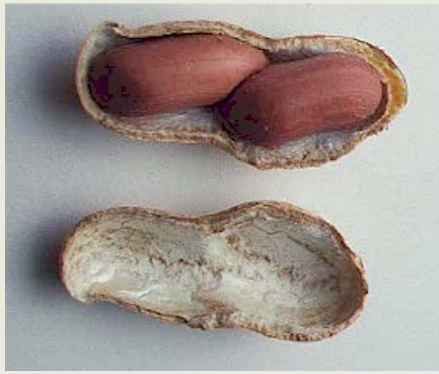
Nuts and Bolts of US Regulatory Dossiers for Genetically Engineered Crops



Blight Blocker Peanut Case Study

Elizabeth Grabau
Dept. of Plant Pathology, Physiology and Weed Science, Virginia Tech

SCRA Workshop, December 6-8, 2011

Outline

- Introduction and rationale: impact of Sclerotinia blight on peanut industry
- Background on peanuts
- Resistance gene and transformation
- Characterization of Blight Blocker lines
- Timeline and costs
- Experience with regulatory agencies
- Obstacles and lessons learned

What are Blight Blocker peanuts?

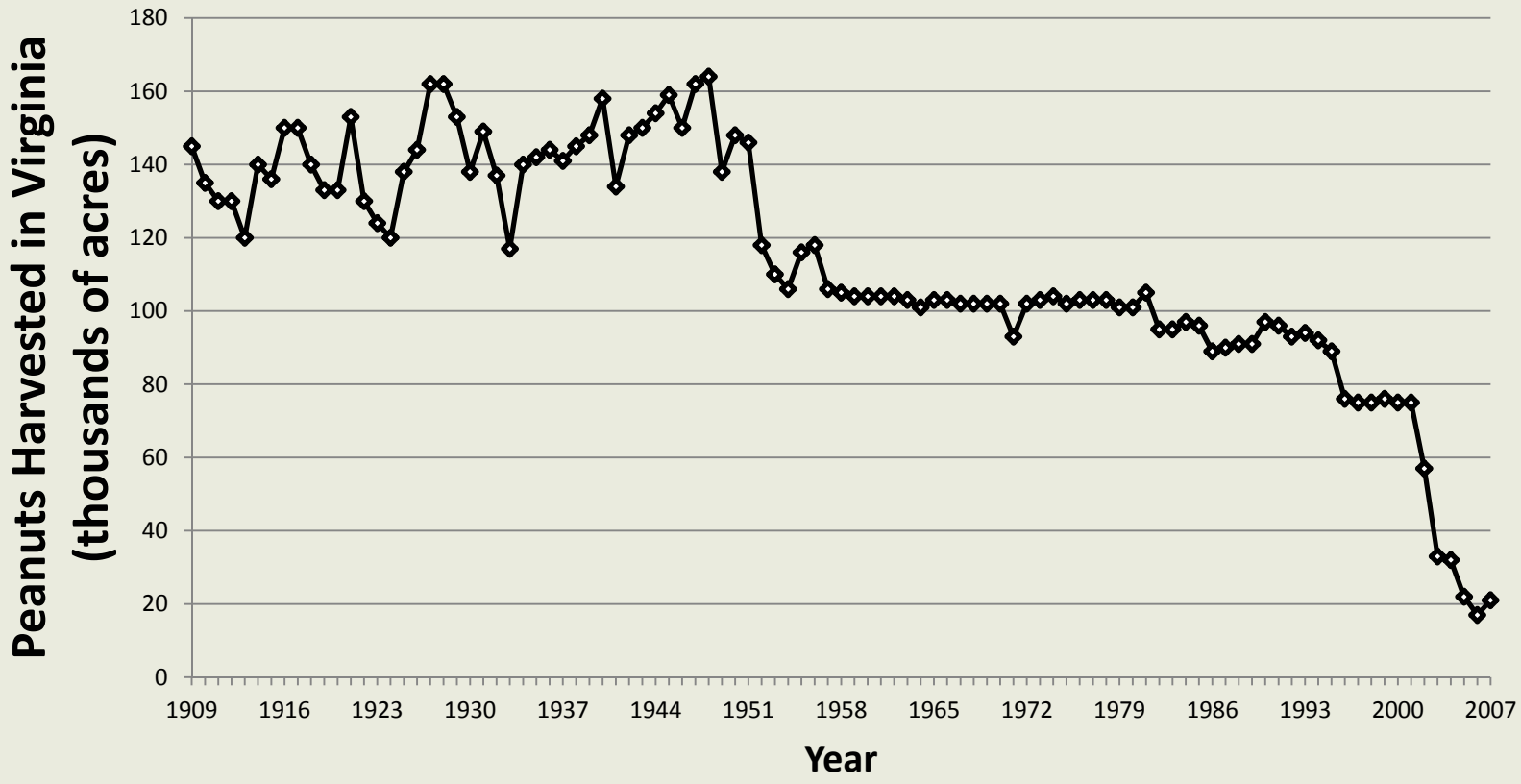
GE peanuts developed at Virginia Tech that demonstrate:

- Excellent resistance to Sclerotinia blight
- Increased yields under disease pressure
- Characteristics identical to parent peanut lines

Why develop Blight Blocker peanuts?

- **Sclerotinia blight** is a devastating fungal disease of peanuts in Virginia-Carolina region and elsewhere
- No naturally-occurring resistance
- Chemical control by fungicides is costly, only partially effective and has potential impact on humans and environment

Background to the problem: declining peanut industry



Sclerotinia blight is caused by a fungal pathogen, *Sclerotinia minor*

sclerotia

S. minor produces **Oxalic Acid**, a pathogenicity factor required for infection

Proposed solution to the problem: engineer disease resistance

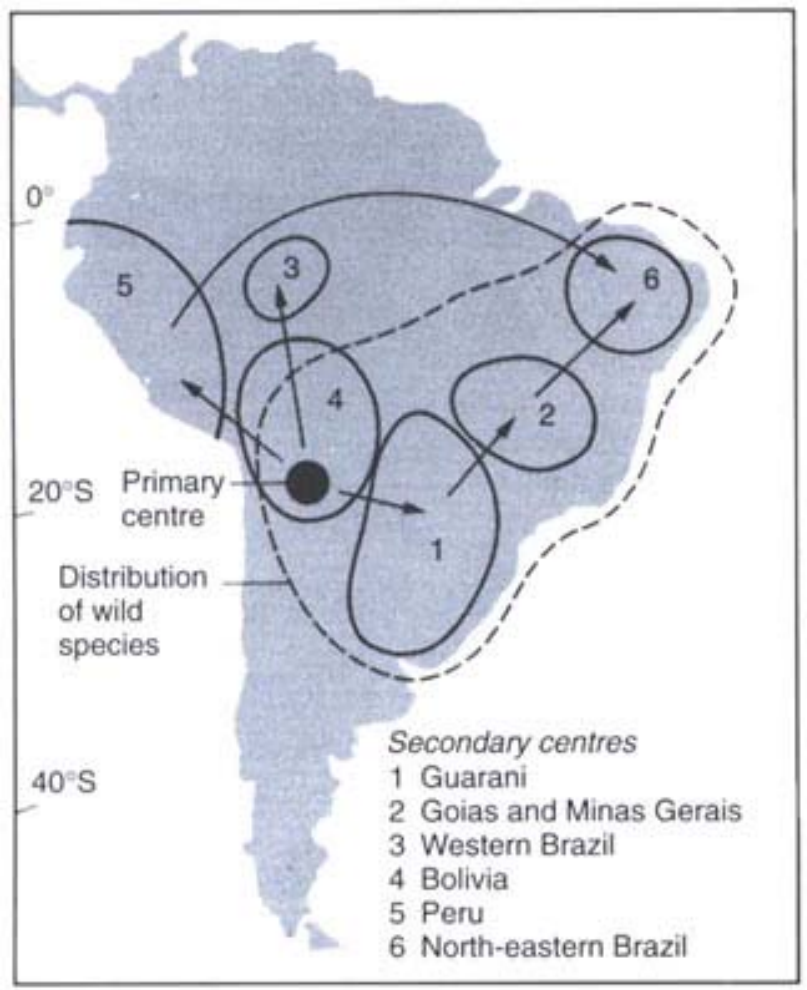
- Use a natural plant product to confer resistance (enzyme oxalate oxidase)
- Express the OxOx enzyme from barley in peanut to degrade oxalic acid

$$\begin{array}{c} COOH \\ | \\ COOH \end{array} + O_2 \xrightarrow{\text{Oxox}} 2CO_2 + H_2O_2$$

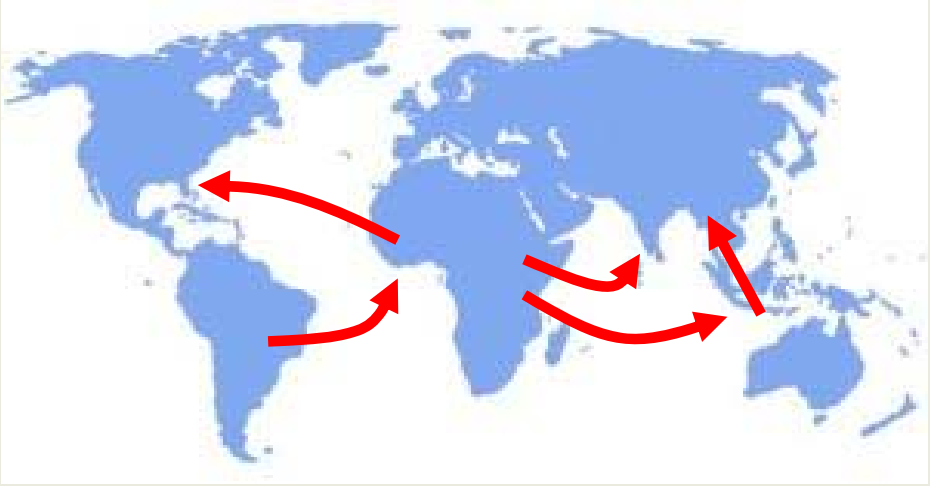
Oxalic Acid

Carbon dioxide
Hydrogen peroxide*

Arachis hypogaea (peanut or groundnut)



Weiss, E.A. 2000 Oilseed Crops.



Kaprovickas. 1969. The Domestication and Exploitation of Plants and Animals.

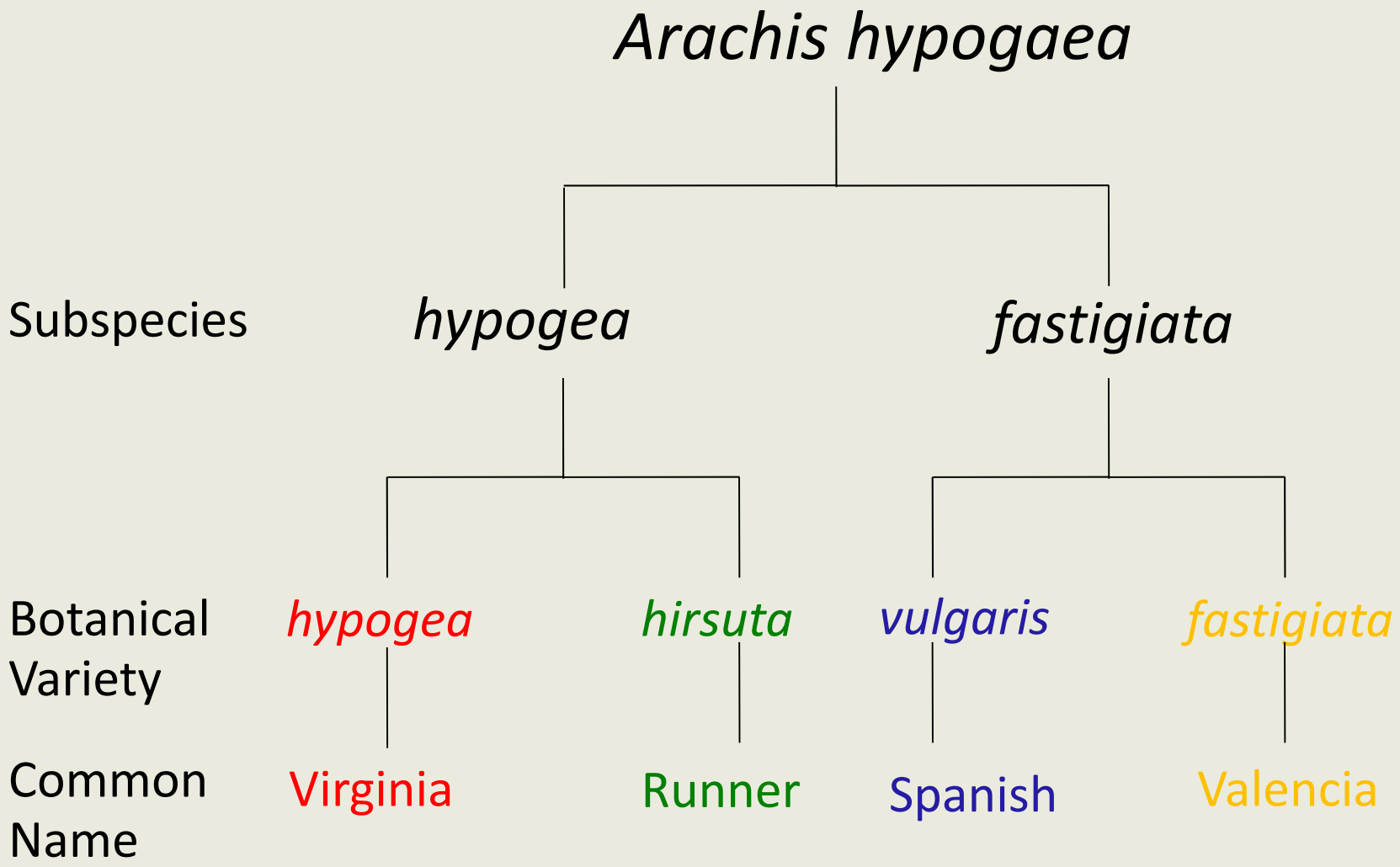
- Domesticated in South America, with oldest artifacts dating from 1500 BC

Cultivated peanut

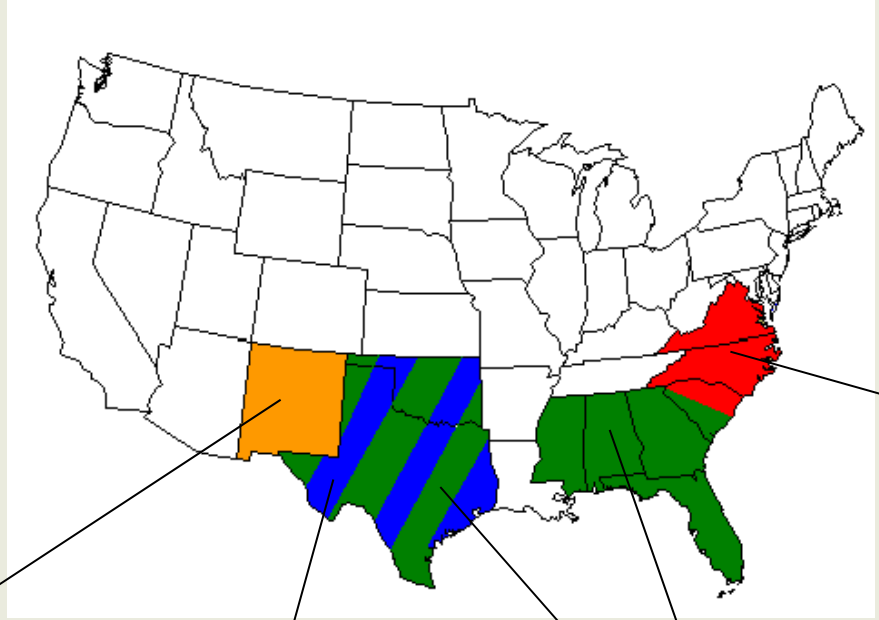
- Legume, not a true nut
- Flowers produce geotropic pegs; peanut kernels develop underground
- Allopolyploid (4X = 40)
- Only naturally interbreeds with *A. monticola*
- Thought to have originated from a hybrid of:

A. ipaensis X A. duranensis with genome duplication

Botanical varieties of peanut



US peanut producing states



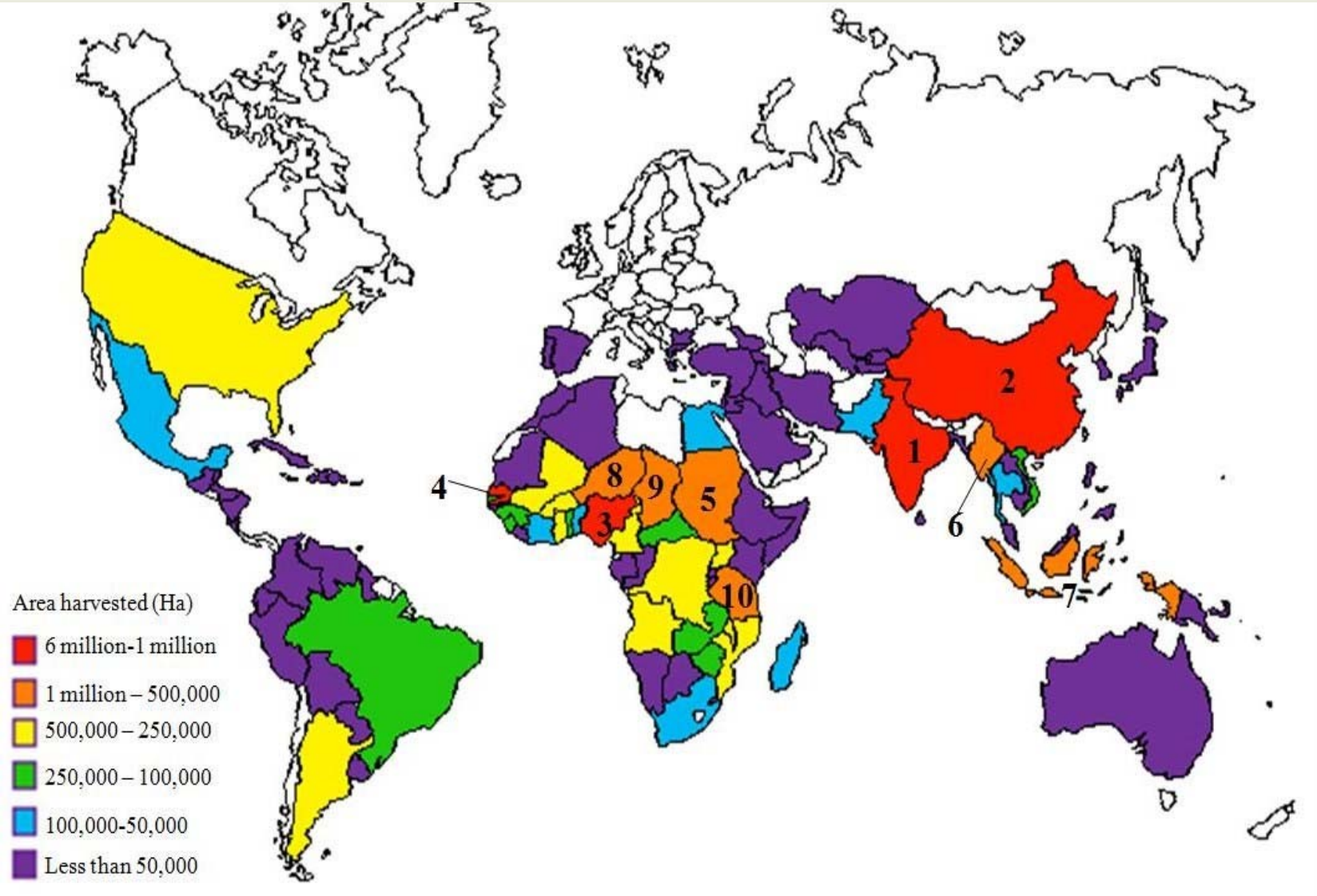
Valencia

Spanish

Runner

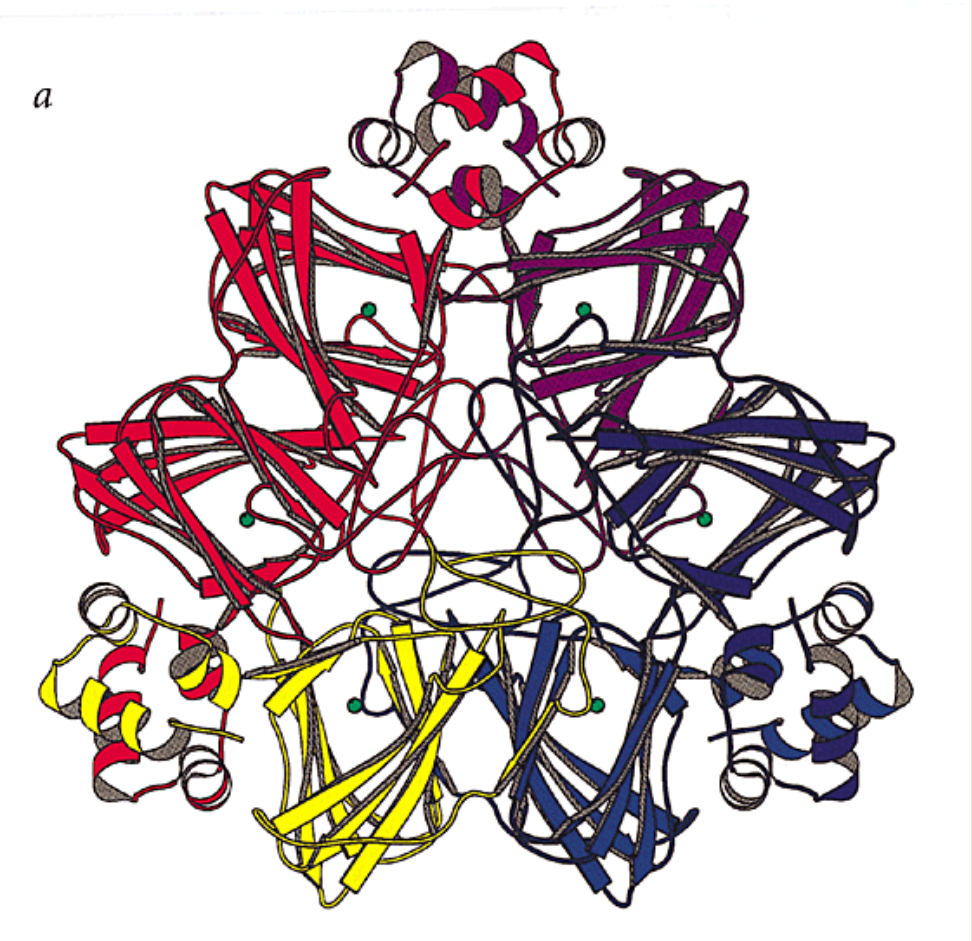
Virginia

Peanut producing countries



Candidate resistance gene: oxalate oxidase

- OxOx enyzme breaks down oxalic acid
- OxOx is also known as *Germin* because it is present in germinating seeds



Woo et al. 2000. Nature Structural Biology

Oxalate oxidase occurs naturally in plants and foods

Oxalate oxidase is found in all cereals

OxOx is increased:

- During germination
- In response to pathogens
- In response to stresses

Expected results of introducing barley OxOx gene into peanut

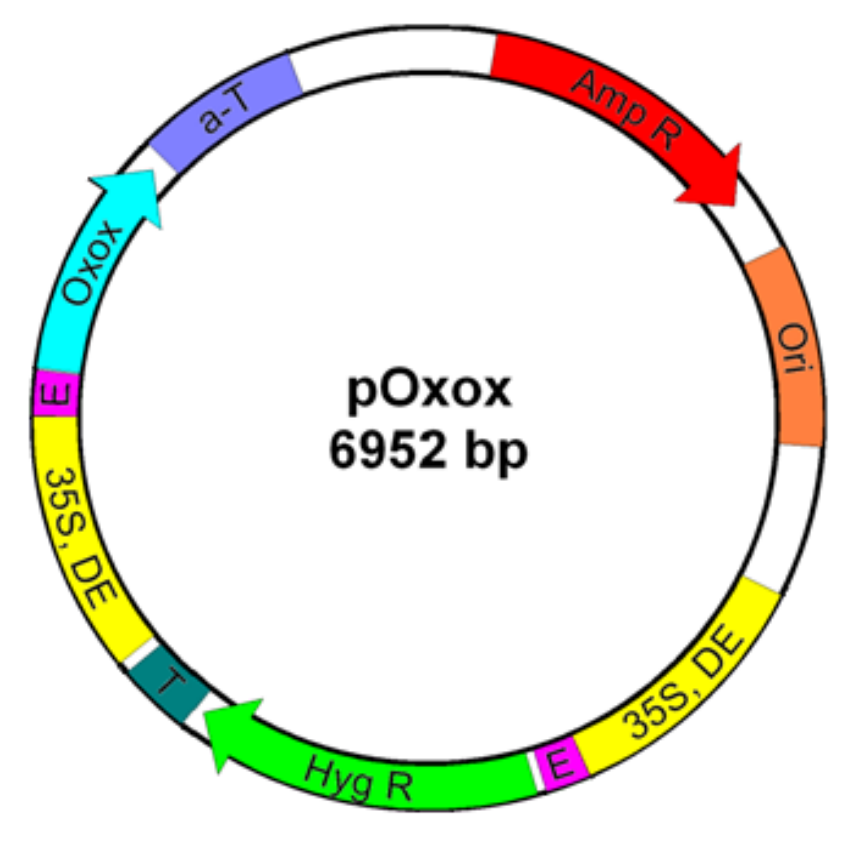
Oxalate oxidase enzyme will prevent or reduce fungal infection by degrading oxalic acid

S. minor ⟶ OA , causes disease and crop loss

Oxalate oxidase

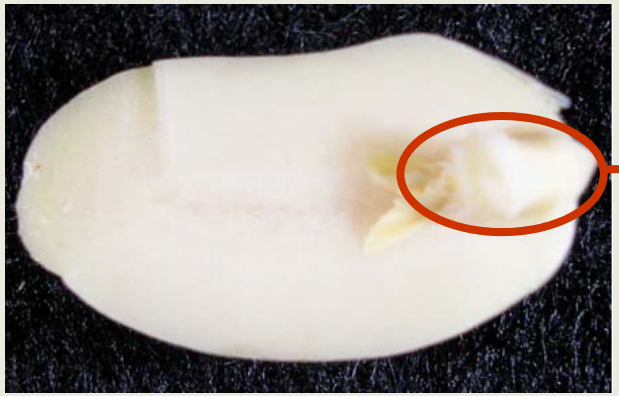
S. minor ⟶ Eliminate OA, no disease, no loss

Transformation construct

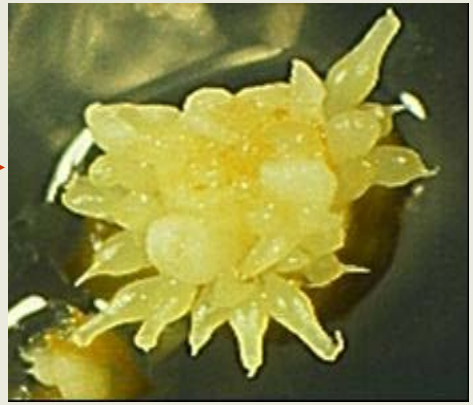


- OxOx cDNA cloned from barley by RT-PCR
- Expression driven by dual-enhanced CaMV 35S promoter
- Hygromycin phosphotransferase used as the selectable marker for transformation

Peanut tissue culture



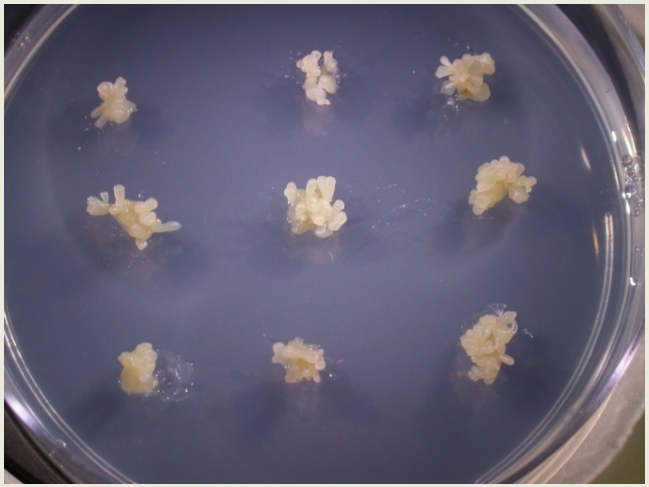
Embryos from mature seed



Embryogenic callus

- Embryogenic callus cultures established from mature seed embryos
- Used 3 popular Virginia peanut cultivars
  - Perry, NC7, Wilson

Transformation by particle bombardment



Used gene gun to transform peanut callus and selected transformed tissue on hygromycin. Manipulated growth regulators to form shoots and roots

Recovery of transgenic plants

Continue the regeneration protocol to recover transgenic plantlets

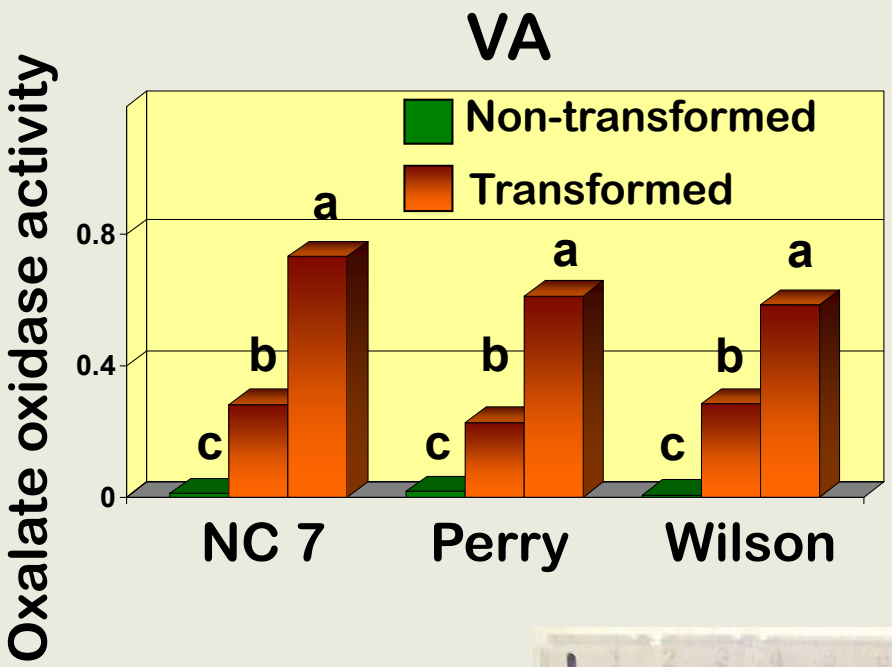
Transfer T_0 plants to greenhouse and test for oxalate oxidase expression

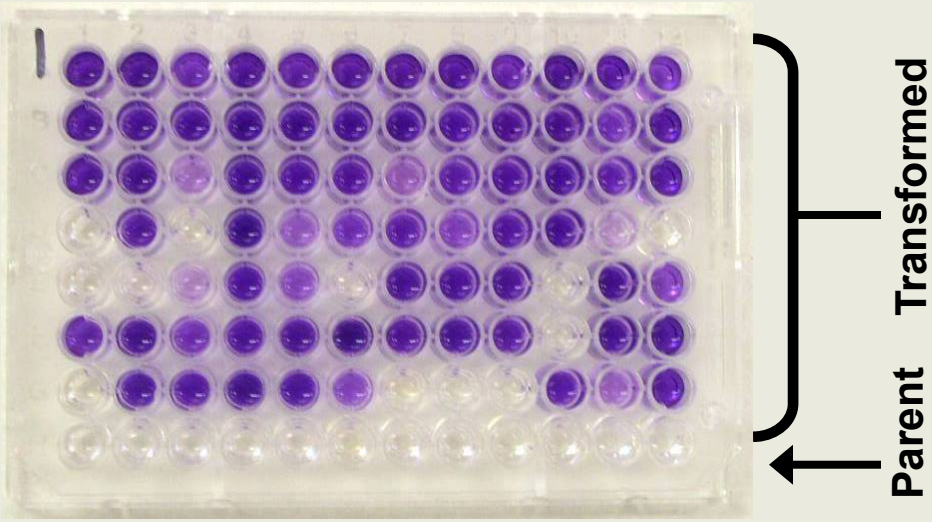
Field testing of Blight Blocker peanuts 2004-2011

<u>Characteristics tested</u>

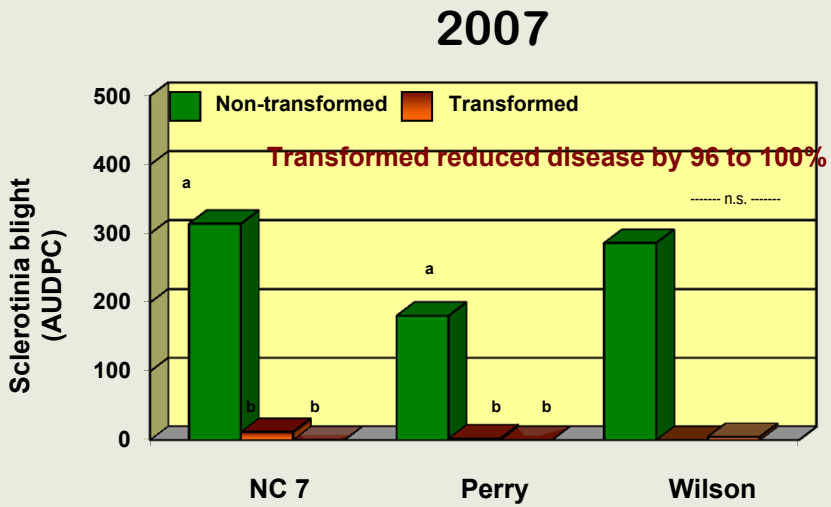
Oxalate oxidase
Yield
Blight ratings
Other diseases
Market/quality traits
Hay analysis
Agronomic data
Outcrossing potential
Volunteers

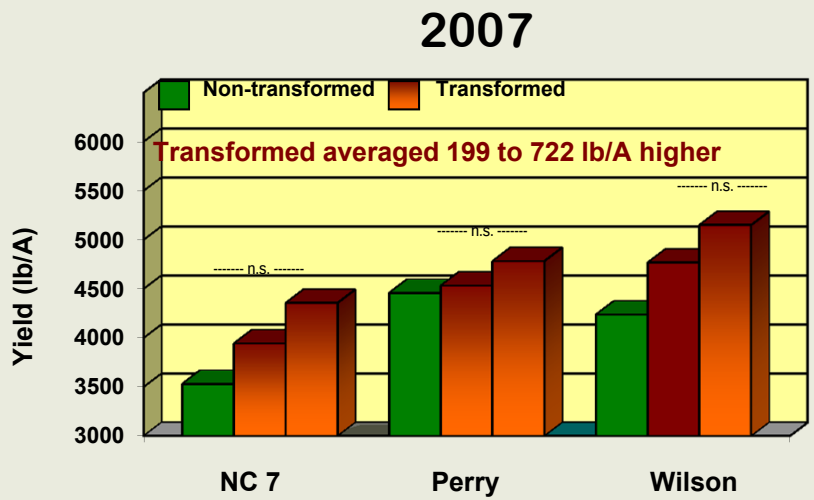
Oxalate Oxidase Expression

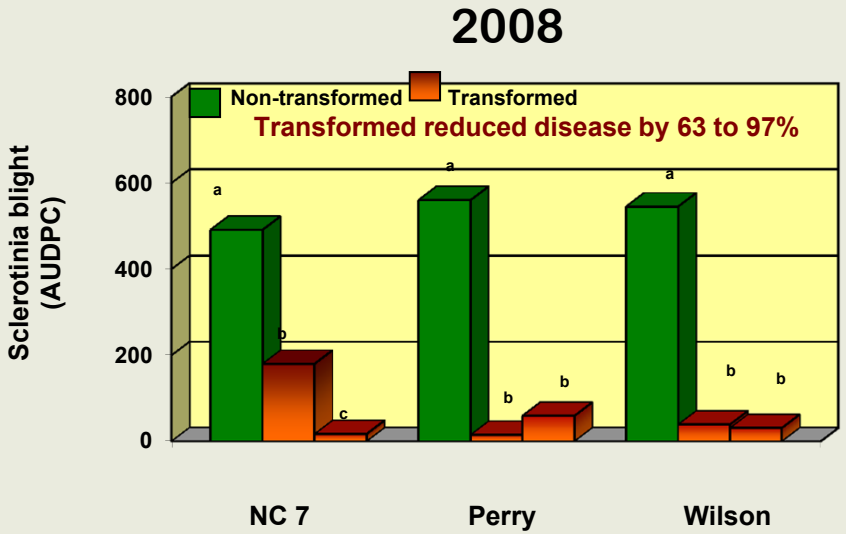


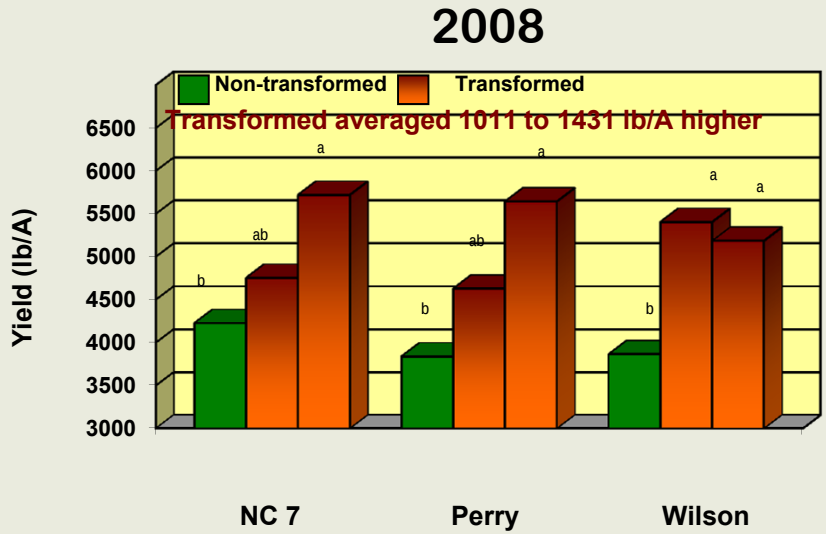


Disease and yield, 2007 and 2008









Market and quality traits

- Grade and value
- Composition
- Pod brightness
- Blanchability

Wilson

W73-27-B-B-B

NC 7

N70-8-24-B-B-B

Perry

P39-7-9-B-B-B

Peanut hay analysis

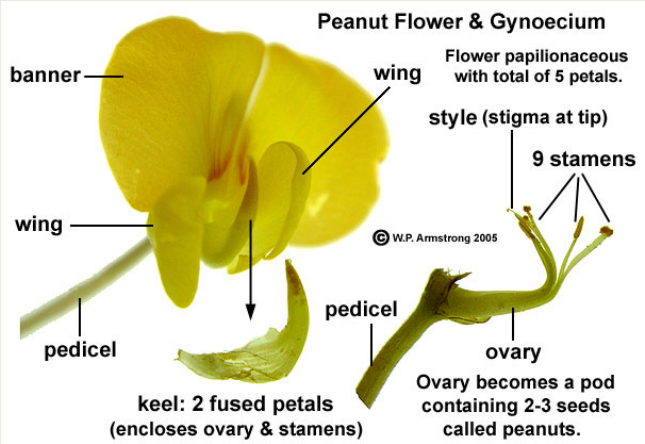
Variety	Neutral detergent fiber (%DM)	Acid detergent fiber (%DM)	Total digestible nutrient (%DM)	Crude protein (%DM)
NC7	48.8	44.5	56.1	7.7
N70-8-24-B-B-B	50.3	45.5	55.5	7.8
N70-6-B-B-B-B	51.9	46.8	54.7	7.7
LSD	ns	ns	ns	ns
Perry	49.8	45.2 b	55.7 a	7.1
P39-7-9-B-B-B	52.1	47.2 ab	54.4 ab	7.1
P53-30-21-B-B-B	54.8	49.8 a	52.7 b	7.0
LSD	ns	3.5	2.3	ns
Wilson	53.8	49.2	53.1	7.3 ab
W14-10-2-B-B-B	53.6	48.7	53.4	7.7 a
W73-27-B-B-B-B	54.9	50.2	52.4	6.7 b
LSD	ns	ns	ns	0.7

Virginia Tech Forage Research Laboratory performed hay analysis. Data are mean of Omega-treated entries in Virginia Trial, 2008. Means followed by the same letter(s) within a column and group are not significantly different (Fisher's LSD, $P \leq 0.05$).

2009 Outcrossing field study

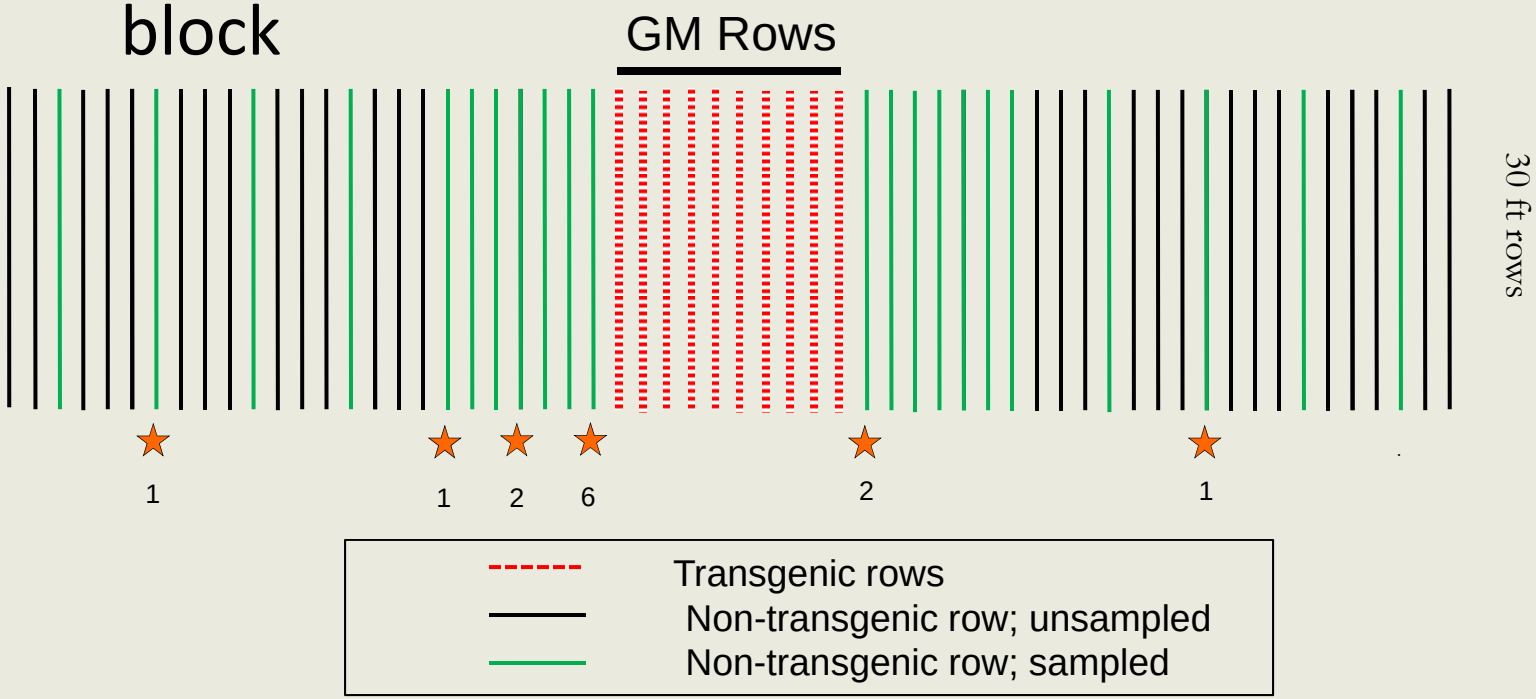
Gene flow in *Arachis hypogaea*

- Primarily self pollination
- Outcrossing by bee pollination:
  Apidae, Bombidae, Halictidae, Megachilidae
- Natural outcrossing rates:
  - in USA: 0-10%
  - in Virginia: 0-2.8%



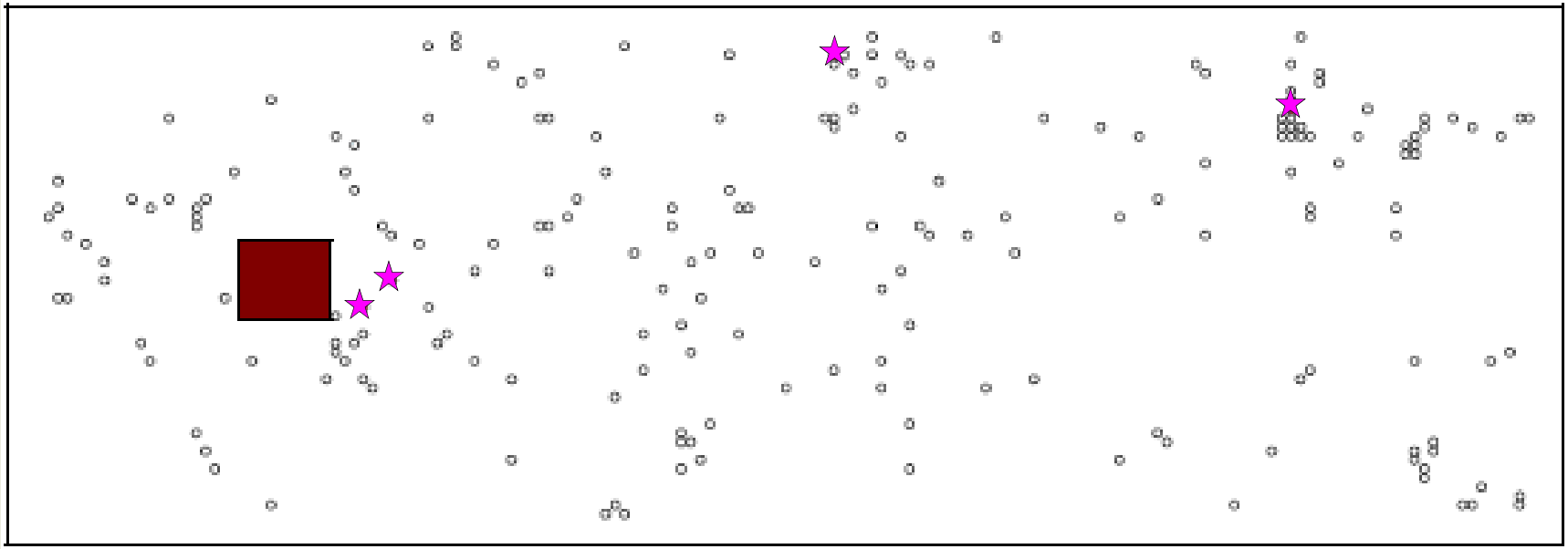
Outcrossing assay

- Where viable GM pollen transferred to non-GM peanuts, transfer detected by assaying seeds for OxOx
  - simple, inexpensive colorimetric assay
  - 372 progeny tested per row - 8,184 total seeds
  - 13 positives (0.16%) frequency highest near GM block



Outcrossing results from plants where pollinators observed

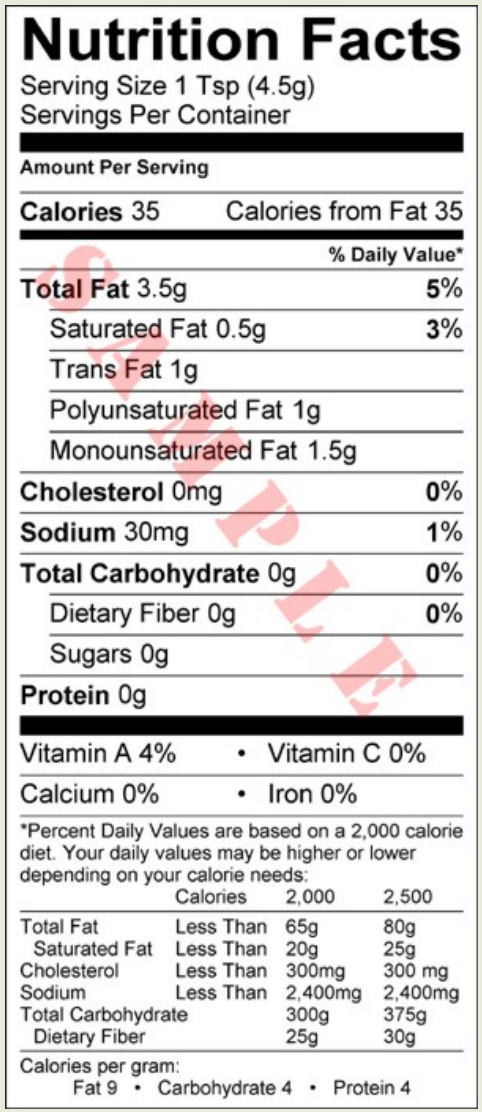
- Flagged plants where pollinators observed (214 plants)
- Recovered 9 to 144 seeds/plant; ave. 60
- 12,716 seeds assayed
- 4 OxOx positive seeds (0.031%) ★
- Low frequency outcrossing, but up to 96 m



Laboratory testing of Blight Blocker peanuts

- Seed composition
- Food label analysis
- Protein detection (western blots)
- Allergenicity potential (compare gene sequences to known allergens)
- Testing for aflatoxin (commercial kit)
- Confirm OxOx transgene: PCR, Southern, enzyme assay
- Stability of trait – multiple generations

Food label analysis



Nutrition Facts

Serving Size 1 Tsp (4.5g)
Servings Per Container

Amount Per Serving

Calories 35	Calories from Fat 35
	% Daily Value*
Total Fat 3.5g	**5%**
Saturated Fat 0.5g	**3%**
Trans Fat 1g	
Polyunsaturated Fat 1g	
Monounsaturated Fat 1.5g	
Cholesterol 0mg	**0%**
Sodium 30mg	**1%**
Total Carbohydrate 0g	**0%**
Dietary Fiber 0g	**0%**
Sugars 0g	
Protein 0g	

Vitamin A 4% • Vitamin C 0%

Calcium 0% • Iron 0%

*Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

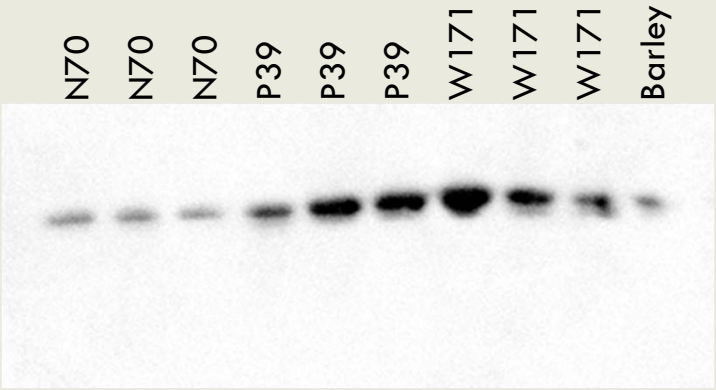
	Calories	2,000	2,500
Total Fat	Less Than	65g	80g
Saturated Fat	Less Than	20g	25g
Cholesterol	Less Than	300mg	300 mg
Sodium	Less Than	2,400mg	2,400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

Calories per gram:
Fat 9 • Carbohydrate 4 • Protein 4

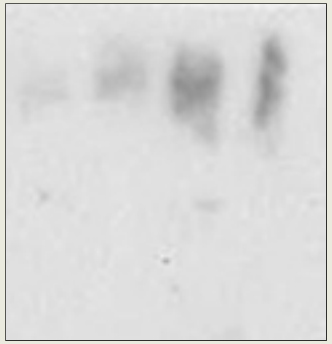
SAMPLE

- Sent 2008 peanut samples to ABC Research Corporation
- Conducted food analysis of peanut kernels including fat, protein, carbohydrates, calories, minerals, fatty acid composition,
- Label from 100 g sample size
- Due to cost limitations, generated one label per line

Protein expression



Oxalate oxidase is present in all transgenic peanuts tested by Western blotting



Oxalate oxidase can be detected in common food products

Oxalate oxidase is a food protein consumed safely every day (baby food, cereals, malted barley products)
Oxalate oxidase is not a pesticide

In silico allergenicity analysis

Species	Know Allergen in the Cupin Superfamily	Identity to Oxox (%)	>35% in 80bp sliding window
Soybean	Beta-conglycinin (7S globulin)	14.8	NO
	Glycinin (11S globulin)	12.5	NO
Peanut	Ara h 1 (7S globulin)	13.7	NO
	Conarachin (7S globulin)	10.6	NO
	Arachin (11S globulin)	16.7	NO
	Ara h 3 (11S globulin)	15.3	NO
Species	**Known Cereal Allergen**	**Identity to Oxox (%)**	**>35% in 80bp**
Barley	Gamma-3 hordein	15.3	NO
	Alpha-amylase	16.4	NO
Wheat	Gamma glutenin	13.0	NO
	Omega-5 gliadin	9.1	NO
Rye	Omega secalin	10.6	NO

Aflatoxin analysis of seed

Cultivar	Aflatoxin (ppb)				
	2005	2006	2007		2008
	VA	VA	VA	NC	VA
NC7	1.75	1.97	0.32 a	0.48 b	1.97
N70-8	2.14	1.89	0.00 b	0.93 a	2.32
N70-6	1.77	1.65	0.00 b	-	-
Perry	1.46	1.55	0.39 a	0.86 b	2.73
P39	1.48	1.74	0.00 b	1.20 a	0.57
P53	1.41	1.89	0.03 b	-	-
Wilson	1.76	2.18 a	0.56 a	0.90	2.25
W73	0.58	1.41 b	0.02 b	1.51	2.99
W14	-	0.72 c	0.01 b	-	-

Means followed by the same letter(s) within a column and group are not significantly different (Fisher's LSD, P≤0.05).

Grower/Industry Support

"While we realize that this registration process can be lengthy, we do hope that it can be expedited so that our growers can begin to reap the benefits of the Blight Blocker peanuts. "

Sponsored funding for transgenic peanuts

• 1998 - Initial funding from Virginia Tech for peanut tissue culture	$ 41,400
• 2000-2011 - Annual support from Virginia and N. Carolina Peanut Growers (Nat. Peanut Board)	$ 410,457
• Peanut Council (1 yr.)	$ 25,000
• Virginia Ag Council (6 yrs.)	$ 111,650
• So. Region IPM (3 yrs.)	$ 119,998
TOTAL	**$708,505**

Personnel support for transgenic peanut project

P.I.s

Dr. Elizabeth Grabau

Dr. Pat Phipps

Postdocs

Dr. Malcolm Livingstone

Dr. Darcy Telenko

Dr. Jiahuai Hu

Dr. Matt Hunt

Graduate Students

Shanna Chriscoe

Nicole Juba

Holly Hills

Technicians

Jaime Hampton

Jenny Jenrette

Haijie Liu

Barron Keeling

Coordinated Framework

Responsibility is shared by three agencies:

Animal and Plant Health Inspection Service

Biotechnology Regulatory Service

- Protect America's agricultural industry from diseases and pests
- All transgenic plants are "**regulated articles**"
- Notification/permitting process for field trials, interstate movement
- Petition for deregulated status

Interactions with BRS

- Annual notification for field testing, 2004-2011
- Pre-petition consultation visit to BRS, September 2006
- 2nd consultation at BRS, January 2010
- Blight Blocker peanut petition submitted, March 2010
- Received response (deficiency) letter, October 2010

Interactions with BRS

- Rewriting document for clarity, i.e. describing split plot experimental design, farming practices, flow chart
- Providing additional information such as agronomic data, additional references
- Conducting additional analyses as requested, i.e. PCR to verify presence of hygromycin resistance gene

EPA

- Regulates the sale and distribution of pesticides including Plant Incorporated Protectants (PIPs)
- PIP: any substance introduced into a plant through breeding or biotechnology which is intended to “prevent, destroy, repel or mitigate any pest.”
- **Registration** Process

Pre-submission consultation with EPA

- Visited EPA in January 2007, no response
- Second visit, October 2010, response received Feb 16, 2011
- Acknowledged discussion of data waivers; recommended against seeking waivers
- Suggested a Science Advisory Panel
- Will require toxicity data for PIP registration as biopesticide

FDA

- Ensures proper labeling and safety of all foods and animal feeds
  - Except for those regulated by EPA
- Voluntary **consultation** process for transgenic plants
- No consultation yet for Blight Blocker

Obstacles to commercialization

- Science is technically difficult, expensive and time-consuming
- Intellectual property issues are a barrier
- Complex, bureaucratic regulatory process
  - Extremely time-consuming
  - Often little guidance from agencies
  - Expensive - need funding for tests, programs to support data collection
  - Scientific community needs regulatory educational opportunities
- No ‘rewards’ to researchers
  - No avenues for publication, grants, recognition

Lessons Learned

- Seek guidance early and often
- Information requested for petitions is not always science-based; often precedent-based
- Regulatory process needs revision, stream-lining
- Training and education in ag regulatory issues would be valuable
- With current regulations, a public institution is unlikely to gain approval for a GE crop without substantial, committed financial and personnel support

Acknowledgements

Pat Phipps, co-PI
Malcolm Livingstone
Jiahuai Hu
Darcy Telenko
Matt Hunt
Haijie Liu
Jaime Hampton
Shanna Chriscoe
Nicole Juba
Holly Hills

National Peanut Board, So. Region IPM, Virginia Ag Council, Virginia and N.Carolina Peanut Growers, Peanut Council, Virginia Tech