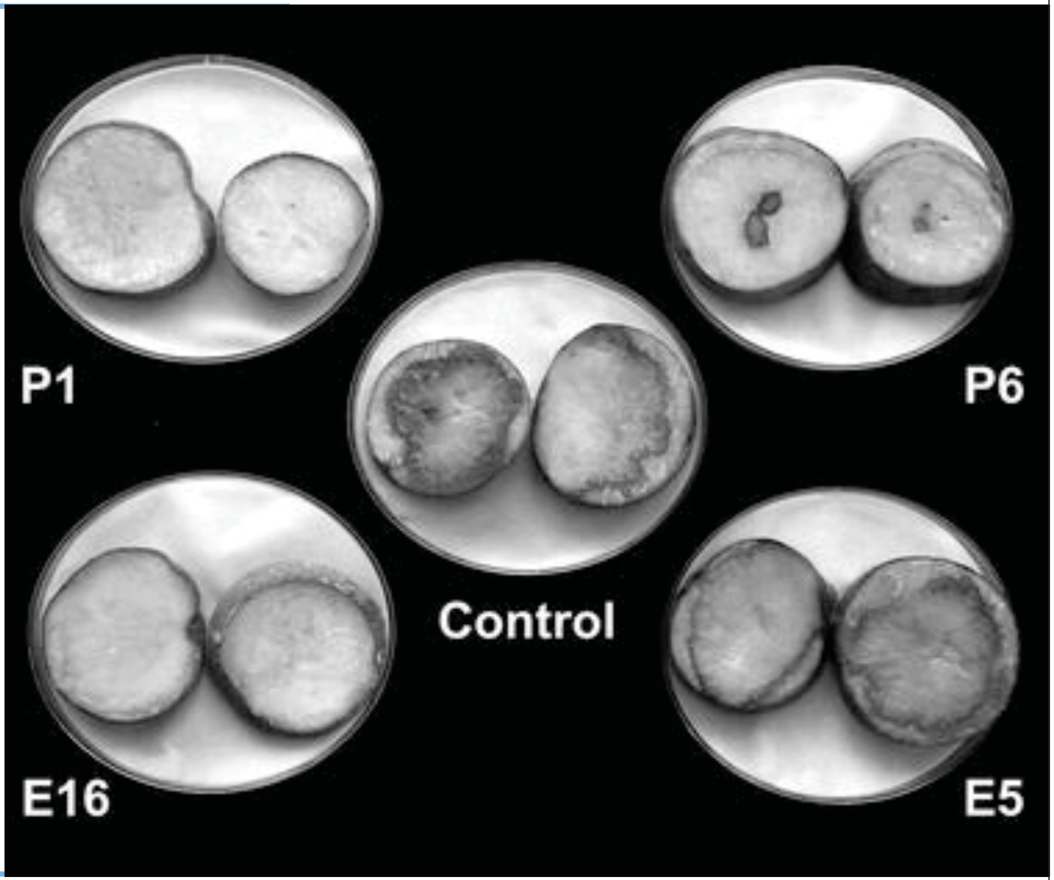
Transgenic Potato Field Trial



More Resistance to Soft Rot for Potato with Shiva-1

Average
Diameter of rot spot (mm)

Line	Day 2	Day 4	Day 6	Score
P1	1.9	2.3	2.3	HR
P3	2.1	3.1	3.8	HR
P4	2.5	3.5	3.8	HR
P6	3.5	7.8	9.2	R
P8	3.9	6,8	8.8	R
E5	53.3	57.5	60.3	HS
E8	49.7	55.3	63.5	HS
E10	35.8	39.8	45.9	S
E12	2.9	5.8	6.8	R
E16	1.4	1.9	1.9	HR
Control	43.8	51.7	55.3	HS

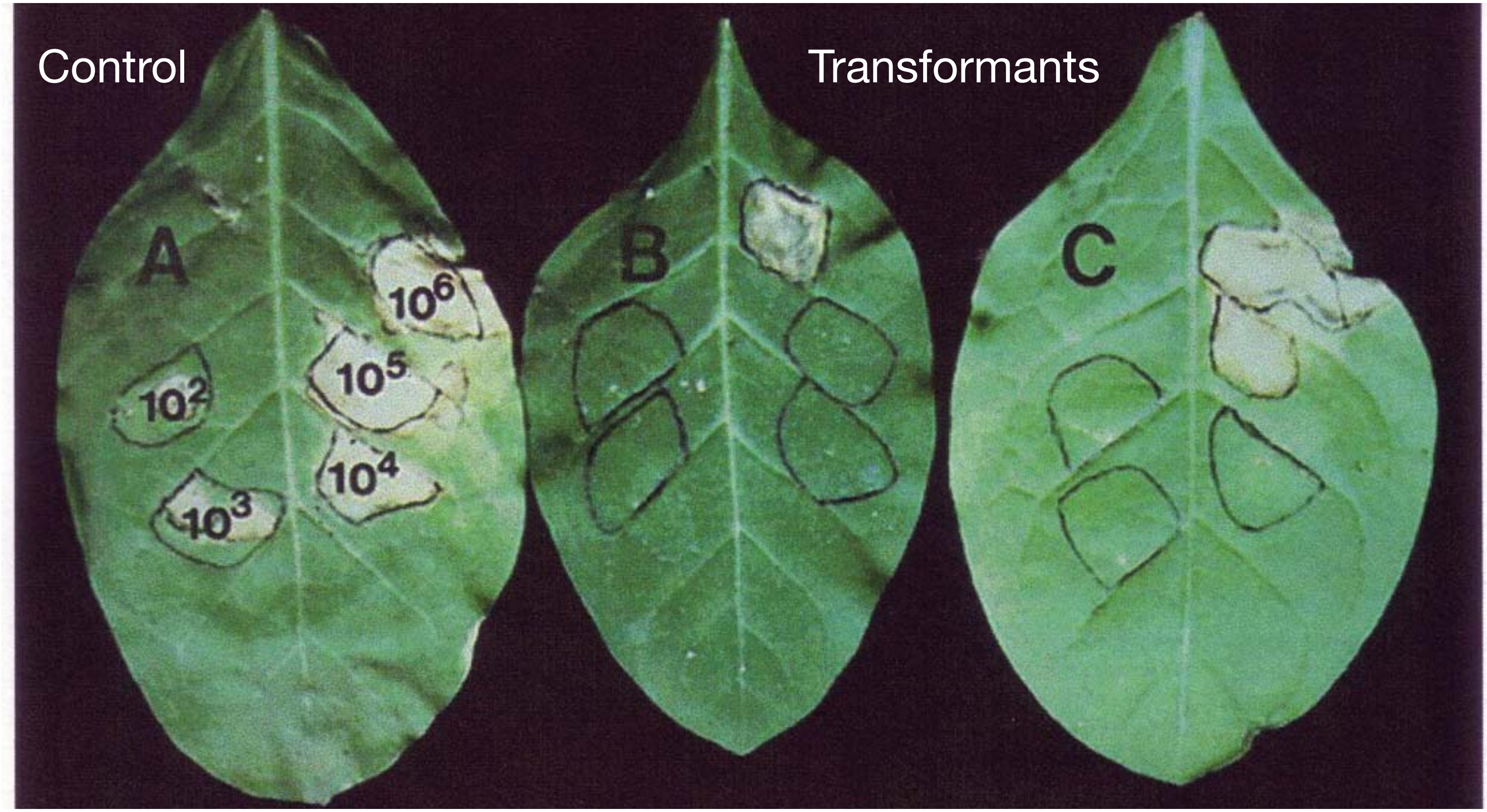


Resistance to Rhizoctonia

Resistance to Phytophthora

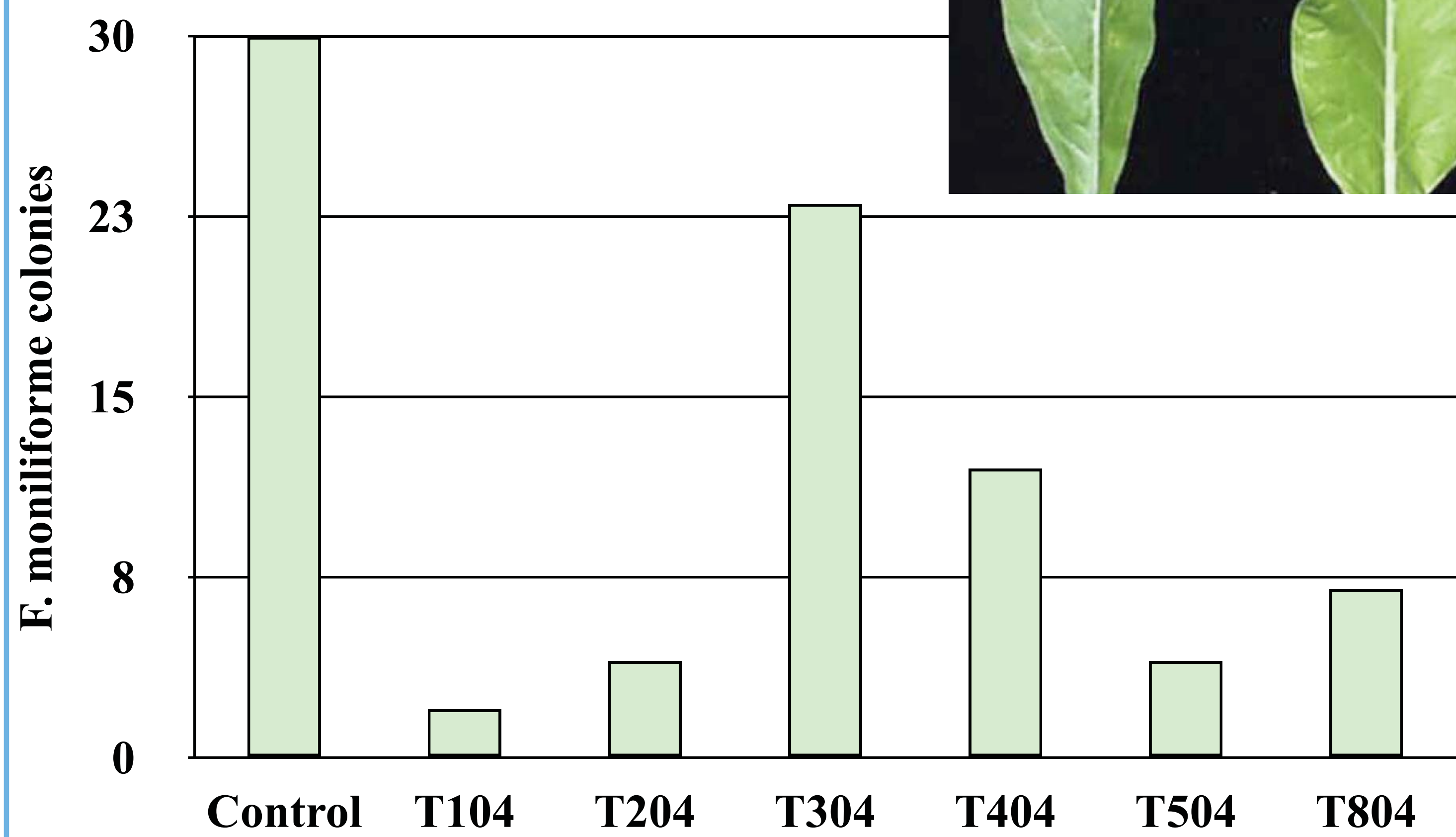
Plants Show Multiple Resistance

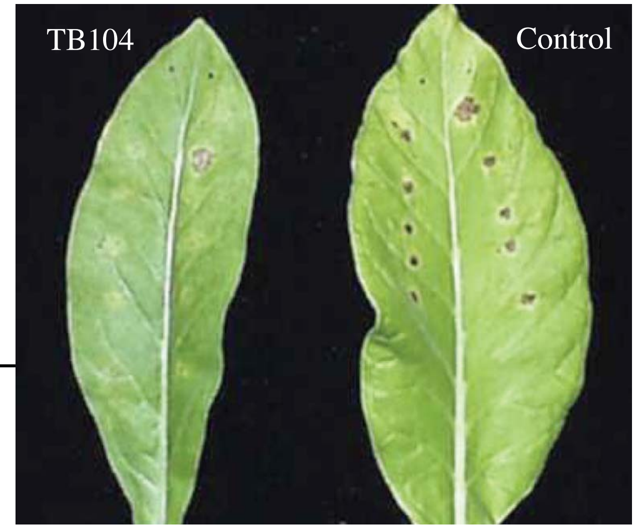
Collaboration with Dr. Arthur Weissinger at NCSU



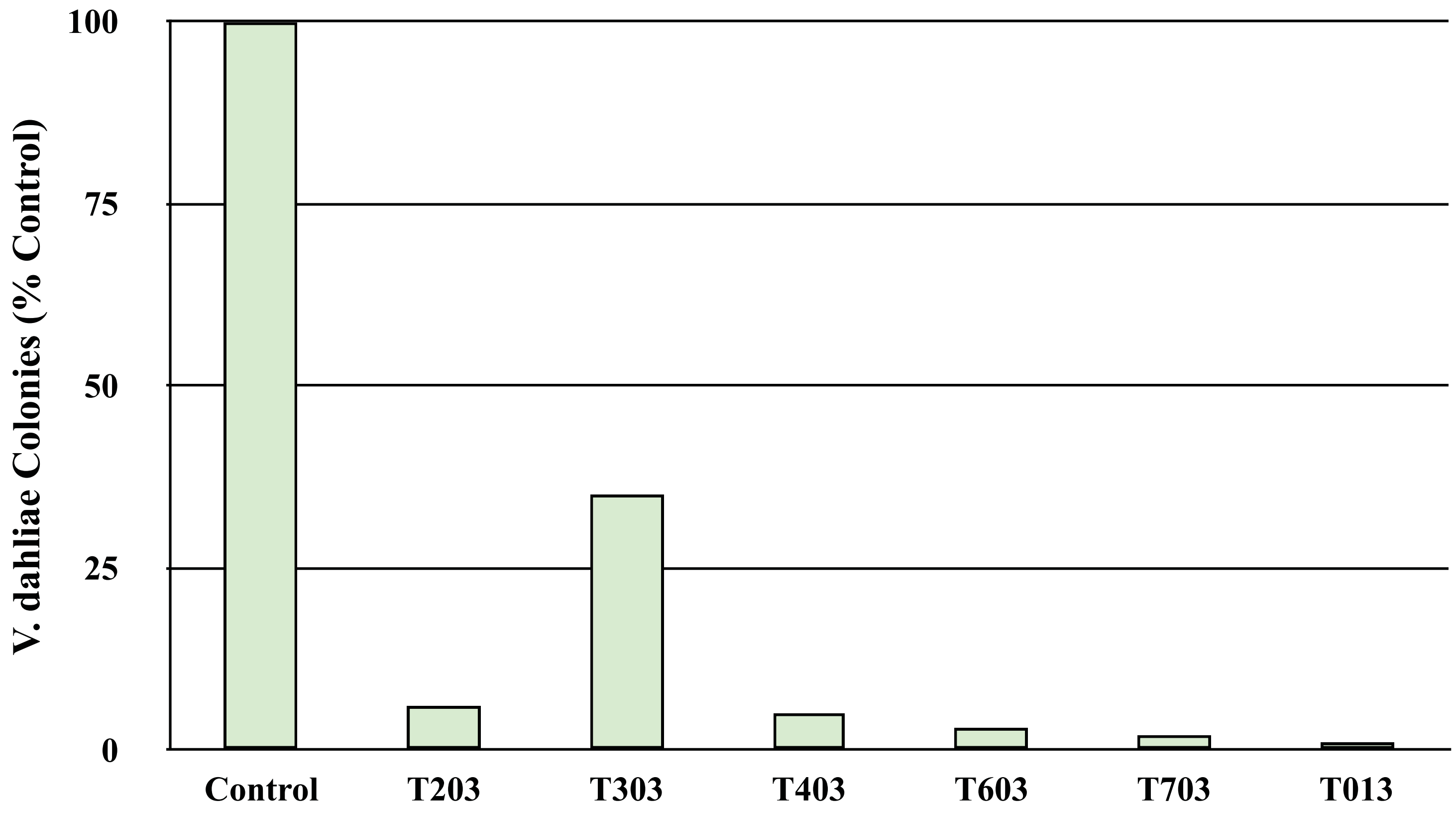
Resistance to Pseudomonas syringae pv. tabaci

Transgenic Tobacco





Transgenic Tobacco



Crown gall (*Agrobacterium* spp.)

On Grape

On Kalanchoe

On Raspberry

Disease Resistant Poplar

Collaboration:

R. Mentag

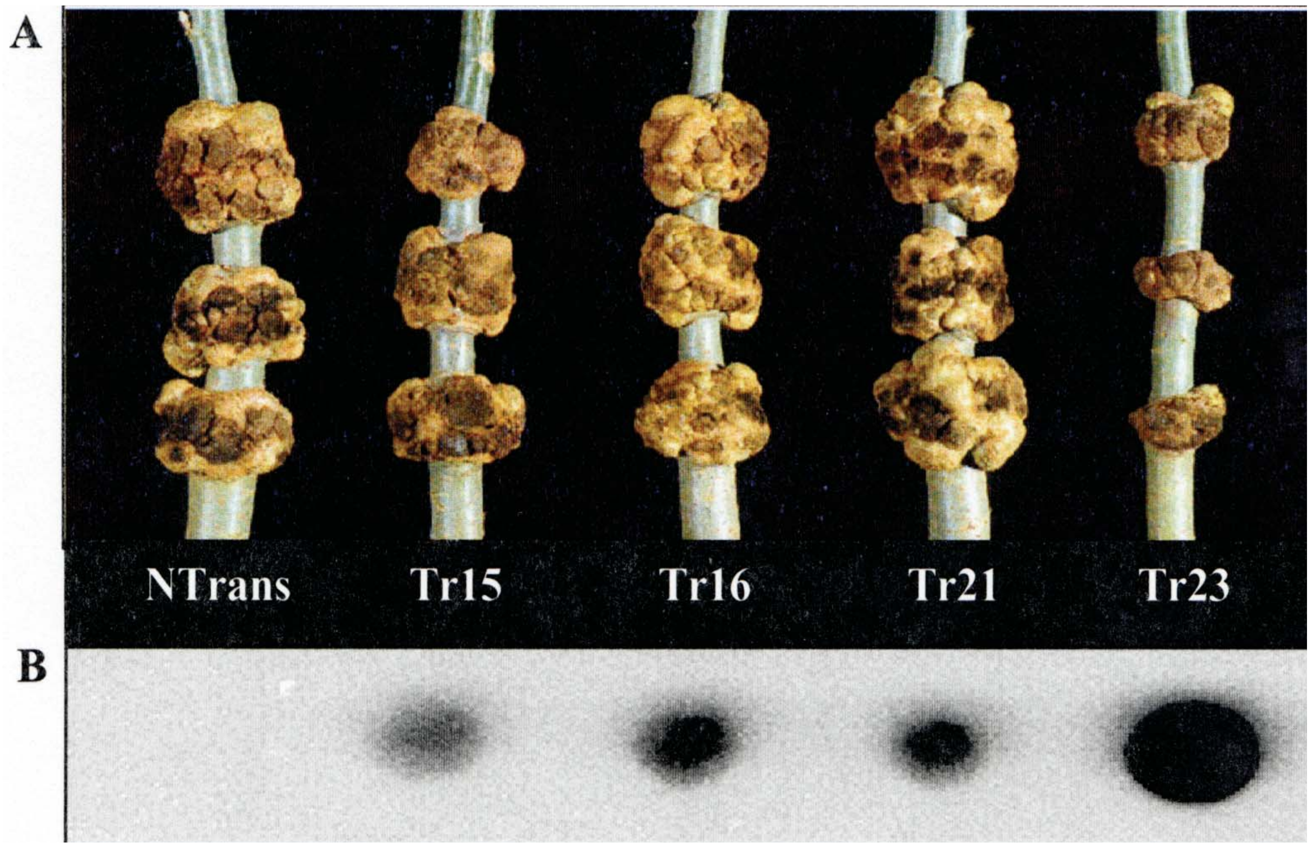
M. Luckevich

A. Seguin

All are with the Canadian Forest Service

A---tumor formation incited by *A. tumefaciens* on control and four transgenic poplar lines 18 weeks after inoculation.

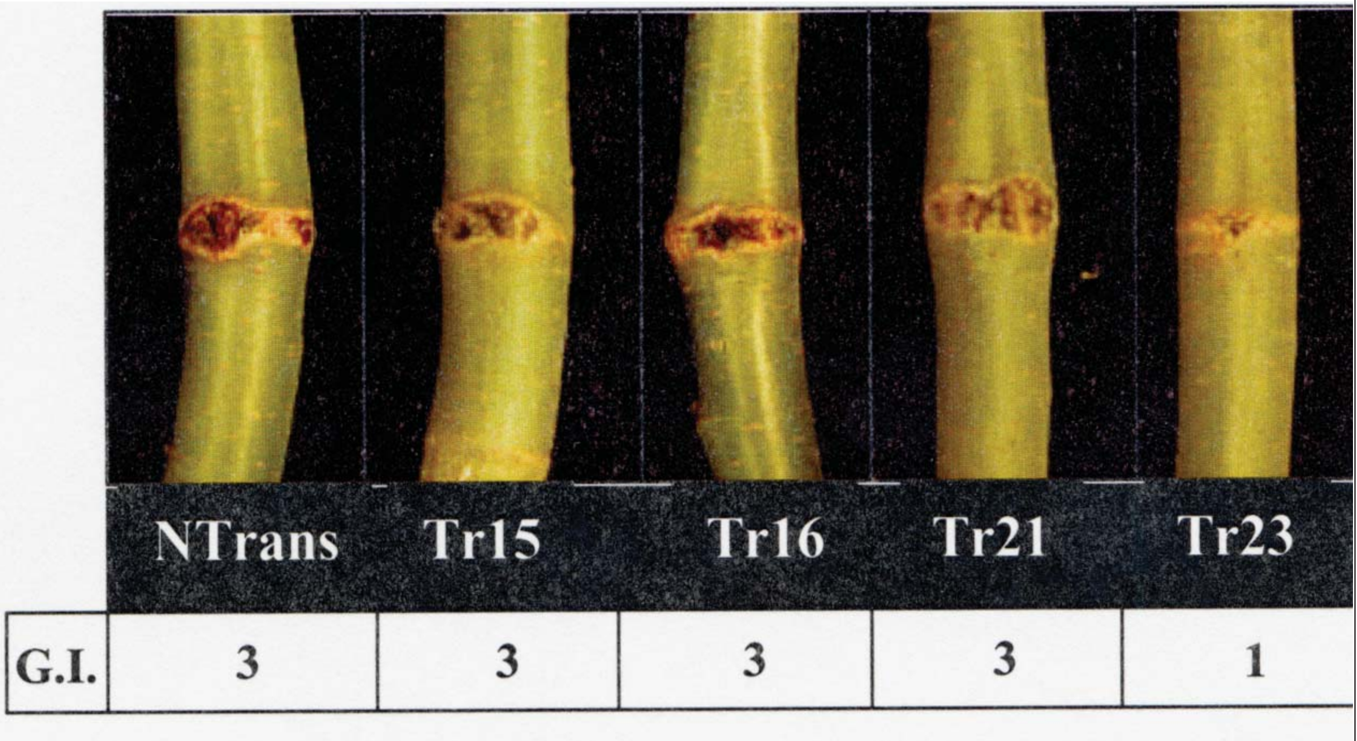
B---dot blot analysis of total RNA using a 4E1 probe.



Tumor Formation on Poplar

Canker Formation in Poplar

Disease symptoms caused by *Xanthomonas populi* on stems of control and four transgenic poplar lines. Lateral canker extension is expressed as girdling index (GI) two months after inoculation



Citrus Canker

Replacement Value is $1,000/tree

Florida Department of Agriculture and Consumer Services - Division of Plant Industry

Comprehensive Report on Citrus Canker in Florida

Through 15 October 2007 Revised* ** Approximate numbers (subject to final reconciliation)*

Background

Since 1995 citrus canker has been detected in 24 Florida counties: Brevard, Broward, Charlotte, Clay, Collier, De Soto, Glades, Hardee, Hendry, Highlands, Hillsborough, Indian River, Lee, Manatee, Martin, Miami-Dade, Monroe, Okeechobee, Orange, Osceola, Palm Beach, Polk, Saint Lucie, and Sarasota. Prior to the 2004 hurricane season, canker was confined primarily to South Florida. A history of the disease by county is presented on the following pages. Highlights of the CCEP include:

- 1910 – Canker identified in Florida for first time.
- 1933 – Canker eradicated.
- 1986 – New detection in Manatee County 53 years later
- 1994 – Eradication declared.
- 1995 – Canker detected for a third time in 1995 near Miami International Airport.
- Possibility of canker spread is monitored with routine surveys by federal and state agriculture officials.
- 2006 January 10 – USDA withdraws funds for eradication. All tree removal ceased. Program shifts to a management program, Citrus Health Response Program.
- 2006 August 1 – USDA imposes statewide quarantine prohibiting the movement of citrus unless a limited permit has been issued. See quarantine areas below for more details.

Total trees destroyed to date statewide:

Residential	865,779
Nursery	4,334,154
Commercial/Grove	11,323,298*
	16,523,176

**Commercial Acres: 87,493*

Citrus Health Response Program (Florida Statute FS 581.184)

- Florida legislature replaces the 1900-foot tree removal law (eradiation program) with a law that requires the division to create rules to protect citrus health in Florida. These rules cover the different areas of citrus production including nurseries, growers, harvesters, packers and processors.

Quarantine Areas

- Florida is currently under a statewide quarantine by the USDA and no citrus may leave the state unless the USDA has issued a limited permit. No Florida grown citrus may enter any citrus producing states or territories. No citrus plants or parts may enter or exit Florida.
- Citrus producing states and territories include: American Samoa, Arizona, California, Guam, Hawaii, Louisiana, the Northern Mariana Islands, Puerto Rico, Texas, and the U.S. Virgin Islands
- This restriction includes dooryard citrus. No citrus grown in residential areas may be shipped out of state without a limited permit; at this time there is no mechanism in place for certifying dooryard citrus.
- There are no restrictions on the movement of citrus within Florida, commercial or dooryard.

Blighted pear tree

“Shepherd’s crook”

Blossom blight

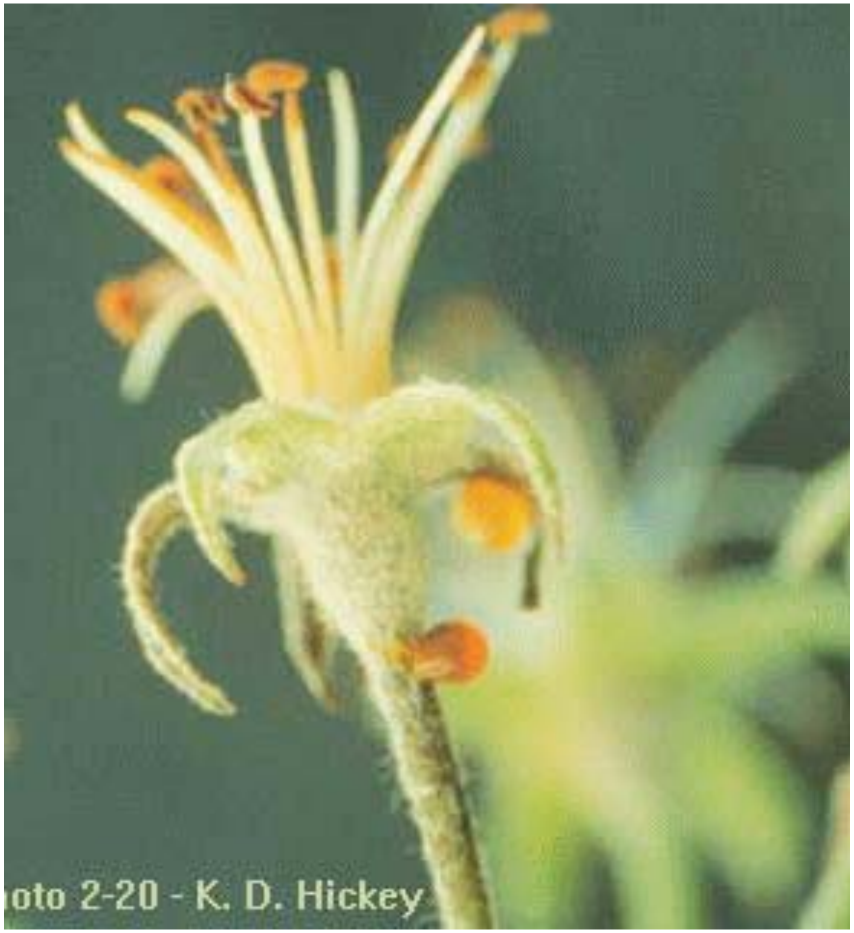
Fire Blight

Bacteria looking like sausages on plant surface

Trauma blight (after hail storm)

Canker (where bacteria overwinter)

Fire Blight (*Erwinia amylovora*)



Bacterial ooze on flower

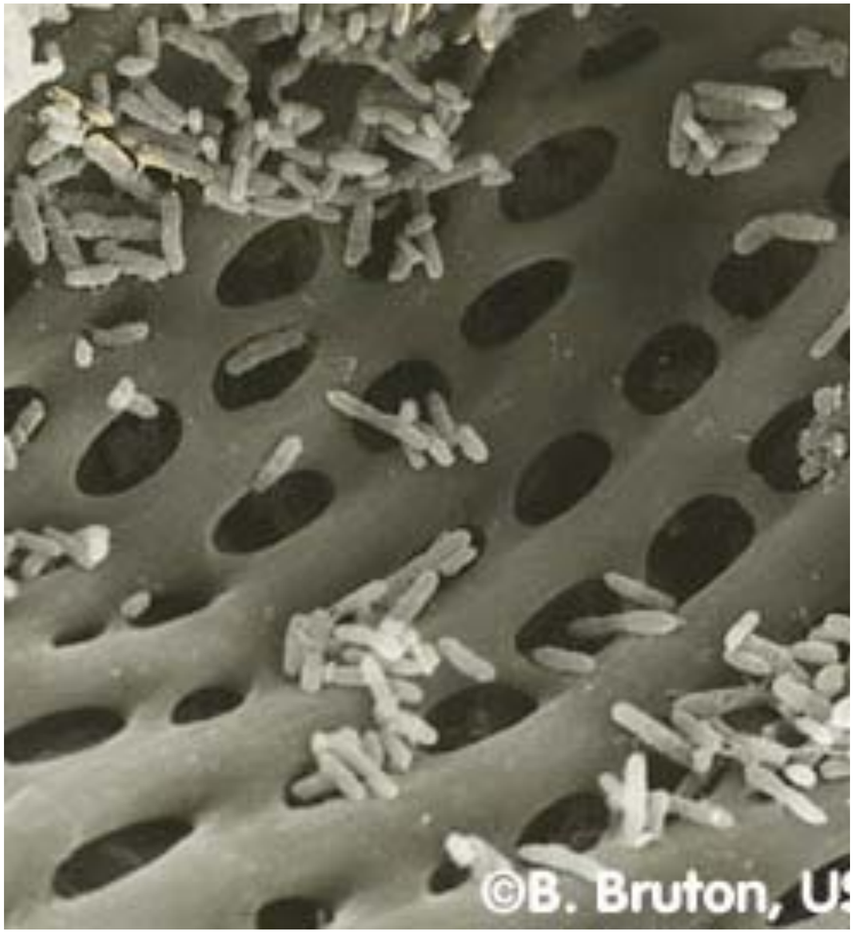
Bacterial ooze on fruitlet

Fire Blight (*Erwinia amylovora*)

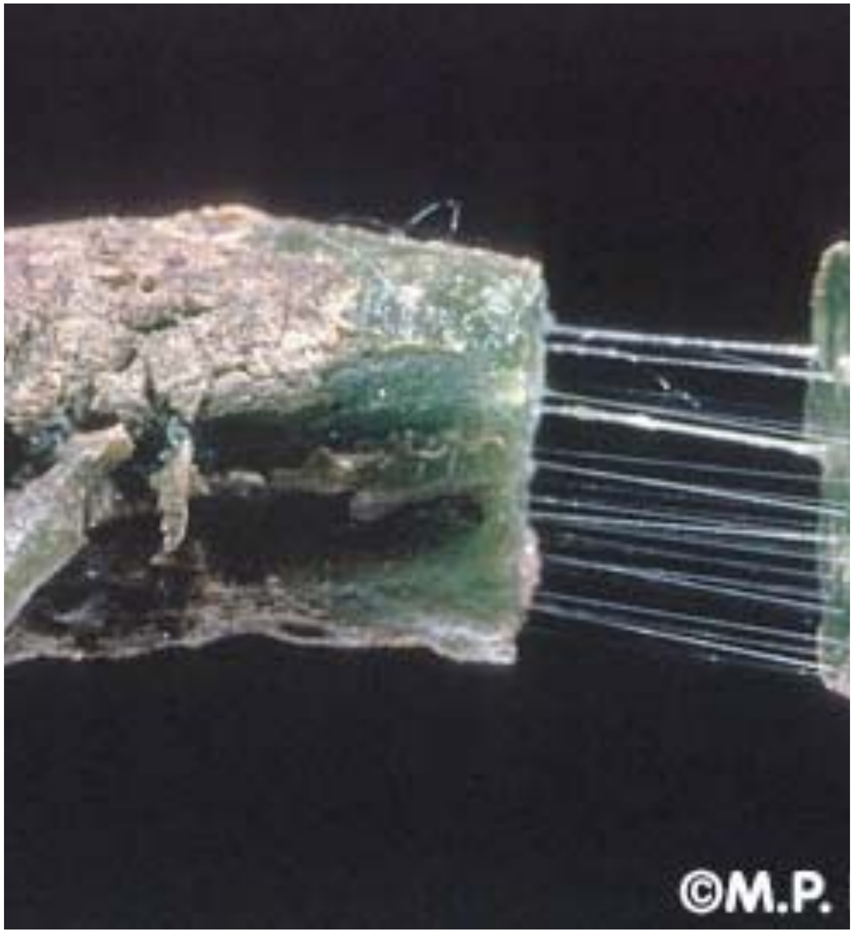
Necrosis and wilting

Extensive wilting
Plant may eventually die

Bacterial Wilt of Cucurbits (*Erwinia tracheiphila)*



Bacteria multiplying at the edge of wounds



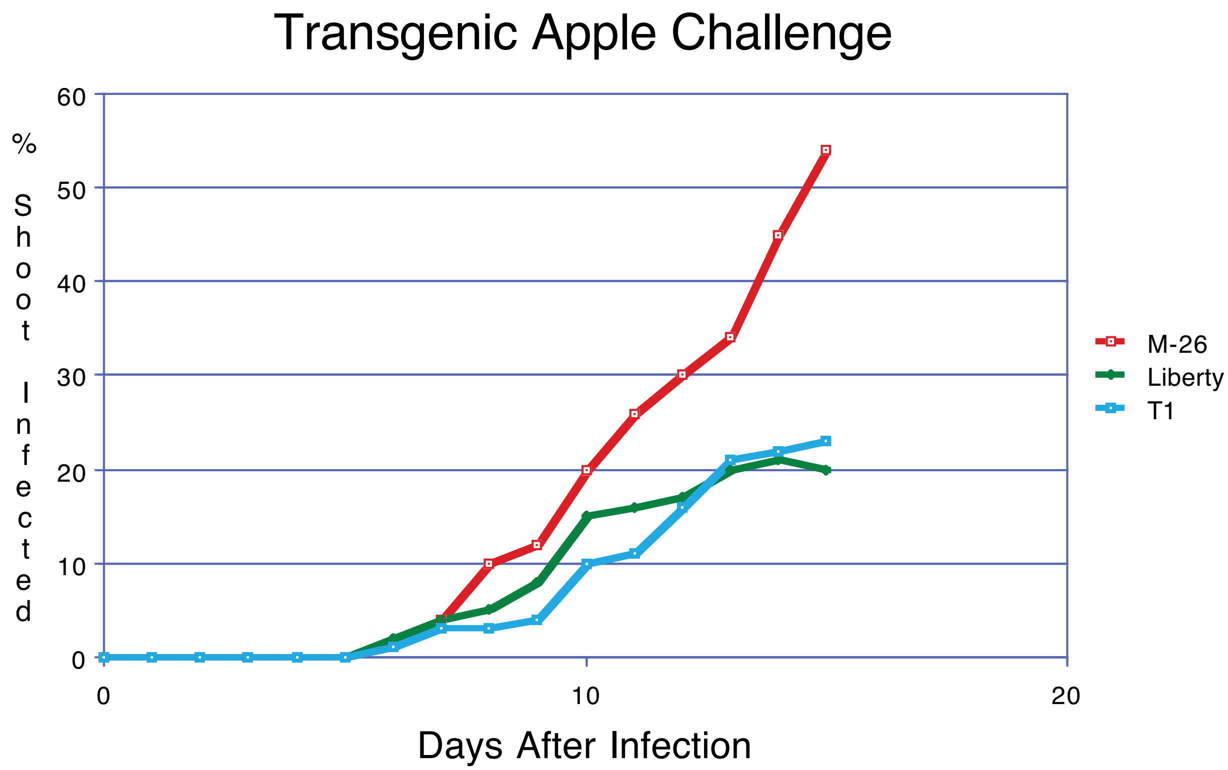
Diagnostic stringing

Bacterial wilt of cucurbits (*Erwinia tracheiphila*)

Spotted Cucumber Beetle

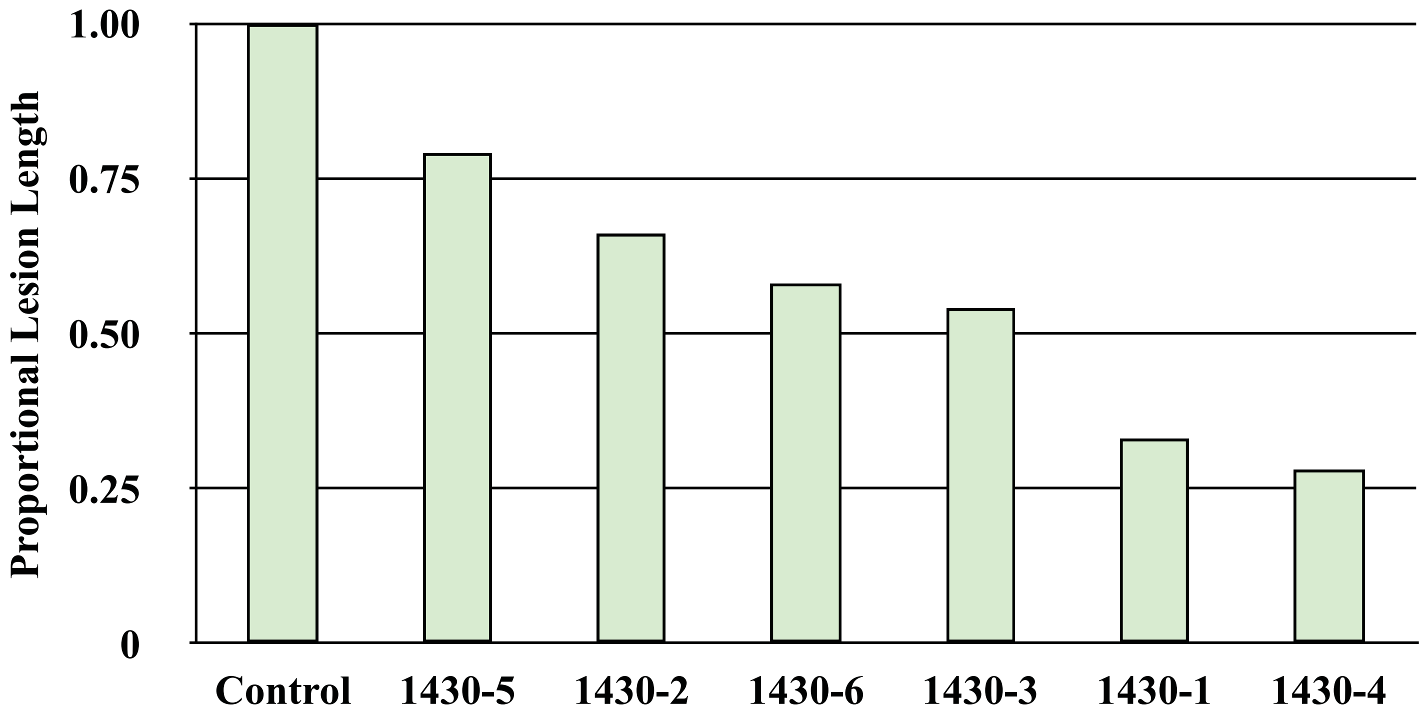
Striped Cucumber Beetle

Bacterial Wilt of Cucurbits Vectors



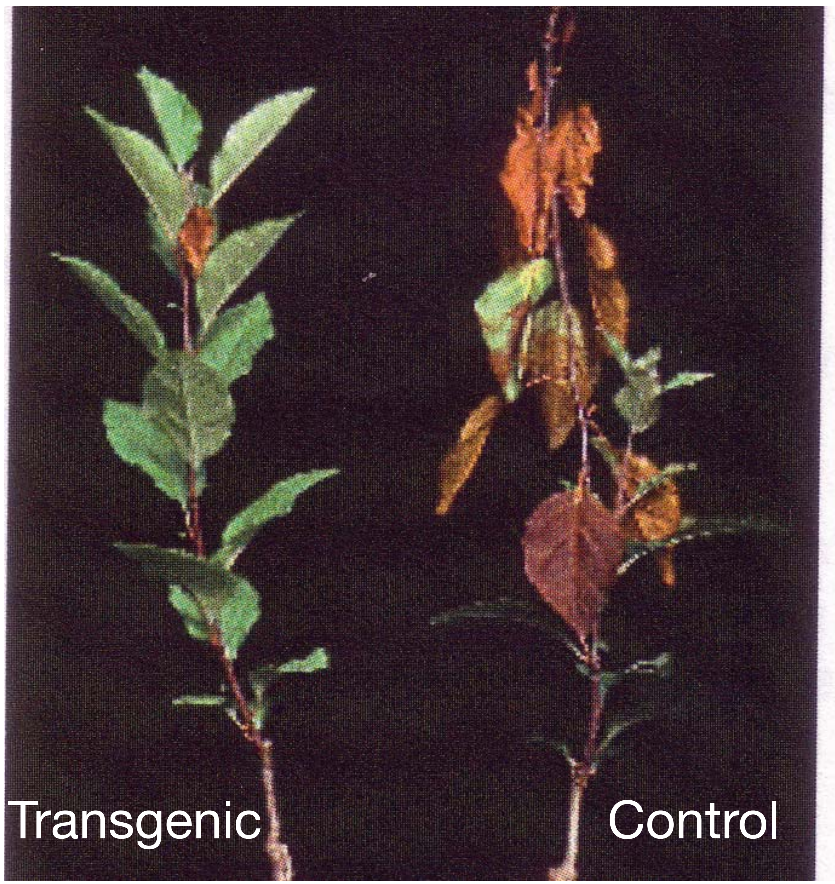
Transgenic Apple Challenge
% Shoot Infected
60
50
40
30
20
10
0
0
10
20
Days After Infection
M-26
Liberty
T1

Disease Suppression in Transgenic Bartlett Pear



*Collaboration with Ralph Scorza at USDA

Resistance to Fire Blight



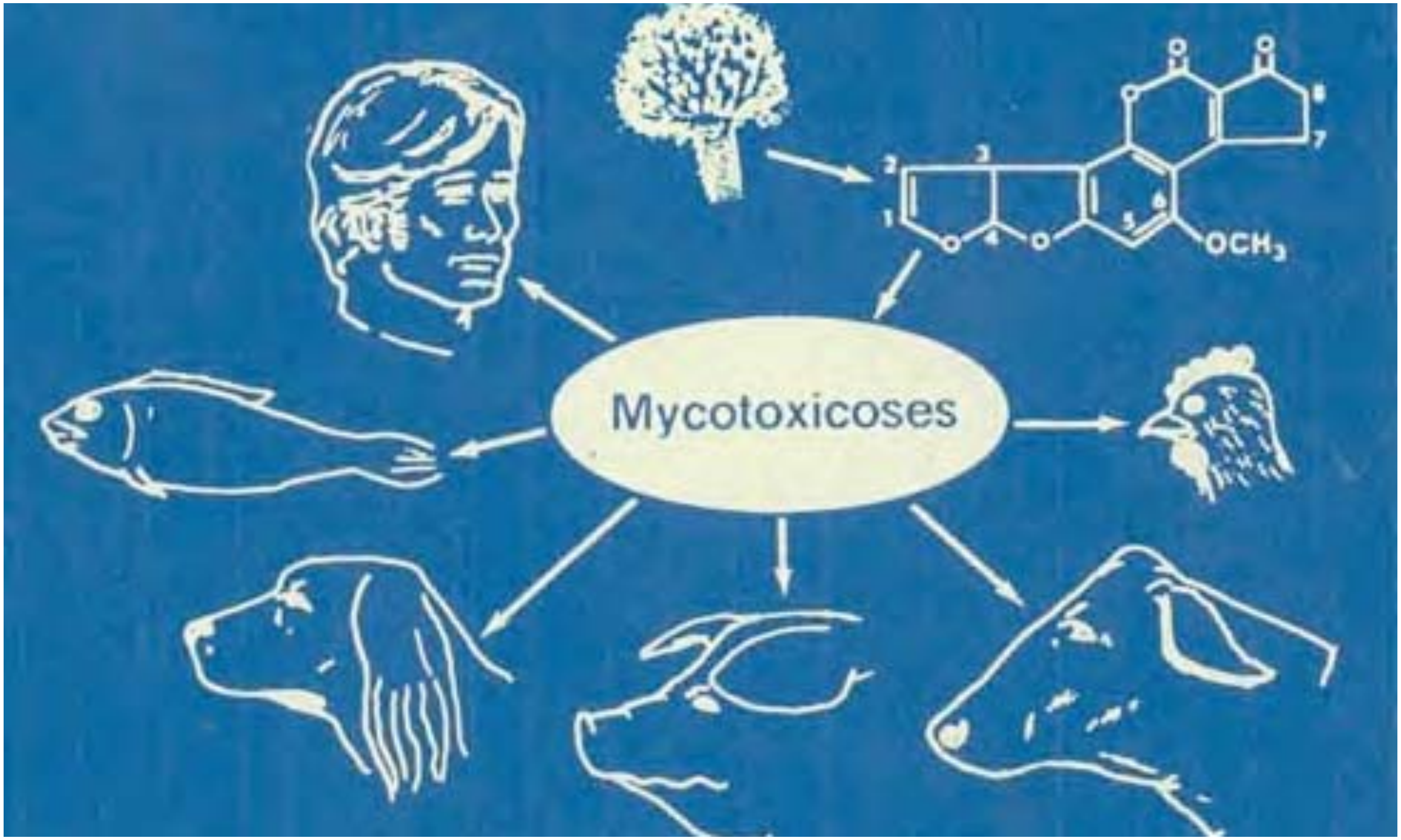
Transgenic Control

Their work showed that in addition to improved disease resistance the transgenic plants were more resistant to insect (Psyllidae) damage by about 4-fold

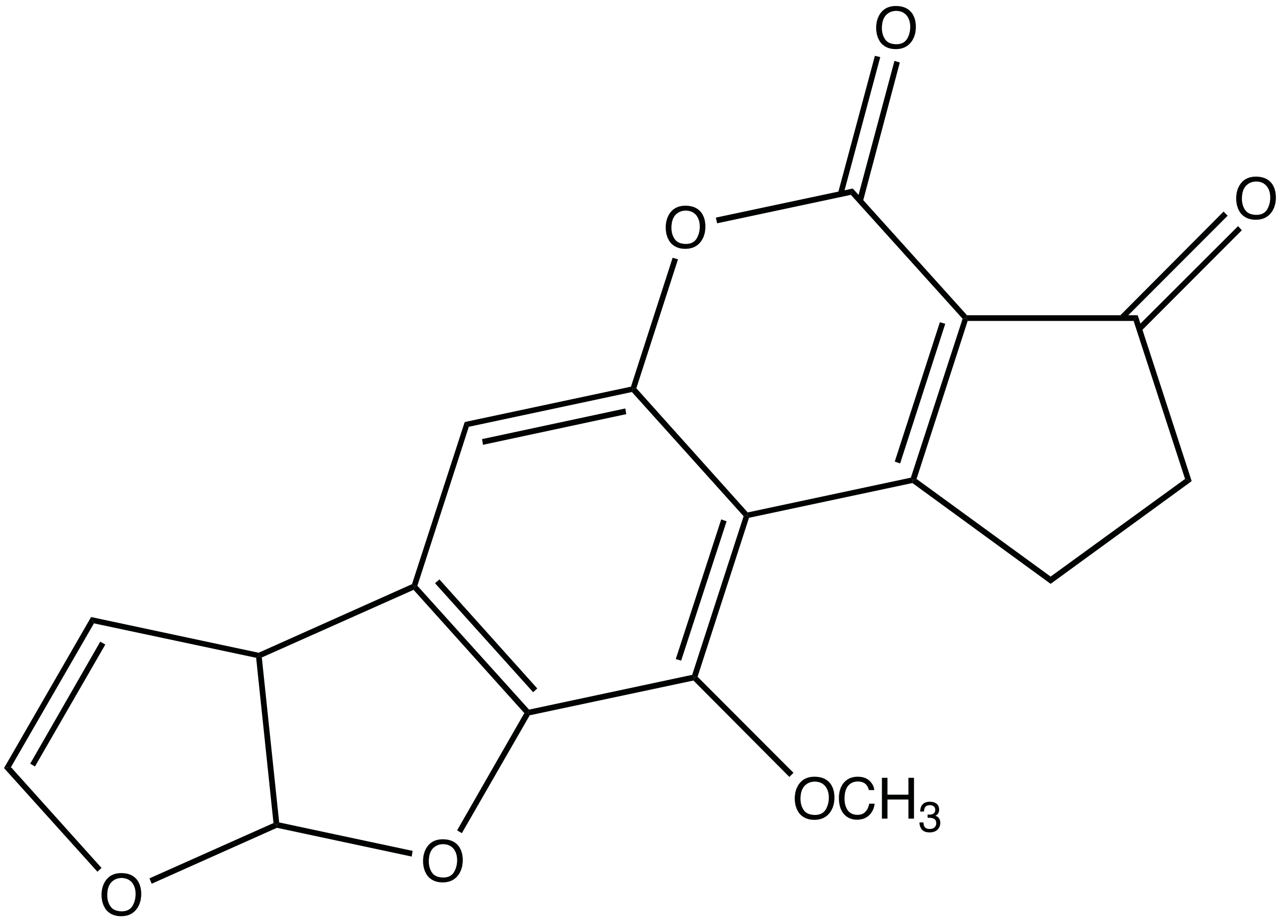
Fungal growth and development of fruiting bodies that will release spores

Aspergillus flavus

Aspergillus Infected Corn



Widespread Genotoxicity and Carcinogenicity



Aflatoxin Structure

One of the most important accounts of aflatoxicosis in humans occurred in more than 150 villages in adjacent districts of two neighboring states in northwest India in the fall of 1974. According to one report of this outbreak, 397 persons were affected and 108 persons died. In this outbreak, contaminated corn was the major dietary constituent, and aflatoxin levels of 0.25 to 15 mg/kg were found. The daily aflatoxin B1 intake was estimated to have been at least 55 ug/kg body weight for an undetermined number of days. The patients experienced high fever, rapid progressive jaundice, edema of the limbs, pain, vomiting, and swollen livers. One investigator reported a peculiar and very notable feature of the outbreak: the appearance of signs of disease in one village population was preceded by a similar disease in domestic dogs, which was usually fatal. Histopathological examination of humans showed extensive bile duct proliferation and periportal fibrosis of the liver together with gastrointestinal hemorrhages. A 10-year follow-up of the Indian outbreak found the survivors fully recovered with no ill effects from the experience.

A second outbreak of aflatoxicosis was reported from Kenya in 1982. There were 20 hospital admissions with a 60% mortality; daily aflatoxin intake was estimated to be at least 38 ug/kg body weight for an undetermined number of days.

Sub-lethal consumption causes liver cancer

Current Tolerated Levels

India;
Brazil;
Europe;
USA

The United States Food and Drug Administration (USFDA) enforces the following action levels for aflatoxins present in human food, animal feed and animal feed ingredients.

Aflatoxin level (in parts per billion)

Commodities and species: 10; 30; 20; 0.05

All products, except milk, designated for humans: 0.5; 30; 20; 0.02

Milk: 20; 30; 20; 0.02

Corn for immature animals and dairy cattle: 100; 30; 20; 0.02

Corn for breeding beef cattle, swine and mature poultry: 200; 30; 20; 0.02

Corn for finishing swine: 300; 30; 20; 0.02

Corn for finishing beef cattle: 300; 30; 20; 0.02

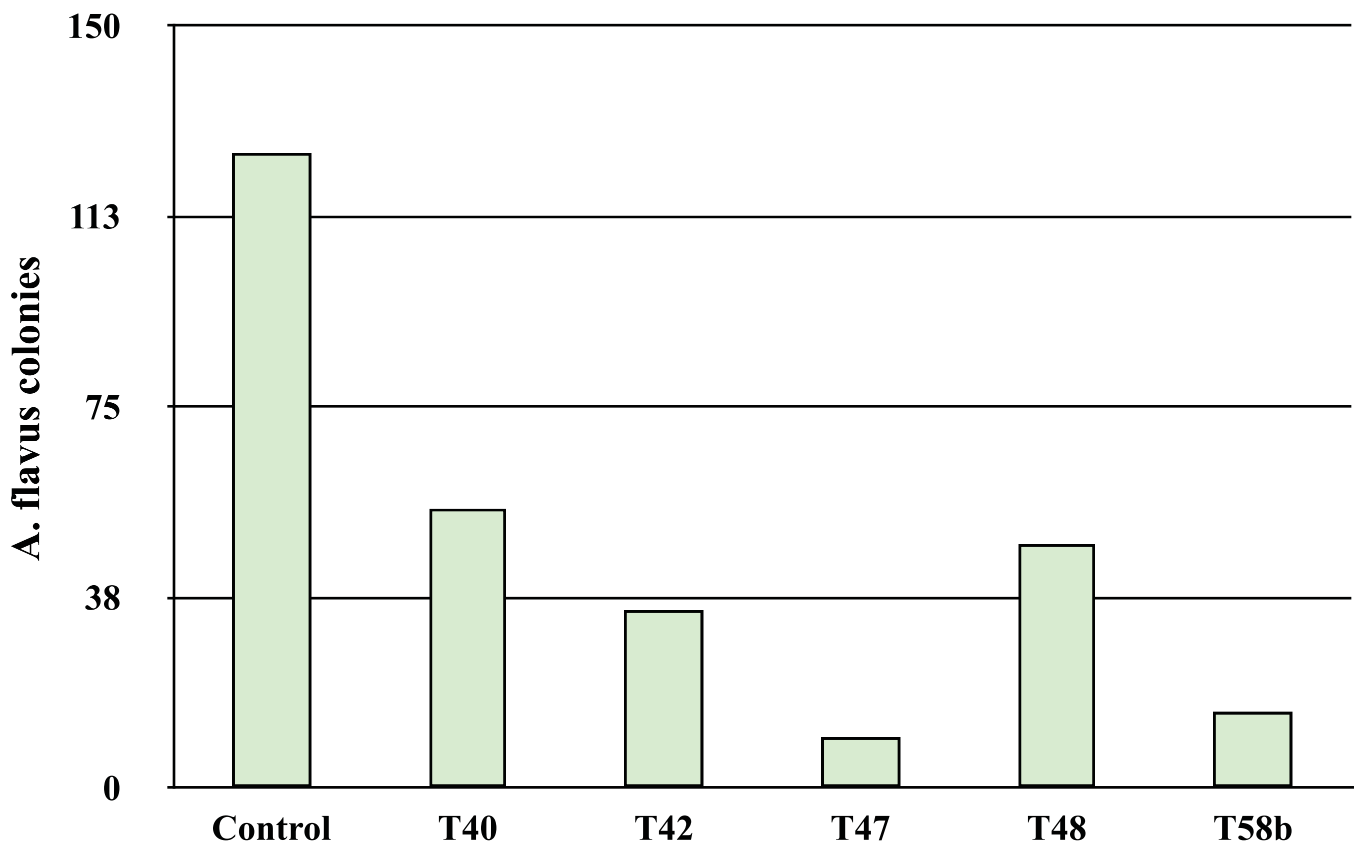
Cottonseed meal (as a feed ingredient): 20; 30; 20; 0.02

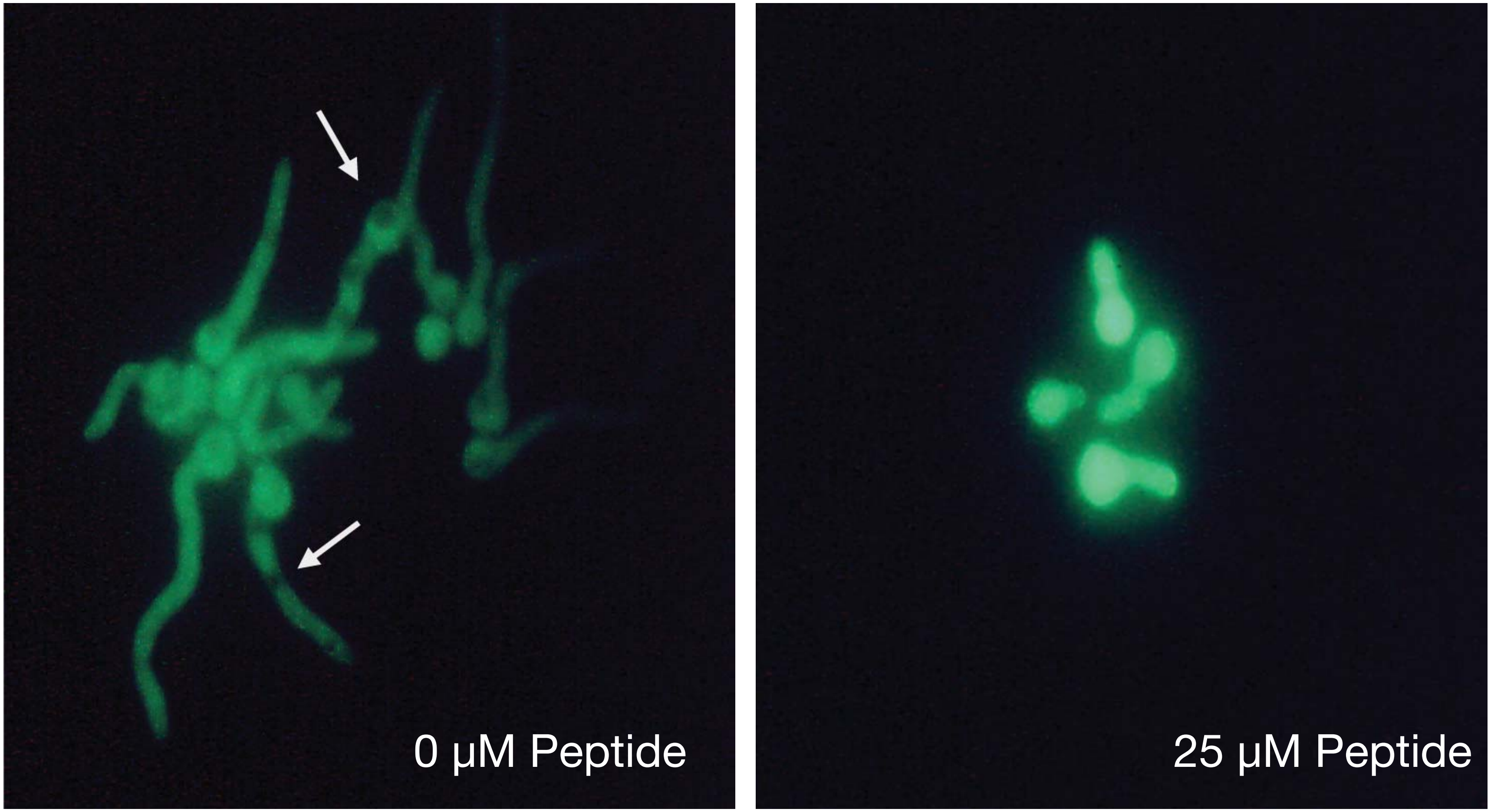
Collaborators:
Dr. TE Cleveland
Dr. Jeff Cary
Dr. K. Rajasekaran

USDA, New Orleans

Aspergillus Infected Cotton

Transformed Embryogenic Cotton





Growth of A. flavus 70-GFP 8 hrs After Continuous Exposure to Antimicrobial Peptide D4E1

Plant[a]	Incidence of severely infected seeds	Seedcoat fluorescence
Control	0.49 ± 0.09	100.0 ± 0.2
C315	0 **	67.8 ± 12.7 **
C333	0.06 ± 0.03 **	89.1 ± 22.2
C343	0.07 ± 0.07 **	45.4 ± 9.8 **

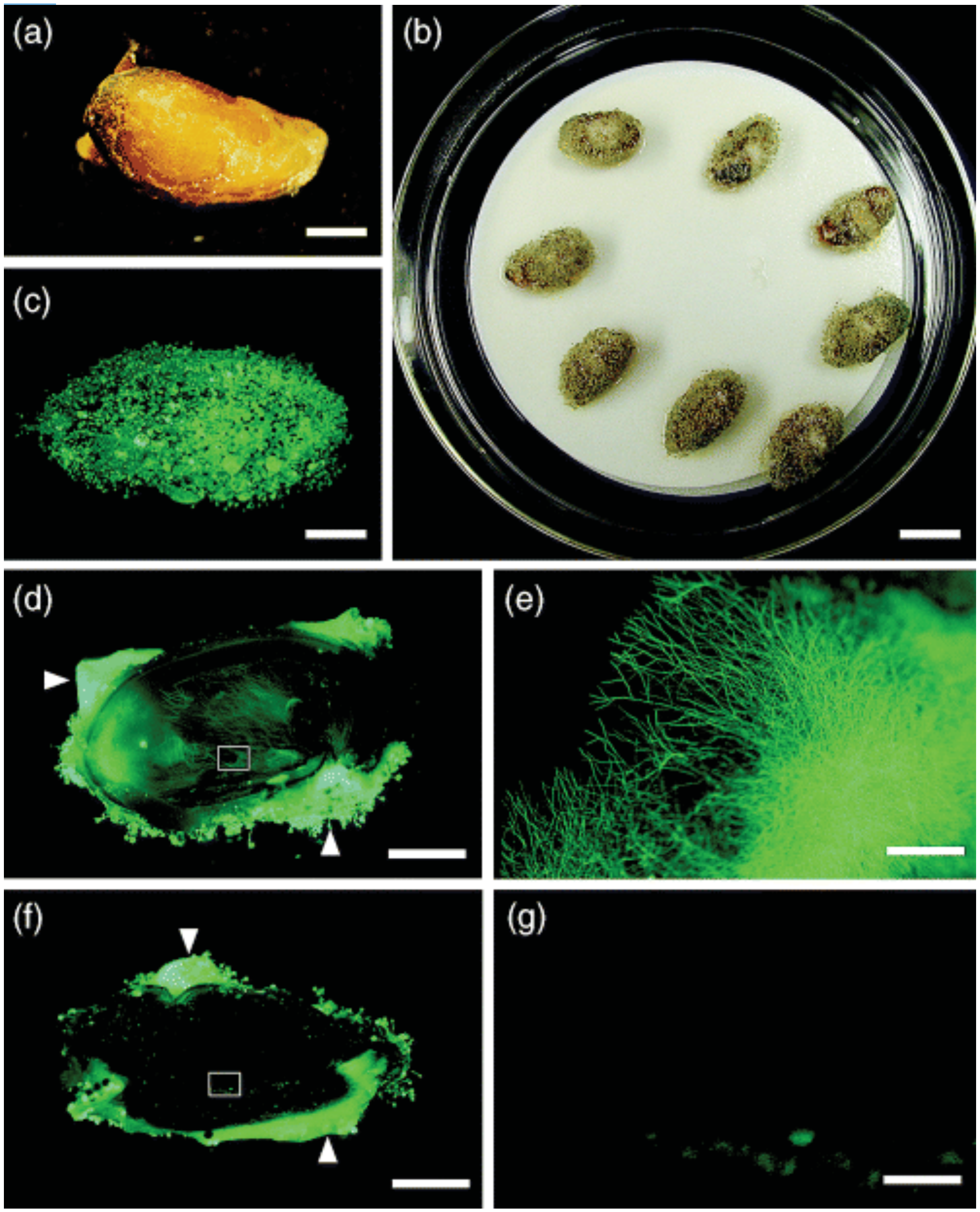
[a] Each plant tested at least three times. Eleven seeds/plant assayed in each experiment.
** indicates a significant difference from control ($P < 0.05$) as determined by the Wilcoxin Rank-Sum Test (Non-Parametric ANOVA)

Colonization of A. flavus 70-GFP of cottonseeds from transformed plants with antifungal peptide D4E1

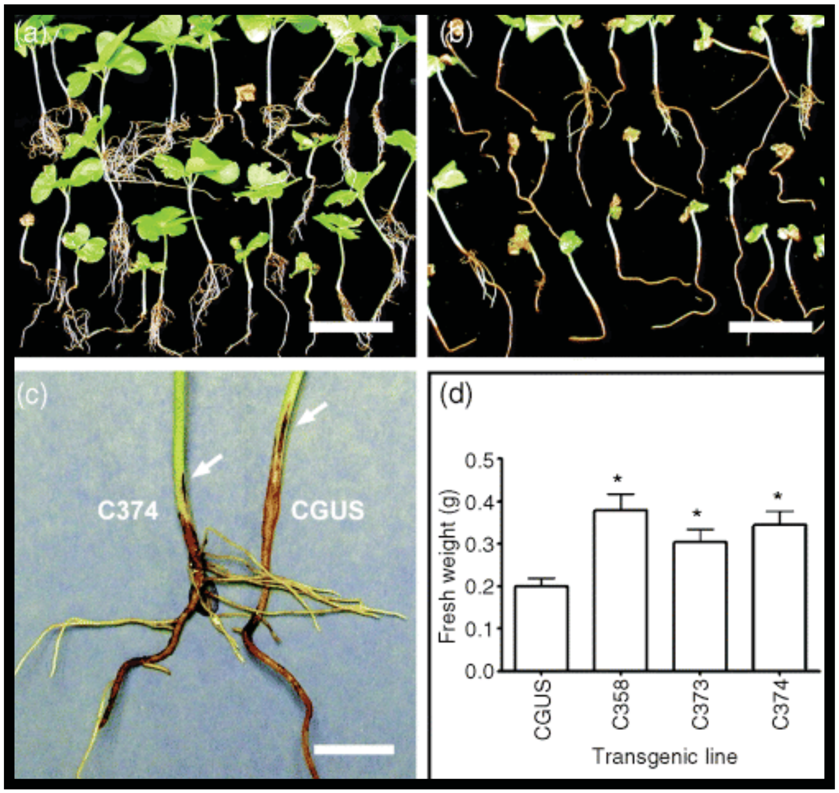
Resistance is Greatly Enhanced

Inhibition Assay of in situ with immature cottonseeds infected with Aspergillus flavus 70-GFP

a) cottonseed prior to inocultaion with A. flavus
b) growth of fungus 7 days after inoculation
c) fluorescence due to fungal growth
d) fluorescence due to fungal growth on control
e) small area marked in d)
f) lack of fluorescence in transgenic
g) small area marked in f)



Resistance to Seedling Pathogen Thielaviopsis basicola



Inhibition Assay of in situ

a) seedlings of transgenic cotton

b) seedlings of transgenic controls

c) comparison of discoloration

d) fluorescence due to fungal growth on control

e) small area marked in d)

f) lack of fluorescence in transgenic

g) increase of fresh weight of seedlings

Work Ongoing in Over 30 Different Plant Species