Ultra-low Gossypol Cottonseed

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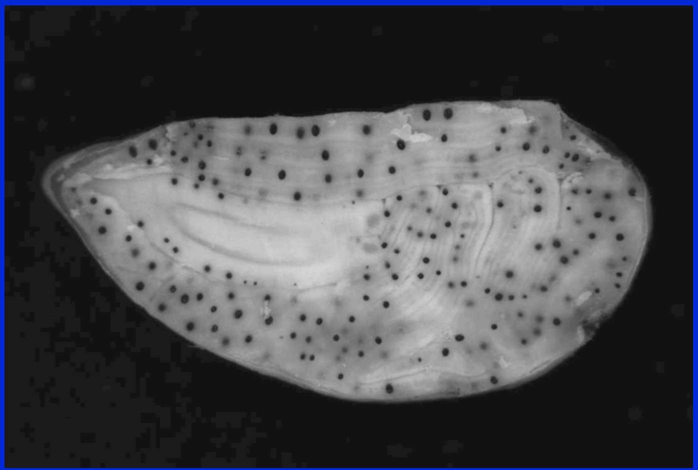
Global Cotton Lint Production in 2011 = 26.1 MMT

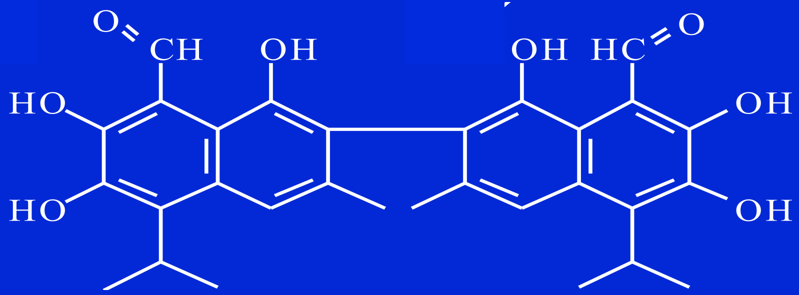
Global Cottonseed Production in 2011 = 48.8 MMT (~11 MMT Protein)

Can meet the protein requirements of 600 million people/year (50 g/day)

Presence of toxic gossypol prevents the use of cottonseed as food & also limits its use as feed

- Protein content of the seed is about 22.5%; good quality protein.
- Its use for human consumption or animal feed is limited by the presence of gossypol in the seed glands.
- Gossypol is toxic to non-ruminant animals, including pigs, chicken and humans. It damages heart and liver.
- Meal used as feed for the cattle – gossypol binds to lysine thus reducing the nutritional value of the meal.





Gossypol

Feed Conversion Ratio (FCR) for Various Animals

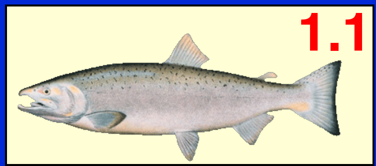
Animal	FCR	Reference
Beef	5.8[a]	Klopfenstein et al., 1991
Pig	3.3[a]	Losinger, 1998; Cromwell, 1991
Duck	2.705[b]	Shalev and Pasternak, 1989
Turkey	2.102[b]	Shalev and Pasternak, 1989
Chicken	2.047[b]	Shalev and Pasternak, 1989
Shrimp	2.0[c]	Tacon, 2002
Tilapia	1.5[d]	McGinty and Rakocy, 1989; Boyd et al., 2005
Salmon	1.1[d]	Villamar, 2002
Channel Catfish	1.0[d]	Lovell, 1990

[a]Feed grain/weight gain ratio

[b]Total feed utilization of live weight for seven week-old broilers

[c]Dry aquafeed/weight gain ratio

[d]Feed/weight gain ratio



Attempts Using Plant Breeding

- McMichael discovered a GLANDLESS mutant of cotton in 1954 (was grown by Hopi Indians of Arizona)
- Seed from glandless cotton was gossypol-free
- National and International breeding programs launched to transfer the trait to commercial varieties
- Animal nutrition studies showed glandless cottonseed to be a relatively good source of feed for pigs, chicken, and shrimp
- Glandless cottonseed was even considered fit for human consumption

Other Human Nutrition Studies

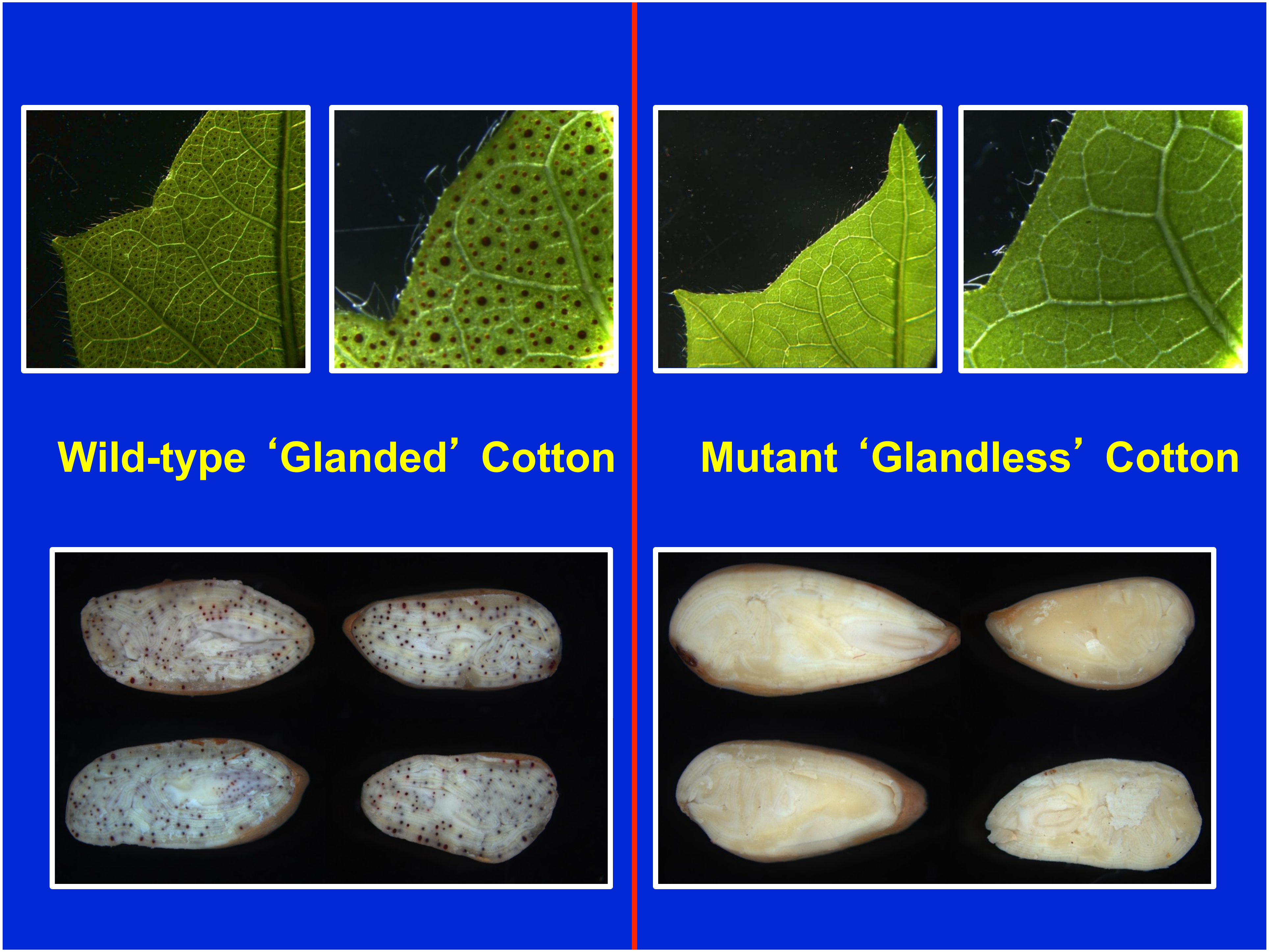
- **INCAPARINA containing 38% CSF used in human nutrition trials (INCAP - a cooperative International Institute established by Central American countries and Panama in the early 1950s)**
  - **Favorable acceptability and no adverse effects in 1-5 year-old children**
  - **Satisfied protein requirement of weanling children**

- **CSF + millet flour blends tested in West African countries**
  - **Blends provided adequate protein for normal as well as malnourished infants and children**

- **Texas Woman's University studies (Betty Alford)**
  - **CSM found safe and nutritious for children**
  - **Cottonseed protein in the form of liquid diets or baked products maintained nitrogen balance with no change in nutrition status in young, adult women**

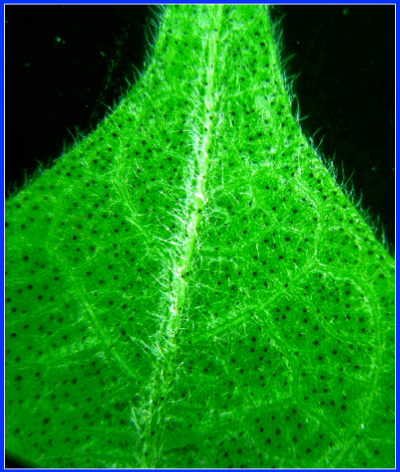
- **Texas Tech University**
  - **Tortillas made with 20-25% CSF improved Lysine and Tryptophan levels as well as leucine-isoleucine ratio**
  - **Improved shelf-life and good acceptability**

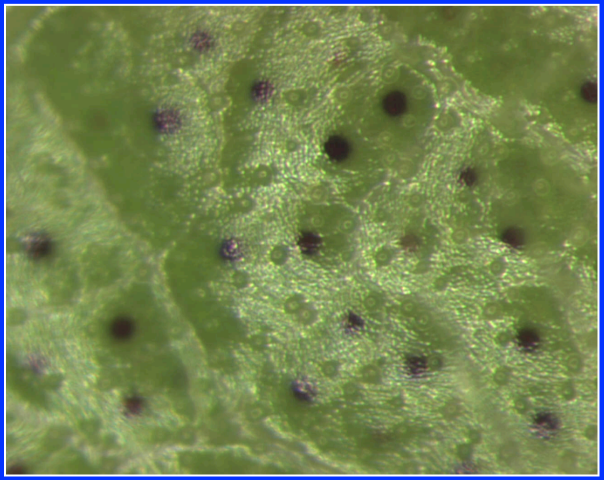
CSM: *Cottonseed Meal;* CSF: *Cottonseed Flour*

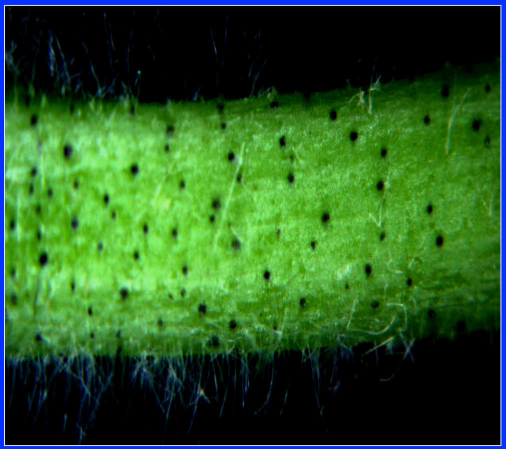

Wild-type ‘Glanded’ Cotton
Mutant ‘Glandless’ Cotton

Gossypol and other related terpenoid aldehydes present in the glands in leaves, stem, and floral parts confer resistance to insect pests

These terpenoids are also induced in response to pathogens and serve as phytoalexins







Traditional Insect Pests that Cause Greater Damage to Glandless Cotton

Cotton Bollworm (*Helicoverpa zea*)
Tobacco Budworm (*Heliothis virescens*)
Beet Armyworm (*Spodoptera exigua*)

Non-traditional Insects that Damage Glandless Cotton

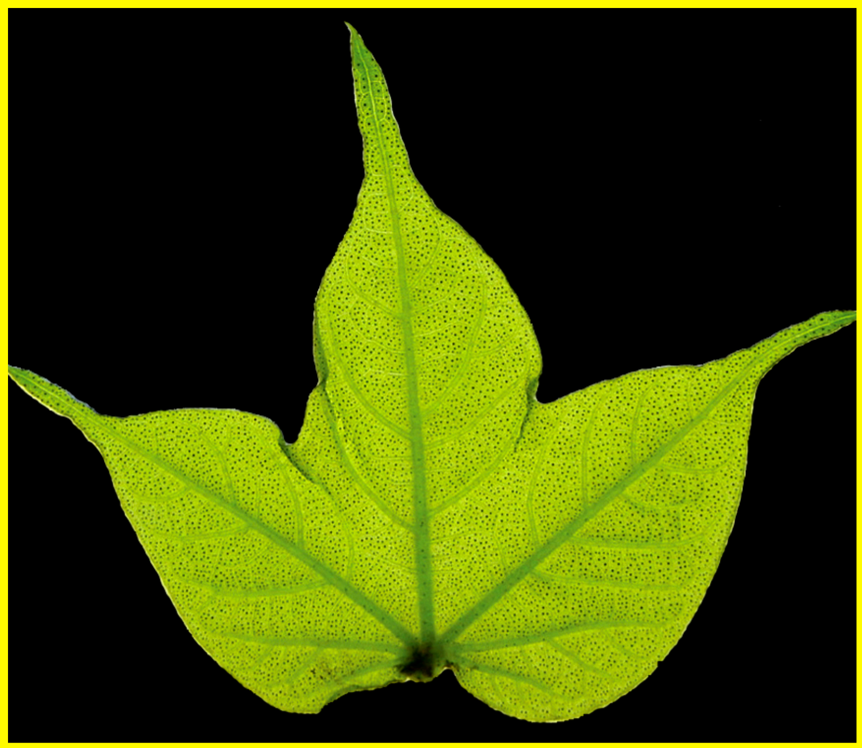
Black Fleahopper (*Spanogonicus albofasciatus*)
Pillbug (*Porcellia sp.*)
Spotted Cucumber Beetle (*Diabrotica undecimpunctata*)
Grape Colaspis (*Maecolaspis flavida*)
Green Dock Beetle (*Gastrophysa cyanea*)
Blister Beetle (*Epicauta vittata*)

- Currently, there is no large-scale cultivation of glandless cotton.

Desired Trait

Seeds:
Gossypol < 450 ppm

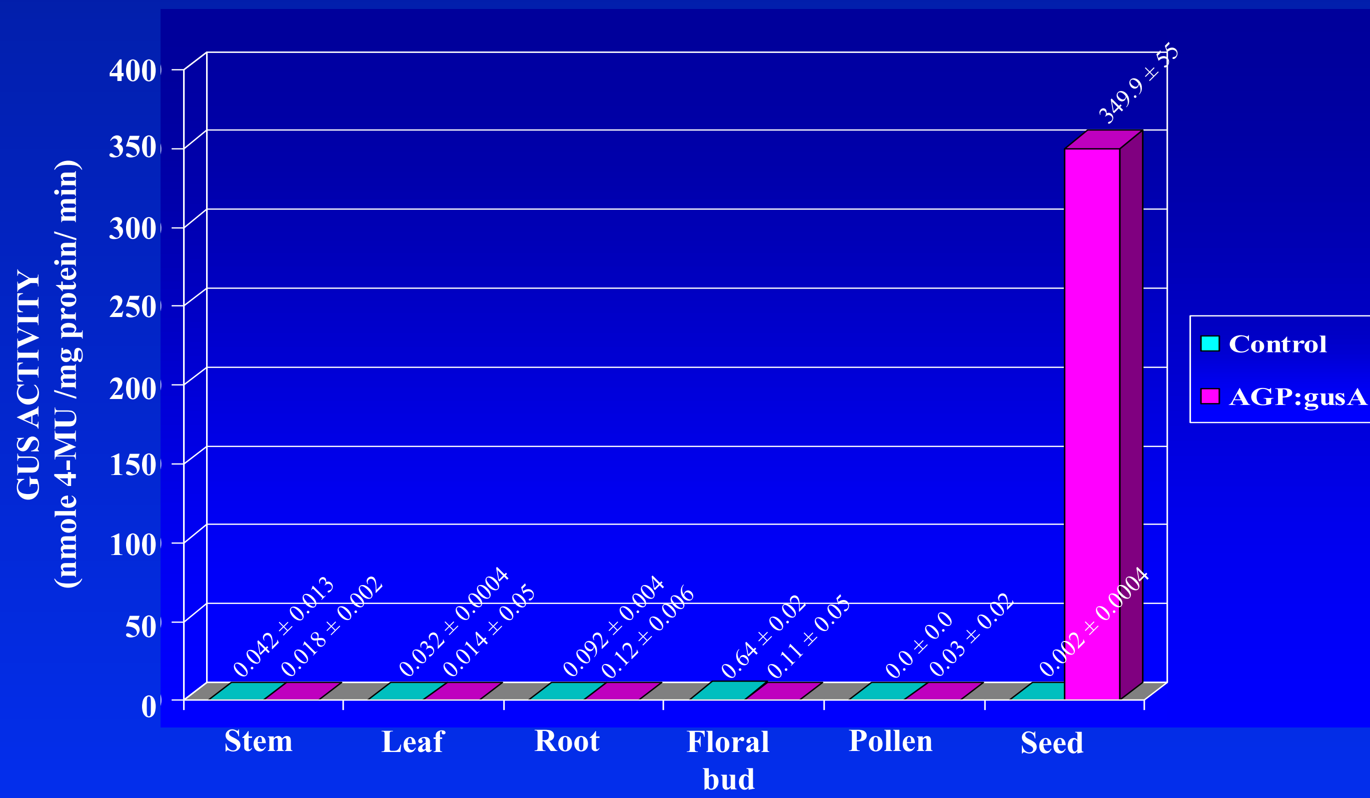
Leaf & other Organs:
Full Compliment of Gossypol and Related Terpenoids for Defense



Is it possible to Use Seed-specific, RNAi-mediated Gene Silencing to generate Ultra-low Gossypol Cottonseed (ULGCS) without Compromising Plants Defense Capabilities???

- Efficient Transformation System for Cotton
- δ-cadinene Synthase Gene
- Seed-specific, α-globulin Promoter
- A Gene Silencing Mechanism (RNAi)

Seed-specific expression of AGP::*gus*A in cotton

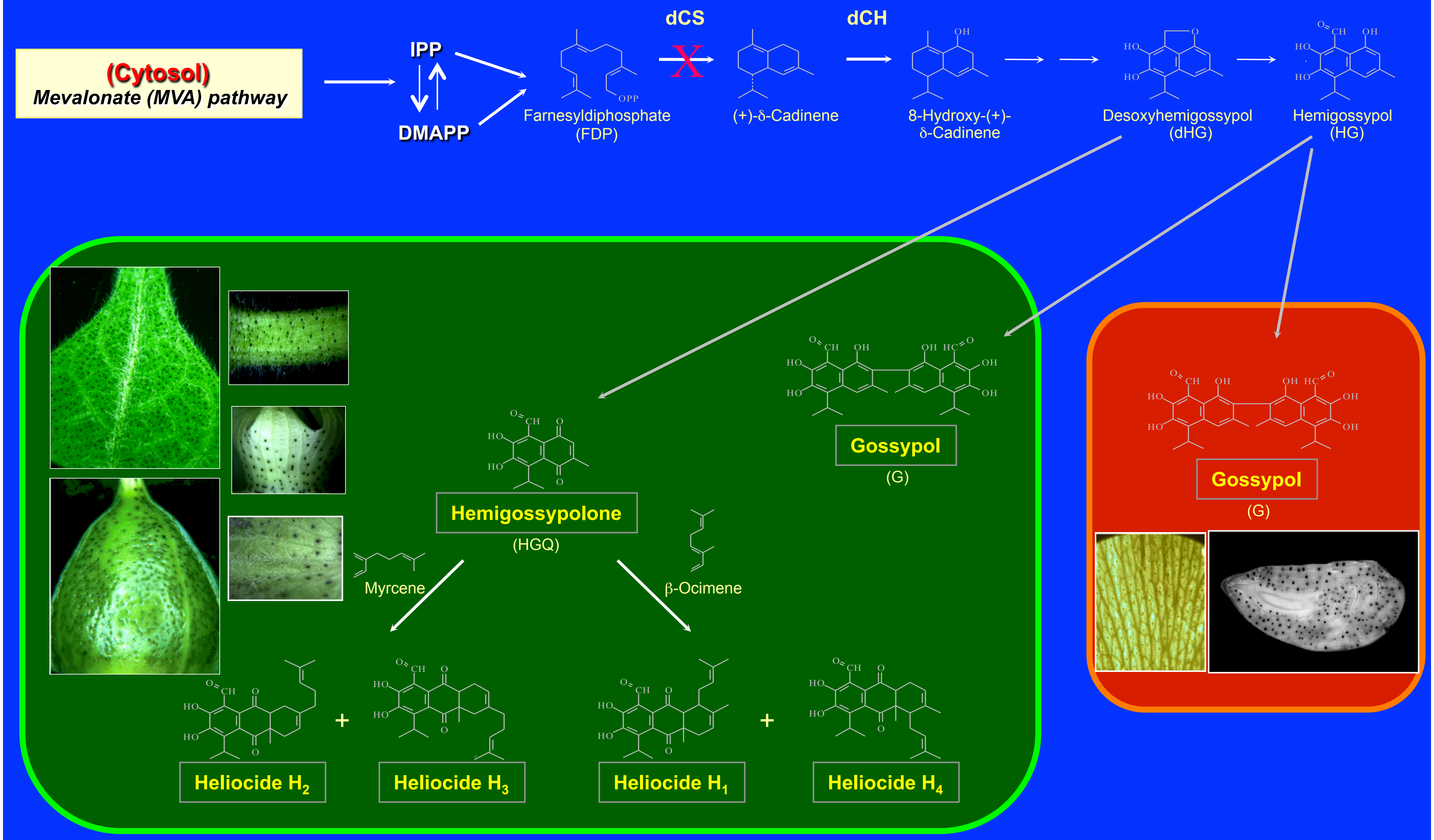


Sunilkumar …… Rathore (2002)

Transgenic Research 11: 347-359

U.S. Patent #7,626,081

Biosynthetic Pathway for Gossypol and Related Terpenoids



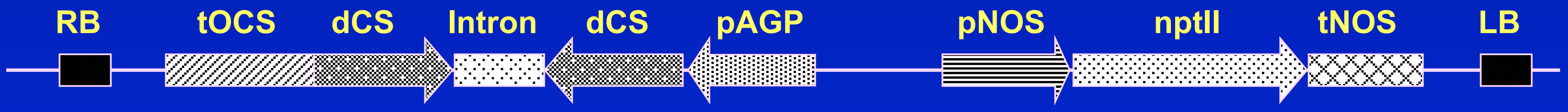
dCS = δ-cadinene synthase

dCH = δ-cadinene hydroxylase

Selection of trigger region for gene silencing

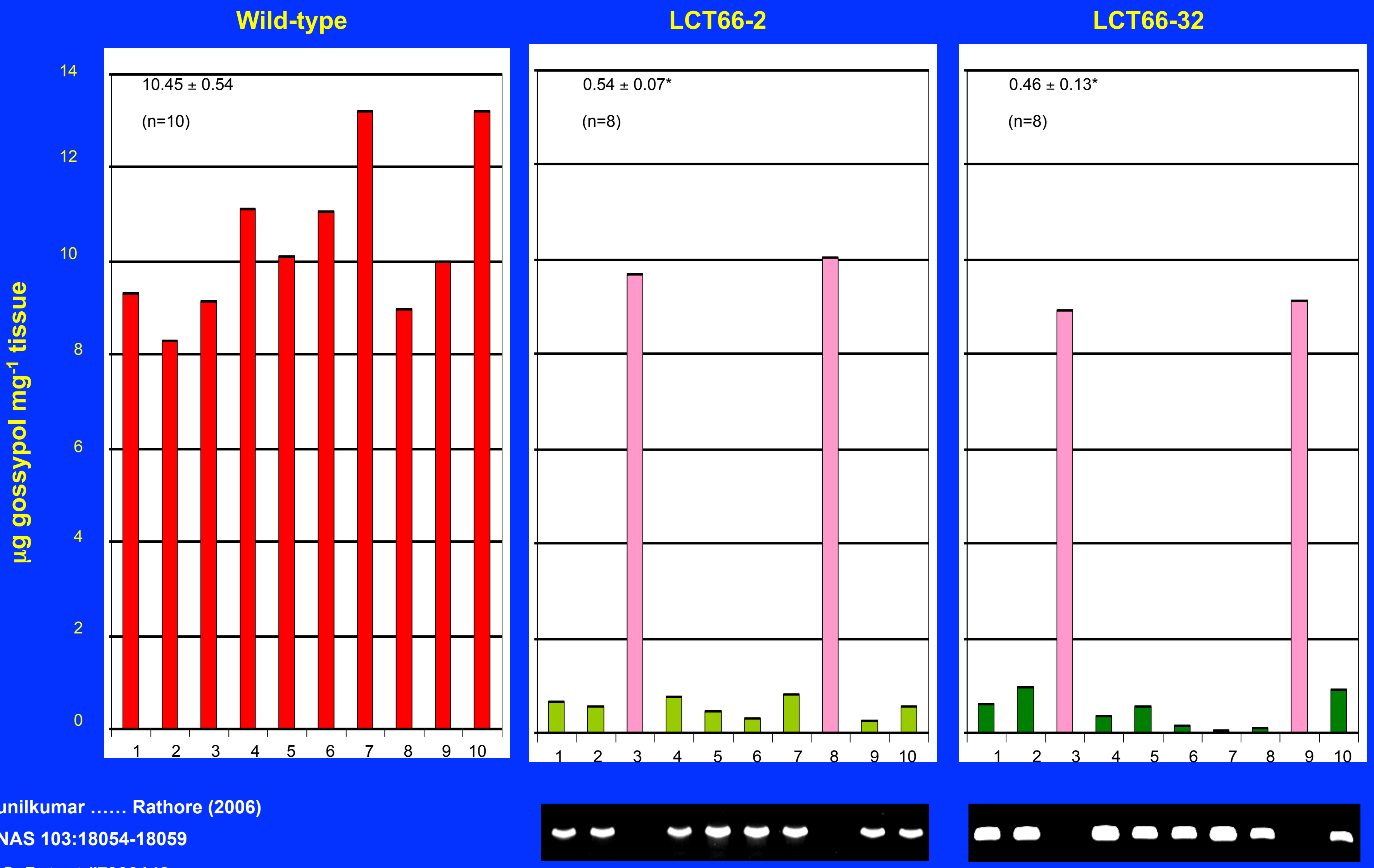
δ-cadinene synthase gene	Genbank accession no.	Plant source	Homology with the trigger sequence (%)	Homology with Complete Coding sequence (%)
Cad1-C14 (XC14)	U23205	*G. arboreum*	99.8	99.8 (1661/1665)
Cdn1-C4	AF270425	*G. hirsutum*	98.8	98.0 (1631/1665)
Cdn1	U88318	*G. hirsutum*	98.5	97.6 (1625/1665)
Cad1-C2	Y16432	*G. arboreum*	96.4	95.9 (1597/1665)
Cad1-C3	AF174294	*G. arboreum*	96.2	96.1 (1467/1527)
Cad1-C1 (XC1)	U23206	*G. arboreum*	96.0	96.0 (1599/1665)
Cad1-A	X96429	*G. arboreum*	80.9	81.4 (1348/1656)

Hairpin RNAi construct to silence δ-cadinene synthase gene



- Used pHannibal and pART27 vector systems developed by Waterhouse, CSIRO [Wesley *et al*. (2001) Plant J. 27: 581-590]
- *Agrobacterium* strain - LBA4404

Gossypol Levels in Individual Wild-type and T1 Cottonseeds

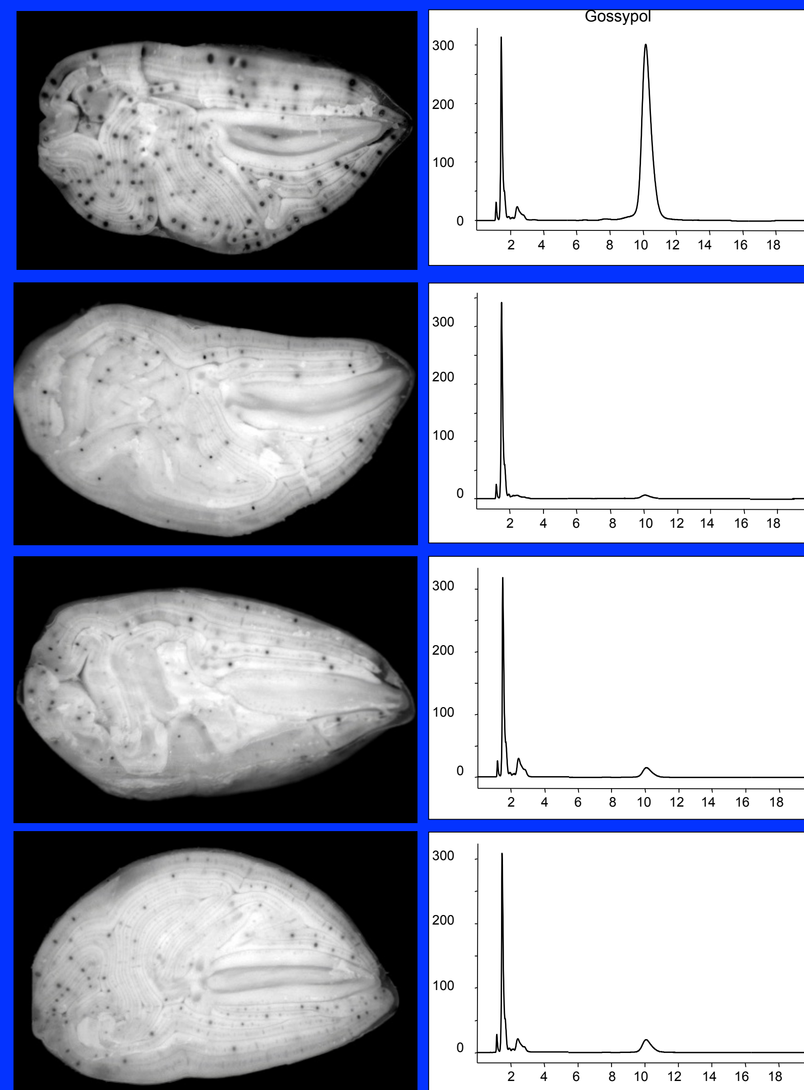


Sunilkumar …… Rathore (2006)

PNAS 103:18054-18059

U.S. Patent #7999148

T1 Cottonseeds from Line LCT66-32 and Individual HPLC Chromatograms for each Showing Gossypol Peaks

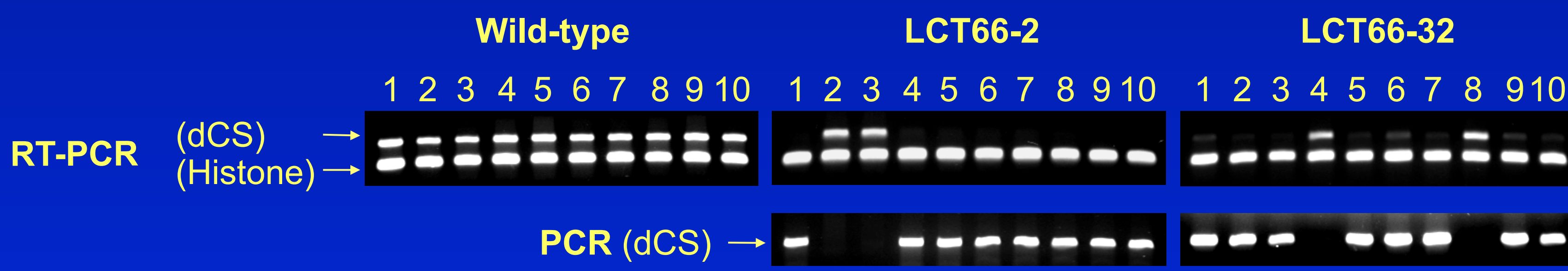


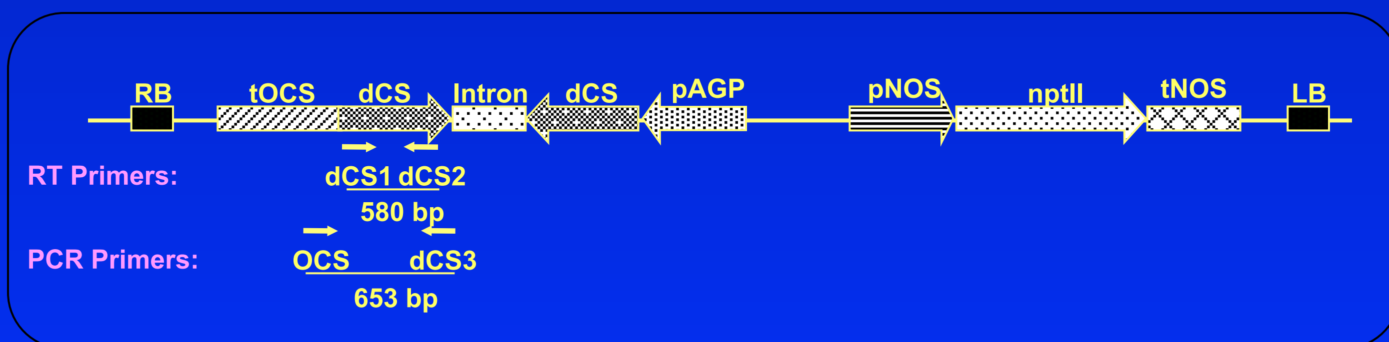
Sunilkumar …… Rathore (2006)

PNAS 103:18054-18059

U.S. Patent #7999148

Levels of δ-cadinene Synthase Transcripts in Individual, Wild-type and T1 Transgenic Embryos at 35 dpa

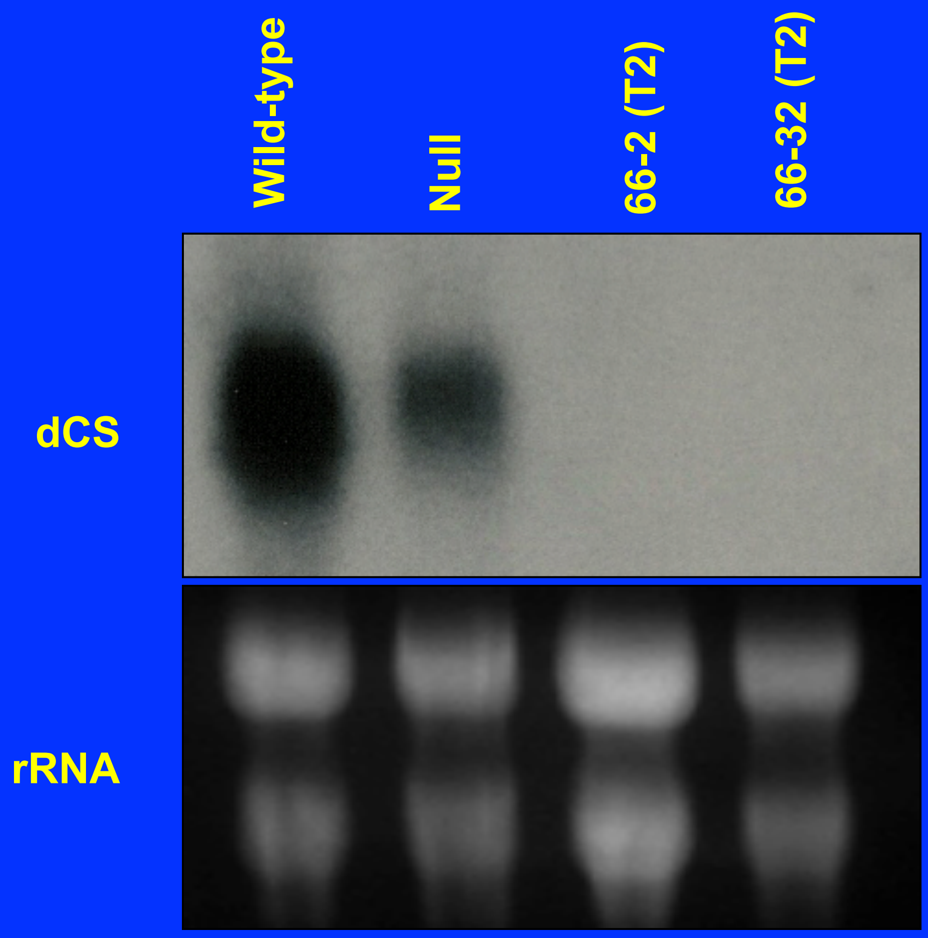




Sunilkumar …… Rathore (2006)

PNAS 103:18054-18059

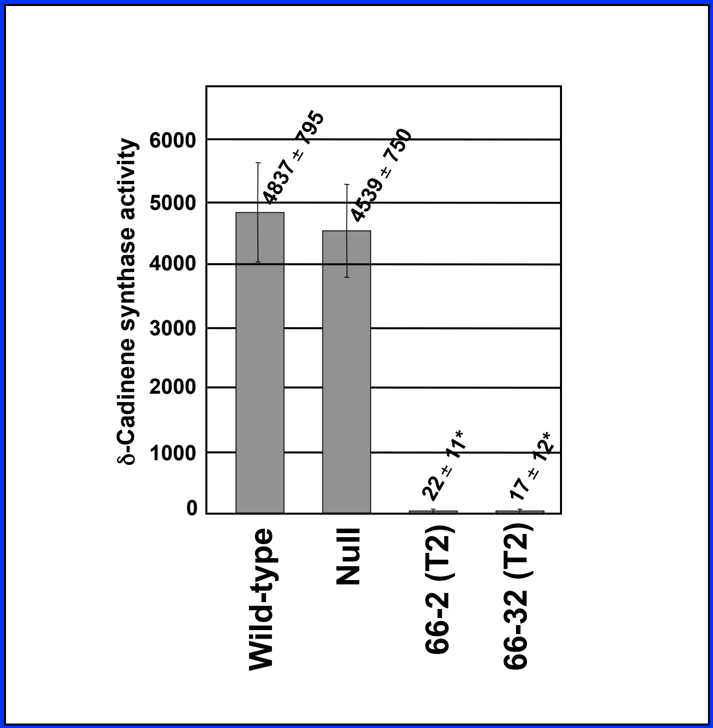
δ-Cadinene Synthase Transcripts in Embryos (35 dpa) from Wild-type, Null Segregant and Transgenic T1 Plants



Sunilkumar …… Rathore (2006)

PNAS 103:18054-18059

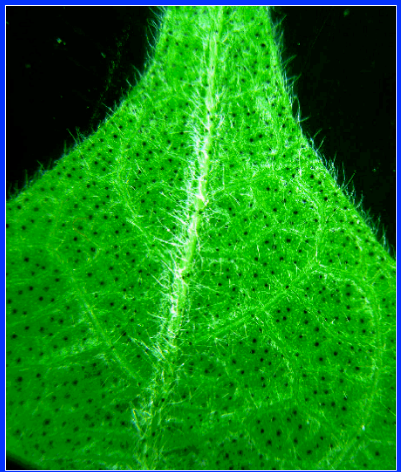
δ-Cadinene Synthase Activities in Embryos (35 dpa) from Wild-type, Null Segregant and Transgenic T1 Plants

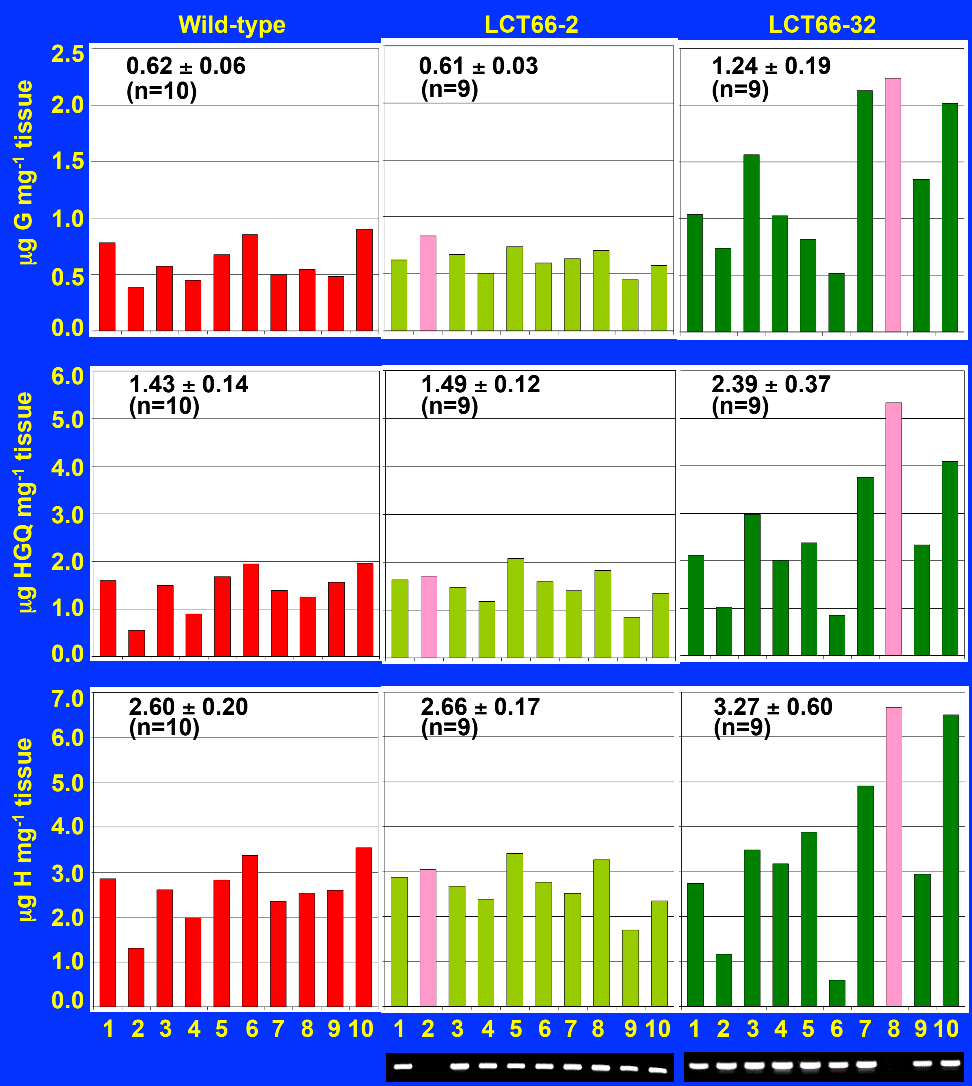


Sunilkumar …… Rathore (2006)

PNAS 103:18054-18059

Levels of Gossypol, Hemigossypolone, and Heliocides in Leaf Tissues from Individual Wild-type and Transgenic T1 Progeny Plants



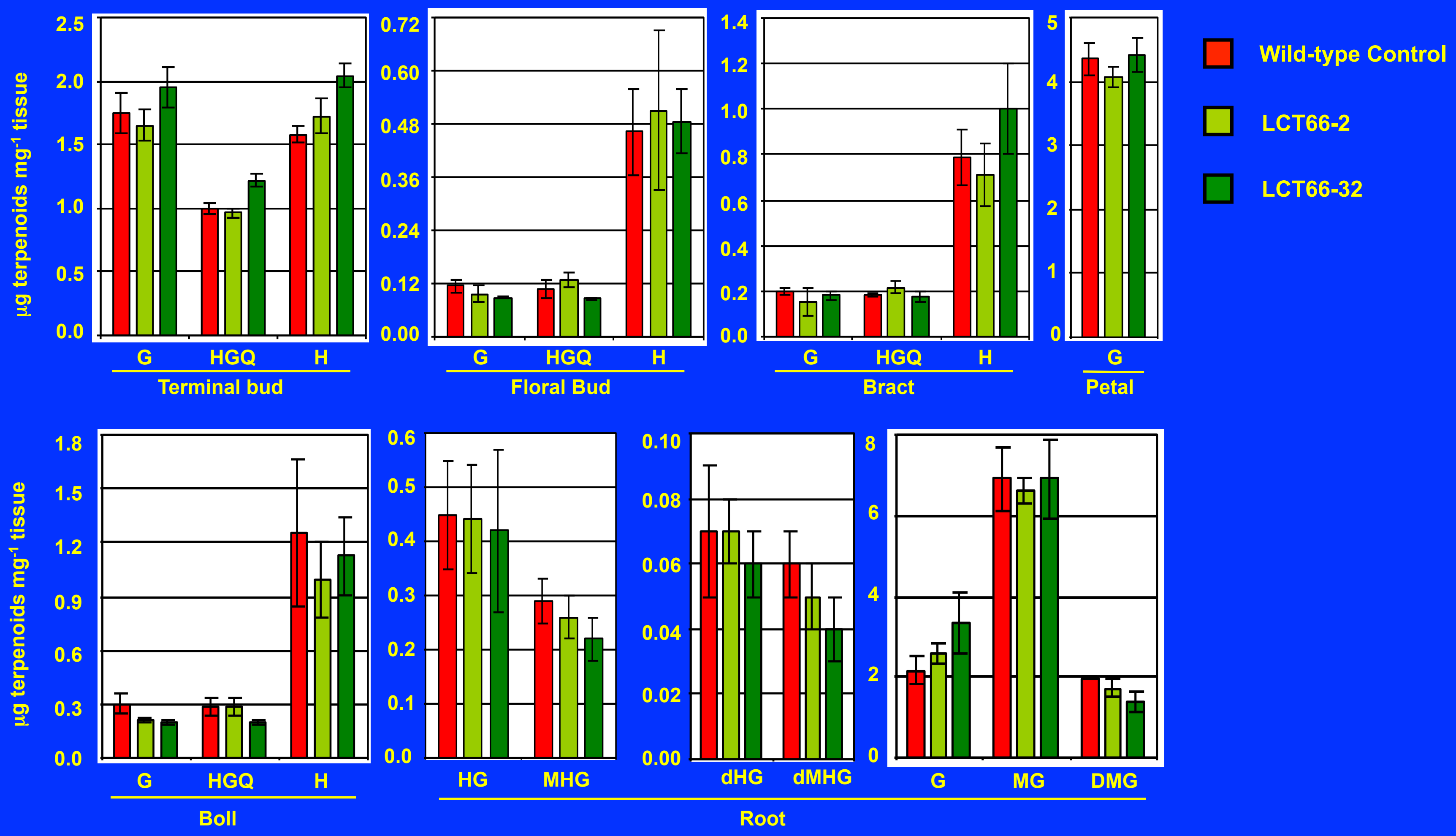


Sunilkumar …… Rathore (2006)

PNAS 103:18054-18059

U.S. Patent #7999148

Levels of Gossypol and Related Terpenoids in Terminal Bud, Floral Bud, Bract, Petal, Boll, and Root Tissues



ULGCS trait is completely tissue specific

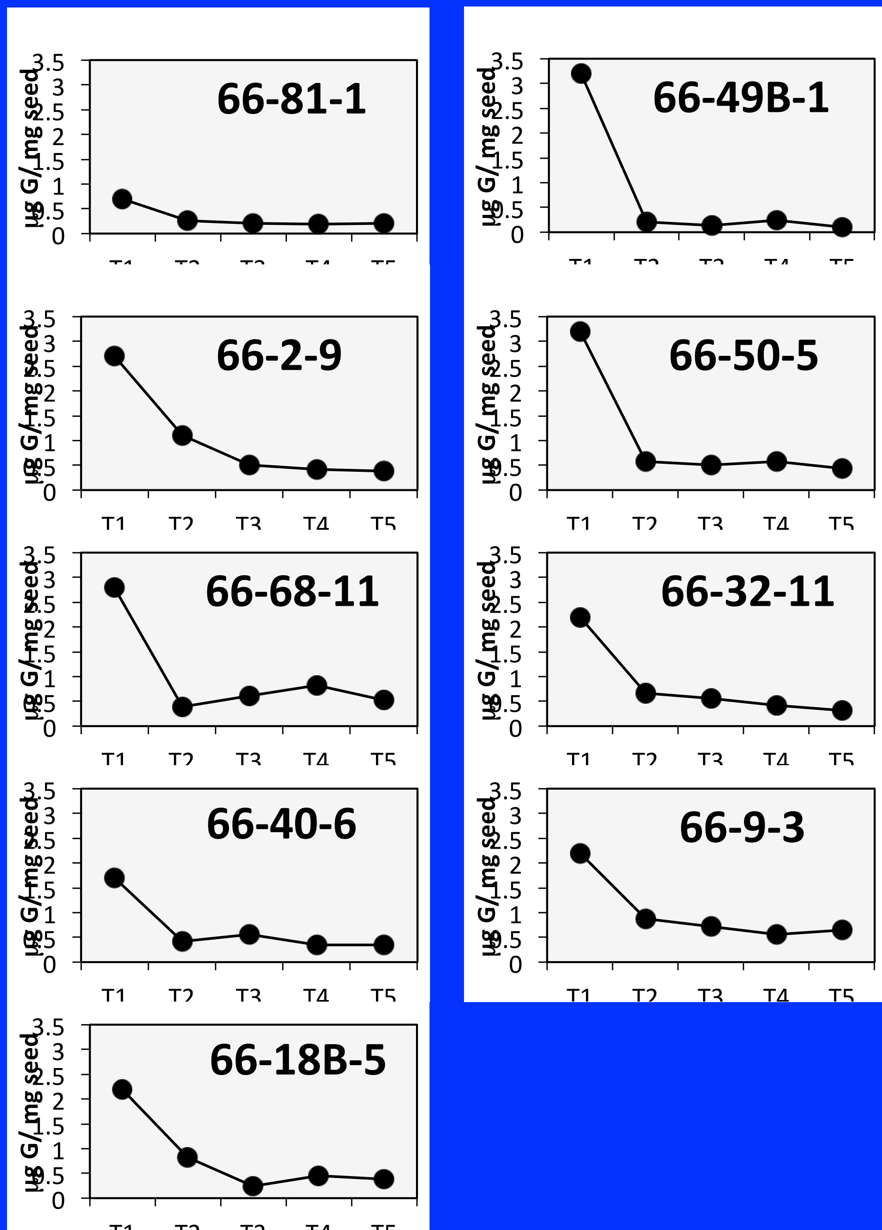
Sunilkumar …… Rathore (2006)

PNAS 103:18054-18059

U.S. Patent #7999148

- Is the RNAi-mediated ULGCS trait stable?

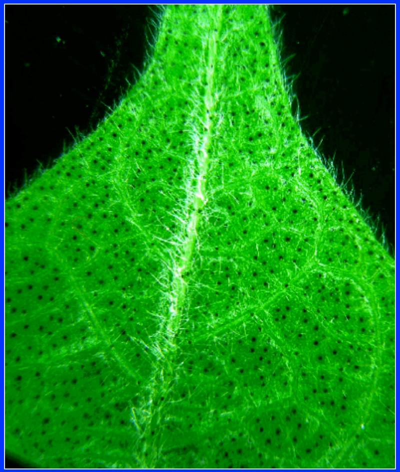
Levels of gossypol (G) in T1, T2, T3, T4, and T5 seeds obtained from greenhouse-grown cotton plants

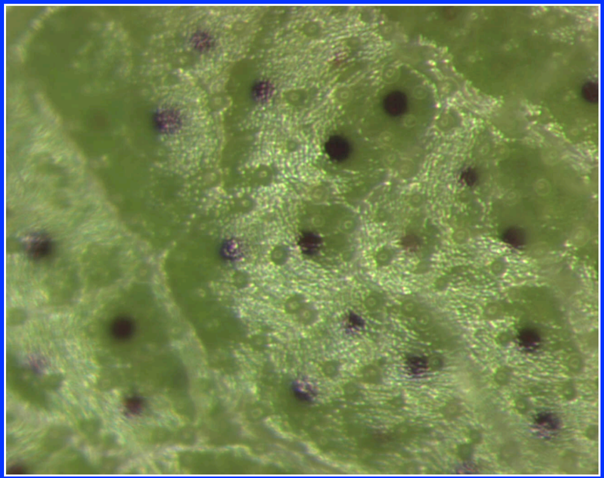


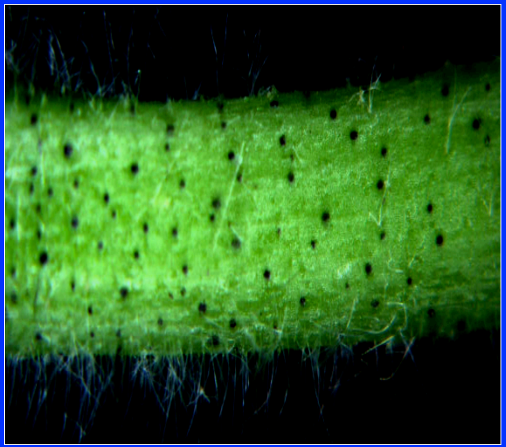
RNAi-mediated, ULGCS Trait is Stable and Heritable

Rathore et al. Plant Biotech. J. (2012)

Gossypol and related terpenoids are also induced in response to pathogens and serve as phytoalexins

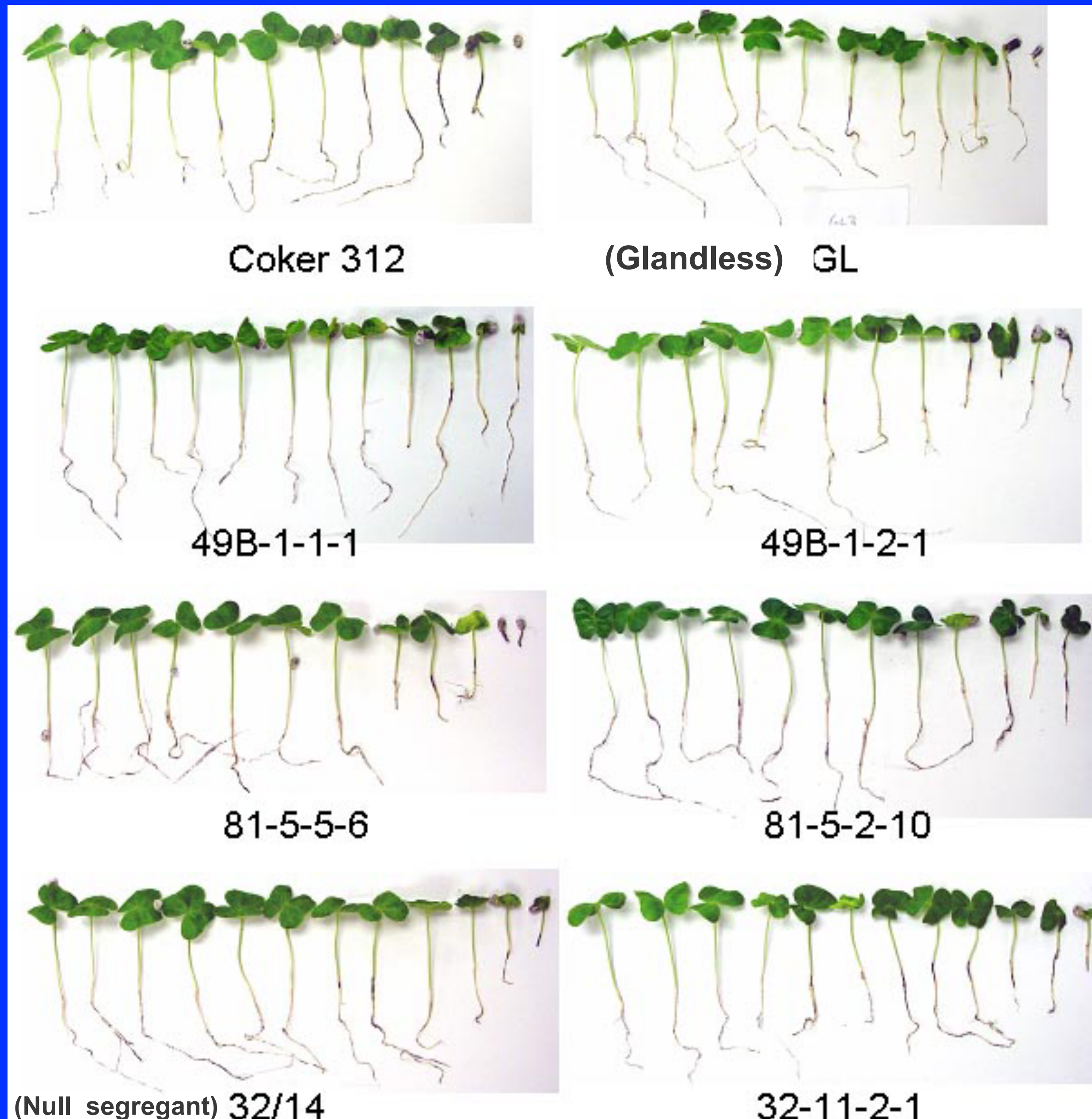






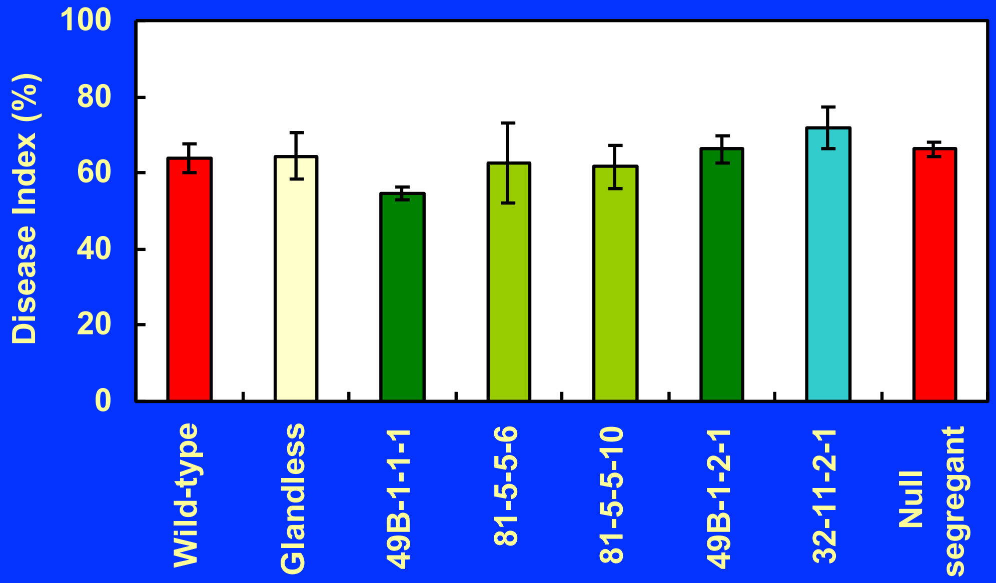
- Are the very young seedlings from RNAi lines more susceptible to cotton diseases?
- Are the very young seedlings from RNAi lines capable of launching terpenoid-mediated defense response when challenged with a pathogen?

Cotton seedlings after five days of growth on *Rhizoctonia solani*-infested soil



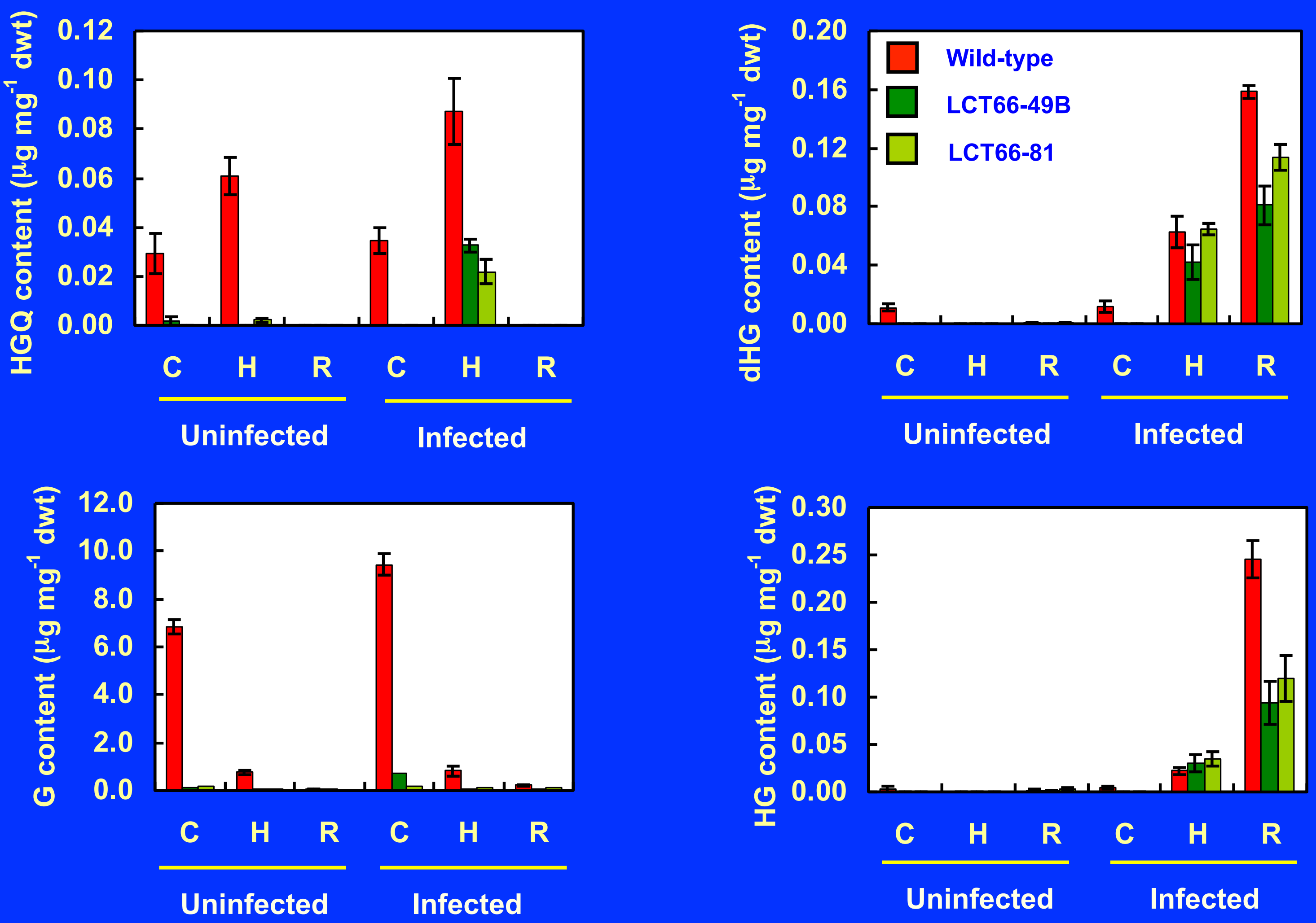
Rathore et al. Plant Biotech. J. (2012)

Susceptibility of the RNAi lines to *Rhizoctonia solani* is not greater than that of the wild-type plants



Rathore et al. Plant Biotech. J. (2012)

RNAi lines are Capable of Launching Terpenoid-based Defense in Response to *Rhizoctonia solani* infection of Roots

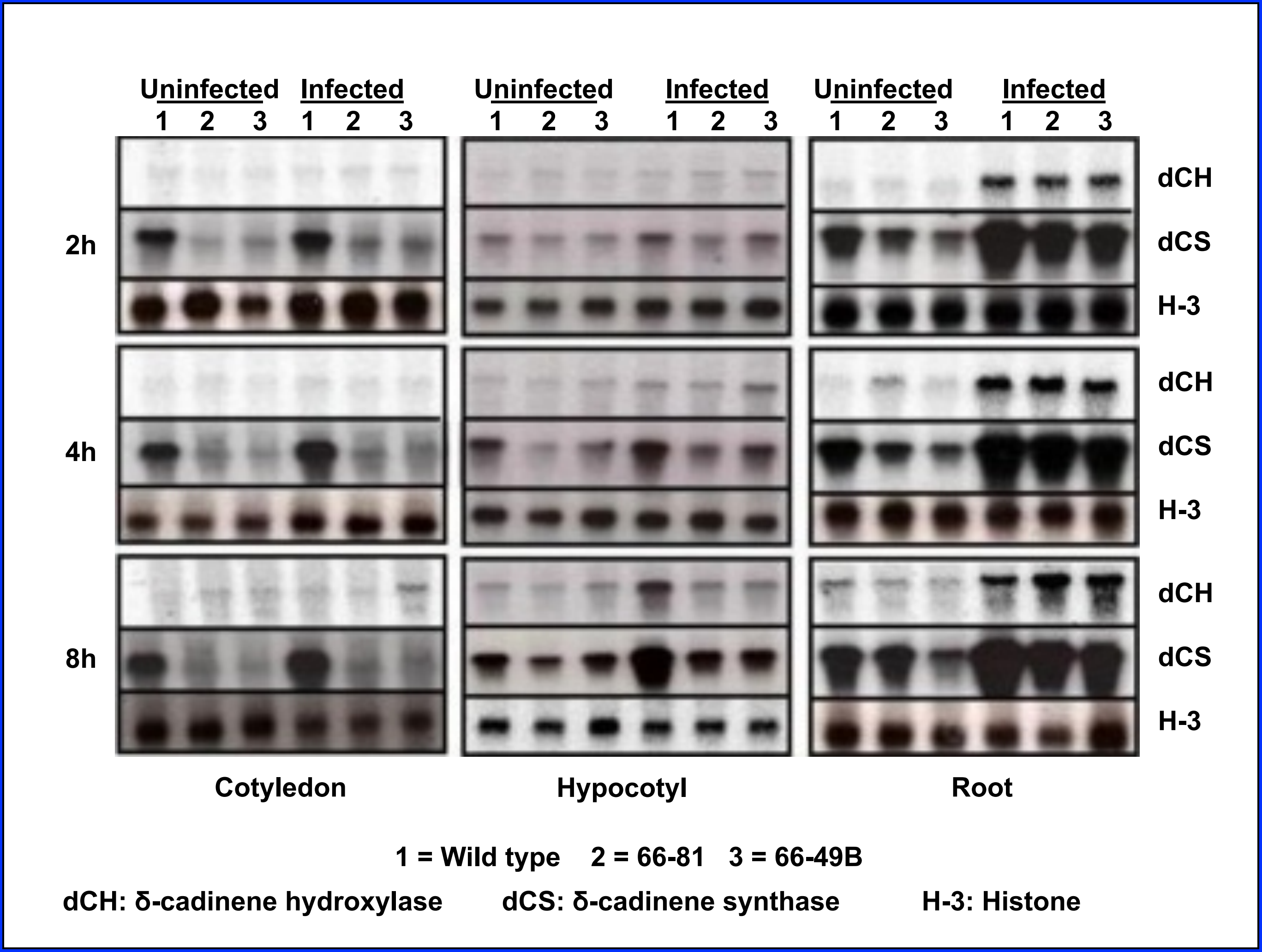


R: Root; H: Hypocotyl; C: Cotyledon

HGQ: Hemigossypolone; G: Gossypol; DHG: Desoxyhemigossypol; HG: Hemigossypol

Rathore et al. Plant Biotech. J. (2012)

δ-Cadinene Synthase (dCS) Transcripts are Induced in the Roots and Hypocotyl of RNAi Lines in Response to a Challenge from *Rhizoctonia solani*



Rathore et al. Plant Biotech. J. (2012)

- Young ULGCS seedlings are capable of launching terpenoid-based defense response

- δ-cadinene synthase gene is induced in ULGCS seedlings in response to a pathogen challenge

- The basic defense mechanism remains functional in the ULGCS seedlings

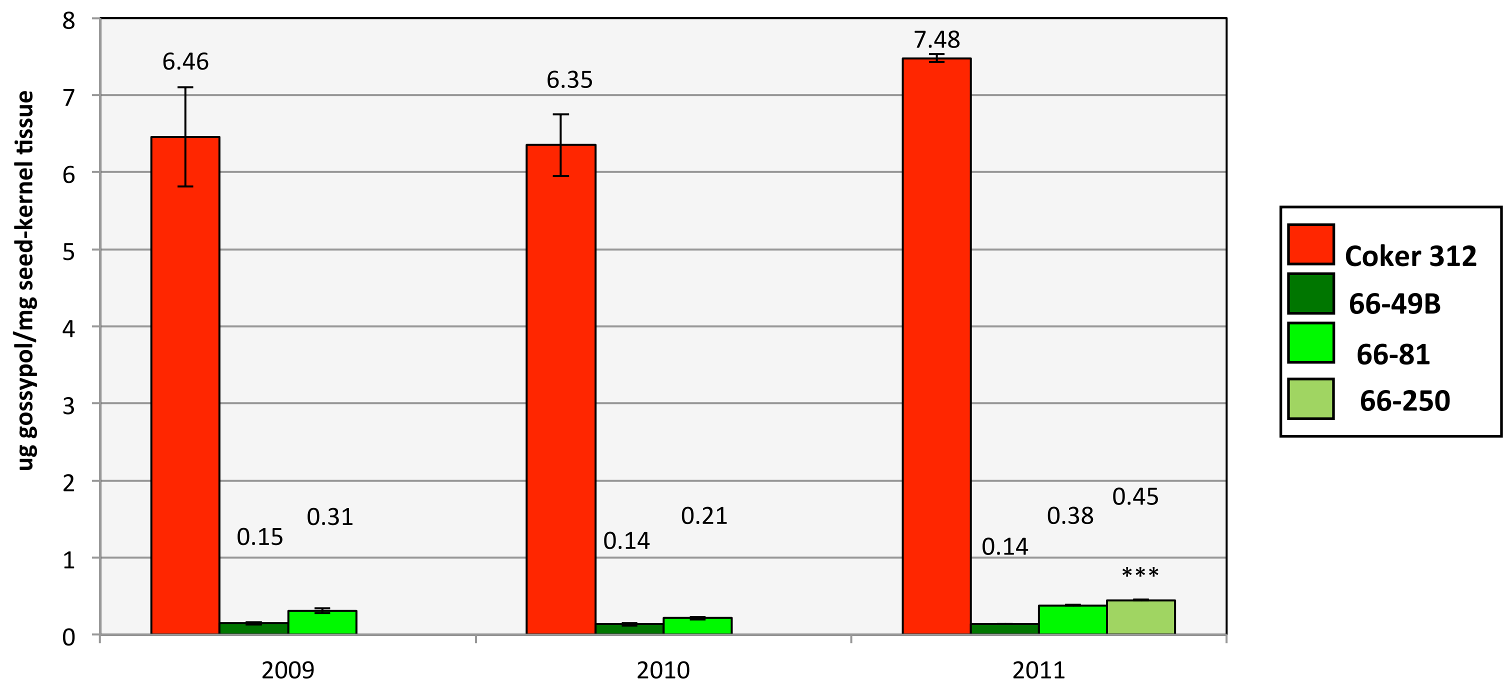
Do RNAi lines maintain the ULGCS trait under field conditions?

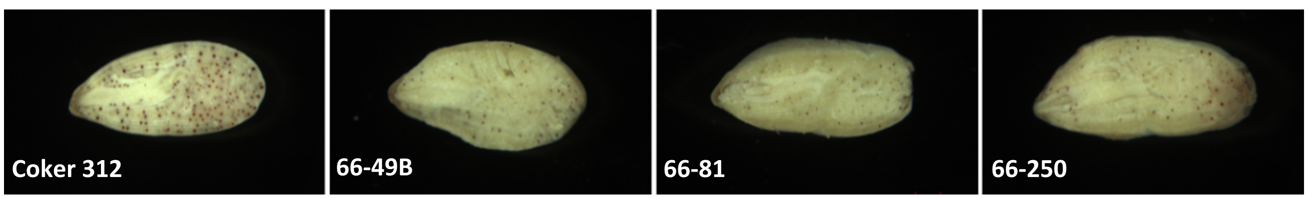
Do RNAi lines maintain normal levels of defensive terpenoids in the non-seed parts of the plant under field conditions?

Ultra-low Gossypol Cottonseed (ULGCS) – Field Trials 2009, 2010 & 2011

- ULGCS Stability
- ULGCS Specificity
- Fiber/seed Yield & Quality

Seed gossypol content of field-grown plants





ULGCS Trait is stable under field conditions

Palle ----- Rathore Plant Biotech. J. (2013)

Levels (μg/mg dry weight) of gossypol and related terpenoids

ULGCS Foliage & Floral Tissues Retain Normal Levels of Terpenoids

		2009			2010			2011			
		Coker 312	66-49B	66-81	Coker 312	66-49B	66-81	Coker 312	66-49B	66-81	66-250
Bracts	G	0.08 (±0.008)	0.06 (±0.003)	0.11 (±0.011)	0.11 (±0.01)	0.10 (±0.03)	0.12 (±0.02)	0.08 (±0.00)	0.06 (±0.00)	0.16 (±0.02)	0.11 (±0.00)
	HGQ	0.15 (±0.02)	0.10 (±0.012)	0.10 (±0.014)	0.17 (±0.04)	0.12 (±0.04)	0.11 (±0.03)	0.25 (±0.04)	0.24 (±0.02)	0.32 (±0.08)	0.38 (±0.02)
	H1-H4	1.25 (±0.05)	0.89 (±0.06)	1.05 (±0.10)	1.78 (±0.32)	1.59 (±0.46)	1.44 (±0.32)	1.51 (±0.25)	1.07 (±0.59)	1.75 (±0.47)	1.82 (±0.09)
	G	5.65 (±0.42)	5.04 (±0.39)	6.41 (±0.91)	3.15 (±0.28)	3.14 (±0.43)	3.47 (±0.19)	3.09 (±0.10)	3.17 (±0.05)	4.01 (±0.08)	3.93 (±0.07)
Floral buds	HGQ	1.11 (±0.14)	0.96 (±0.11)	0.88 (±0.23)	0.45 (±0.09)	0.36 (±0.06)	0.40 (±0.07)	0.51 (±0.06)	0.61 (±0.09)	0.66 (±0.02)	0.87 (±0.04)
	H1-H4	2.39 (±0.09)	1.99 (±0.22)	1.90 (±0.40)	1.47 (±0.32)	1.46 (±0.19)	0.90 (±0.03)	1.89 (±0.13)	2.07 (±0.19)	2.30 (±0.13)	2.40 (±0.12)
	G	1.04 (±0.30)	0.99 (±0.26)	0.82 (±0.03)	1.02 (±0.03)	0.98 (±0.24)	1.15 (±0.12)	0.92 (±0.14)	1.15 (±0.08)	1.32 (±0.31)	1.17 (±0.19)
Terminal part of axillary branch	HGQ	1.62 (±0.16)	1.22 (±0.19)	1.15 (±0.17)	1.24 (±0.06)	0.98 (±0.05)	1.12 (±0.09)	2.22 (±0.21)	1.89 (±0.19)	1.96 (±0.27)	2.27 (±0.12)
	H1-H4	2.84 (±0.27)	1.77 (±0.23)	2.43 (±0.40)	1.64 (±0.14)	1.74 (±0.25)	1.94 (±0.17)	3.19 (±0.43)	2.70 (±0.11)	2.91 (±0.21)	3.67 (±0.16)
	G	0.34 (±0.08)	0.19 (±0.02)	0.48 (±0.13)	0.80 (±0.01)	0.43 (±0.04)	0.97 (±0.04)	0.65 (±0.09)	0.62 (±0.04)	1.12 (±0.12)	0.63 (±0.01)
Leaves	HGQ	1.66 (±0.49)	0.78 (±0.06)	1.37 (±0.37)	2.25 (±0.23)	1.25 (±0.09)	2.09 (±0.08)	4.04 (±0.40)	3.73 (±0.30)	4.38 (±0.18)	3.57 (±0.15)
	H1-H4	2.68 (±0.36)	1.77 (±0.23)	2.46 (±0.76)	1.81 (±0.38)	0.91 (±0.07)	1.14 (±0.09)	2.94 (±0.25)	2.77 (±0.59)	3.51 (±0.47)	2.57 (±0.11)
	G	1.69 (±0.09)	1.37 (±0.08)	1.88 (±0.07)	2.02 (±0.24)	1.22 (±0.05)	1.47 (±0.06)	1.06 (±0.06)	0.86 (±0.02)	1.04 (±0.06)	0.89 (±0.01)
2 day bolls	HGQ	7.29 (±0.05)	6.43 (±0.23)	6.58 (±0.41)	4.29 (±0.28)	3.69 (±0.24)	3.05 (±0.05)	4.95 (±0.41)	4.57 (±0.26)	3.98 (±0.25)	4.32 (±0.03)
	H1-H4	11.03 (±0.14)	9.05 (±0.54)	7.64 (±0.39)	11.27 (±3.58)	9.96 (±1.88)	5.81 (±1.31)	8.66 (±0.73)	8.74 (±0.54)	5.51 (±0.60)	7.62 (±0.38)
Petals	G	5.70 (±0.15)	6.28 (±0.22)	6.25 (±0.25)	4.77 (±0.26)	5.92 (±0.13)	5.97 (±0.05)	3.60 (±0.19)	3.78 (±0.30)	4.15 (±0.18)	4.22 (±0.14)

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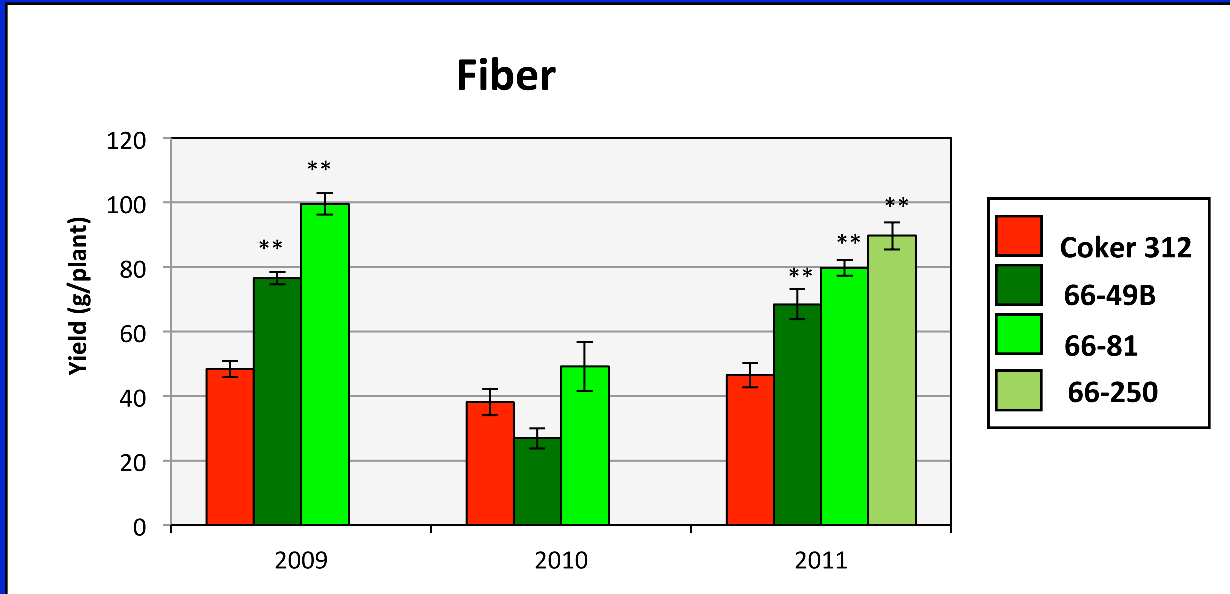
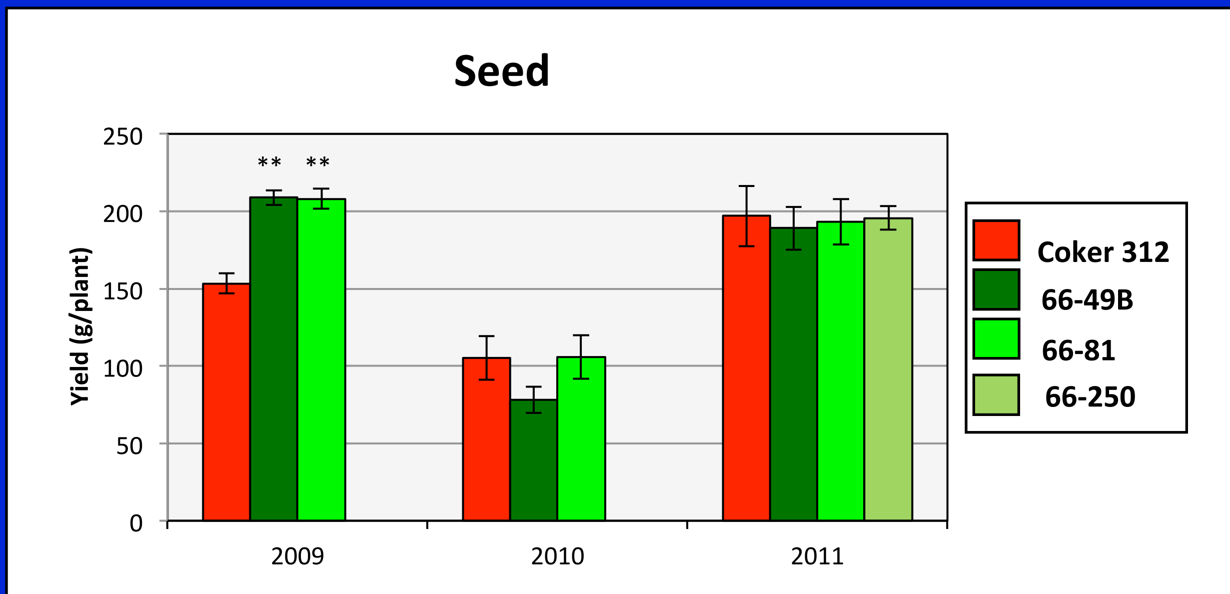
Yield data for field-grown plants





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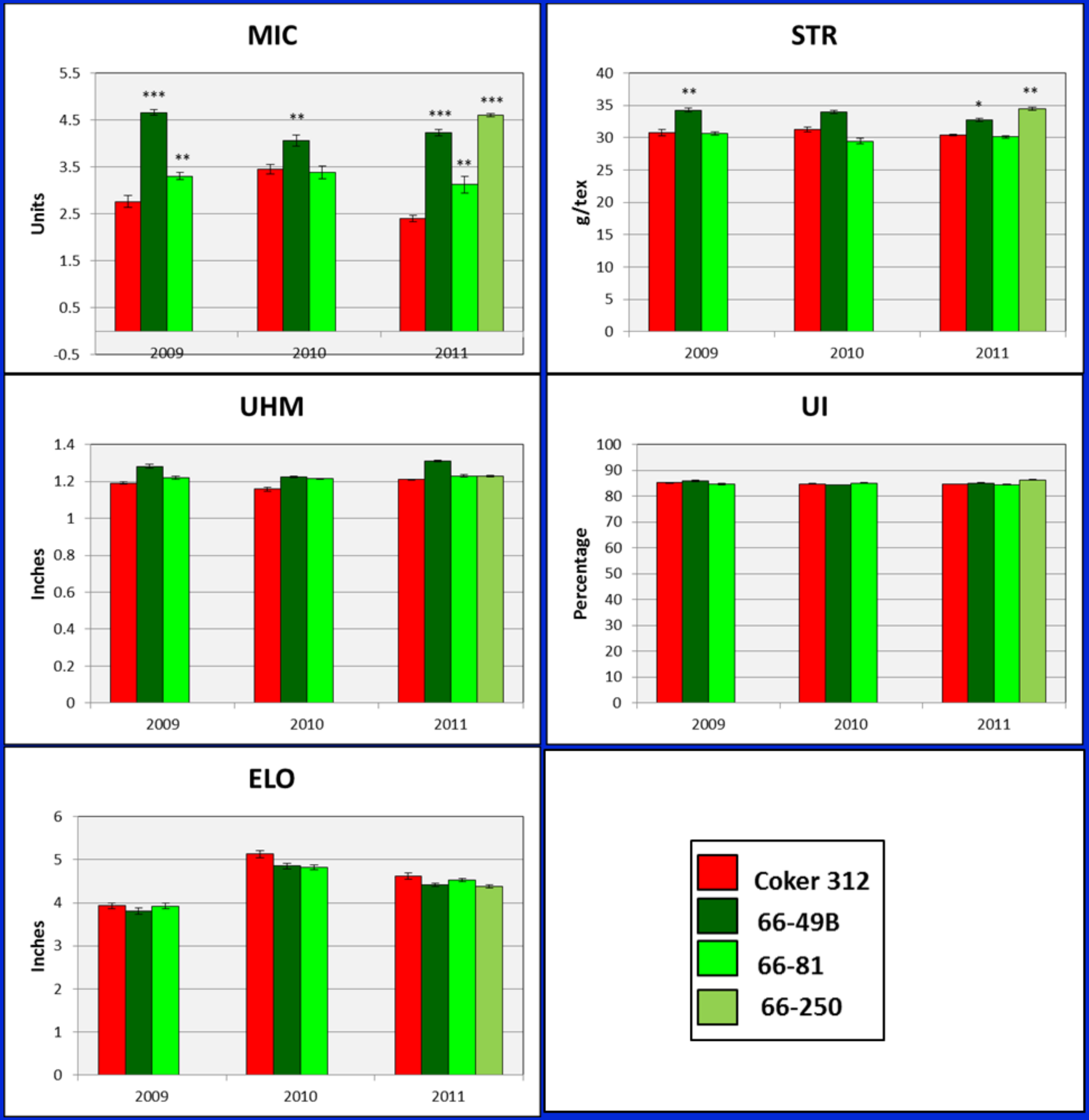
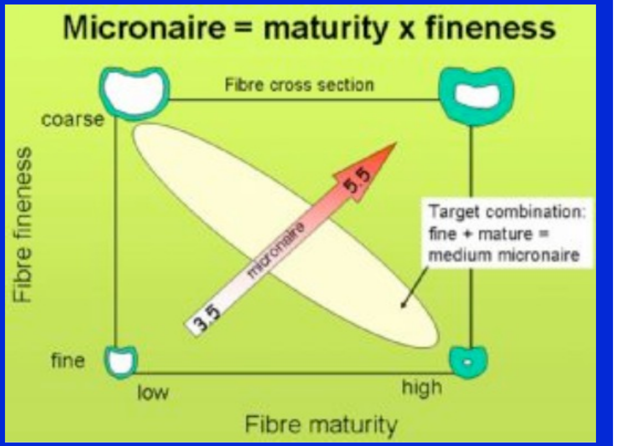
Fiber Quality under Field Conditions





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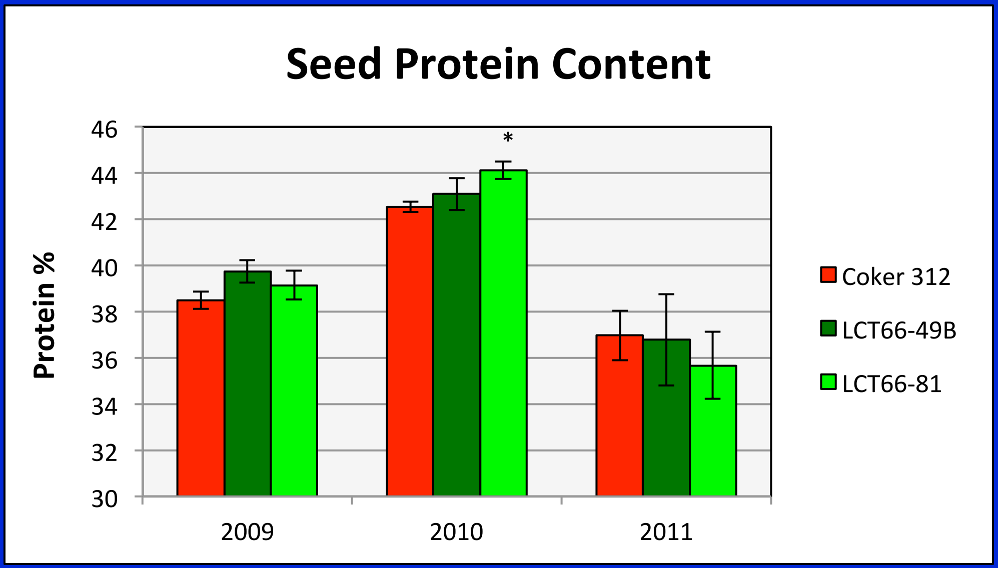
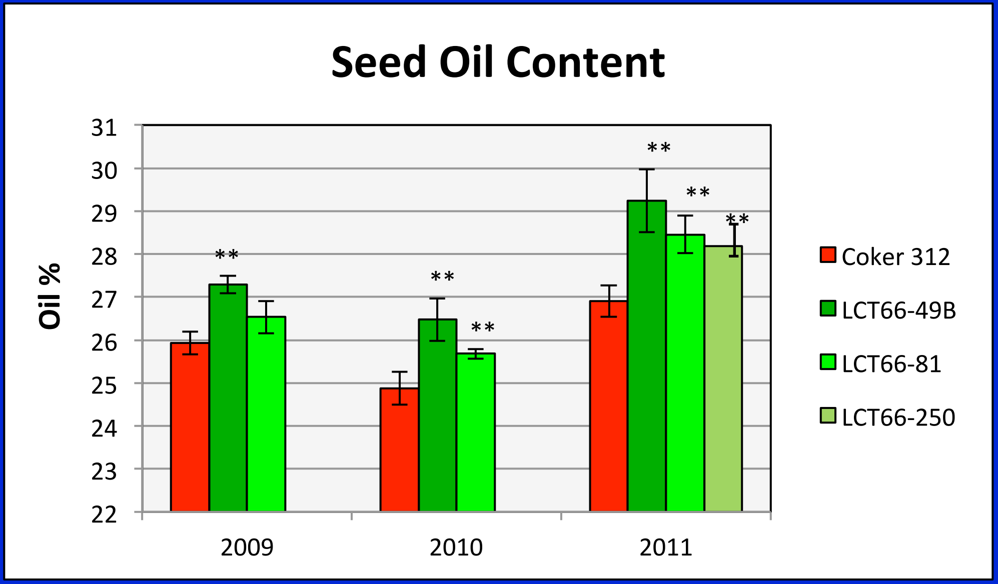
Protein and Oil levels in seeds of plants grown under field conditions





Note that the protein values were measured in seed kernels, while oil content was determined in whole seeds.
Data represent mean ± SE, *P<0.05; **P<0.01, n= 3.

Palle ----- Rathore Plant Biotech. J. (2013)

Compositional Analysis of Acid-delinted Cottonseed from Field (Dry Weight Basis)

Component (%)	LCT66-49B	LCT66-81	Coker 312	Acala GLS	DP 50
Moisture (fresh weight basis)	8.40	9.64	9.05	8.18	5.42
Protein	29.6	29.8	29.5	27.4	21.4
Total Fat	20.5	22.0	20.1	25.3	23.0
Ash	4.05	4.49	4.72	4.35	4.60
Carbohydrates	45.9	43.7	45.7	42.9	51.0
Phytic acid	1.80	1.75	2.08	2.04	1.85
Free gossypol (%)	0.0388	0.0284	0.671	0.00942	0.830
Total gossypol (%)	0.0479	0.0400	0.750	0.0150	0.996

Oil content (NMR)	24.69 ± 0.10	23.45 ± 0.17	22.89 ± 0.16
Total gossypol (%, HPLC, kernels)	0.0147 ± 0.0064	0.0312 ± 0.0026	0.6462 ± 0.0337

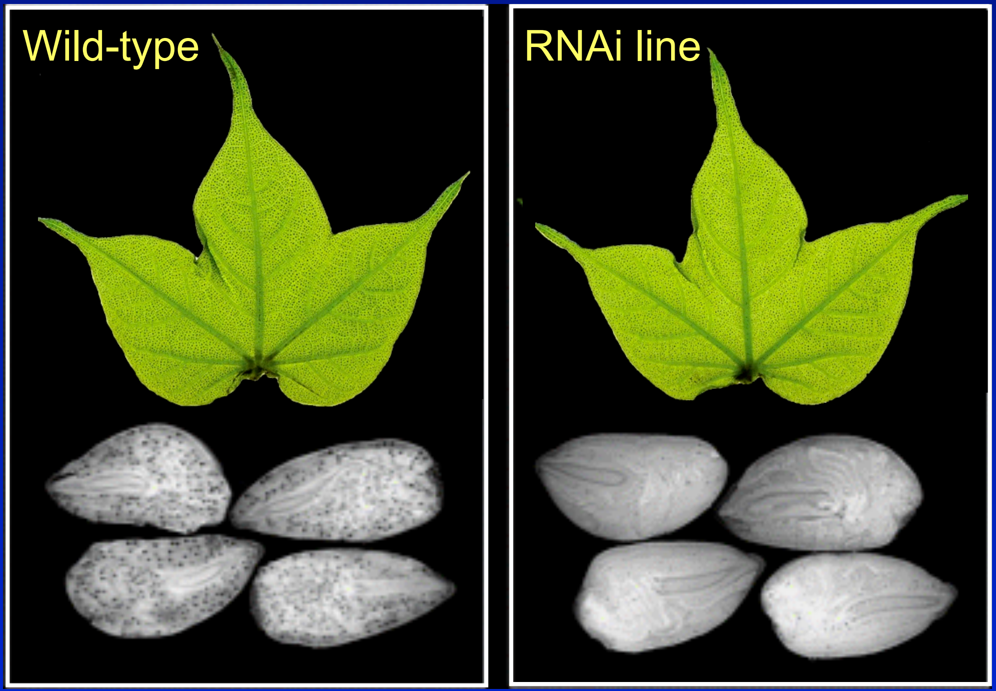
Palle ----- Rathore Plant Biotech. J. (2013)

- **ULGCS trait is stable under field conditions**

- **Fiber- and seed-yields are not negatively impacted in the ULGCS lines**

- **Fiber-quality is not negatively impacted in the ULGCS lines**

- **With the exception of ultra-low levels of gossypol, no other major differences were observed in terms of seed composition**



- Some of the RNAi lines have seed-gossypol levels less than 200 ppm (FDA: < 450 ppm and FAO/WHO: < 600 ppm).
- Non-seed tissues DO NOT show reduction in the levels of gossypol and other defensive terpenoids. Upon germination, newly formed tissues of the plantlet produce normal levels of terpenoids.
- Trait is heritable (T1 –T8 generations) and stable under field conditions.
- Pathogen-induced terpenoid biosynthesis pathway remains functional and the RNAi lines do not show higher susceptibility to pathogens or insects.

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