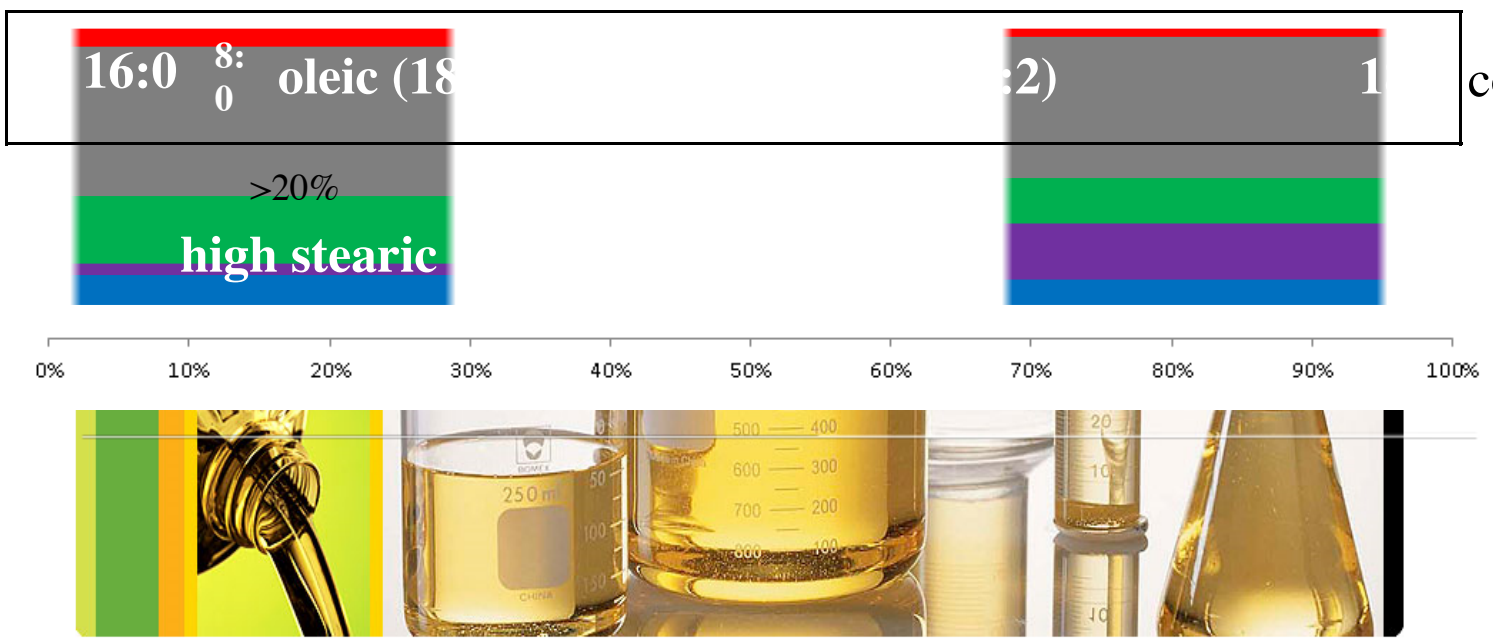
Stearic acid project final report
USB Project # 1420-632-6605

Jason Gillman*, Kristin Bilyeu, Karen Hudson,
Rouf Mian, Grover Shannon and Earl Taliercio

2-15-2017

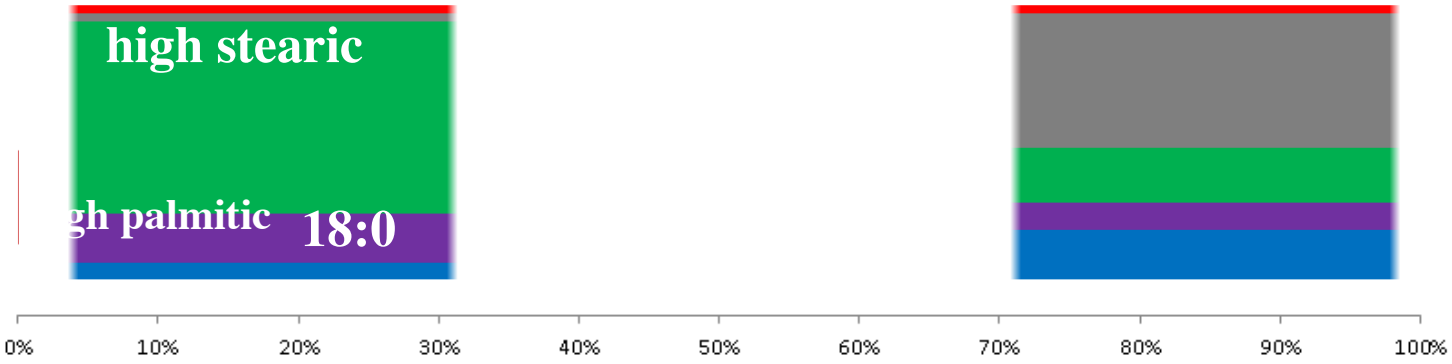
"A rising demand for palm oil has resulted in the clearing of huge tracts of rain forest in Borneo, putting wildlife habitats at risk. Left, access roads and terraced fields in Sarawak."
Nytimes.com Photo: Mattias Klum

Objective: Create more functional soybean oil Containing high stearic acid content



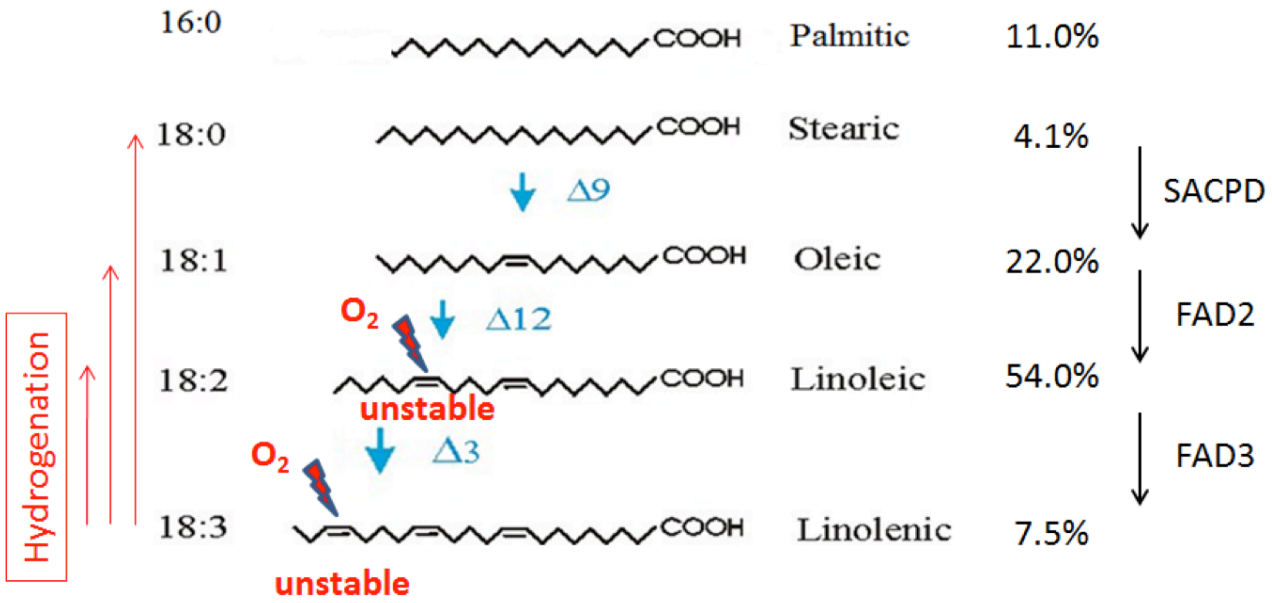
conventional

Other options for high stearic acid soybean oil



Stearoyl-Acyl Carrier Protein-Desaturase

- The high stearic acid trait is controlled largely by mutations in a Δ9-Stearoyl-Acyl Carrier Protein-Desaturase gene (SACPD)
  - Selectively inserts the first double bond at the ninth carbon of C18:0-ACP
  - Homologous to SACPD genes in Arabidopsis (*SSI2*), rapeseed, and castor



SACPD - Multiple genes encode one enzyme step

- Soybean has four SACPD isoforms
- SACPD-A, B, C, D
  - Plastid-localized, soluble enzymes
  - SACPD-A and B are 391 aa long with 98% identity
  - SACPD-C 386 aa long, 63% sequence identity to A or B
  - SACPD-C expressed specifically during seed development
  - SACPD-D is a pseudogene

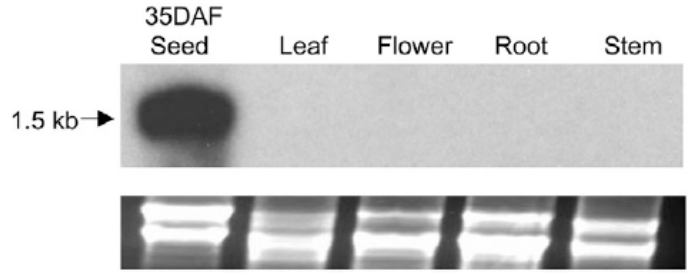
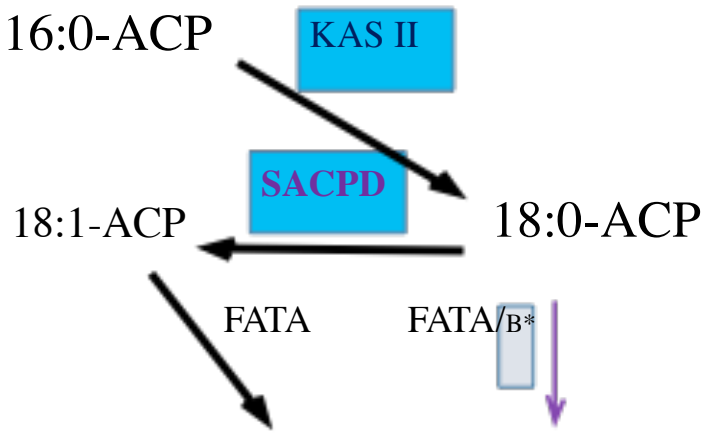
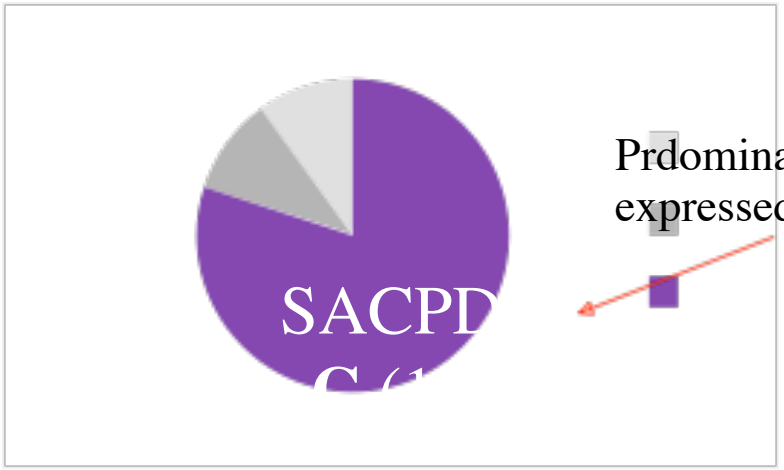


Figure 2. RNA gel blot of *SACPD-C* transcripts in different soybean tissues. Lower panel shows ethidium bromide staining of the gel before blotting to show the relative equivalency of RNA loading. DAF, days after flowering.

Zhang et al (2008)





Many Sources of Elevated Stearic Acid were identified by Project participants

- Many sources of the elevated stearic acid trait have been discovered through mutagenesis methods
- To date, there exists only one naturally occurring elevated stearic mutant (FAM94-41)

Line	Method	% Stearic Acid
FA41545	EMS	15.5
A81-606085	EMS	18.7
A6	Sodium azide*	28.1
A9	Sodium azide	16.3
A10	Sodium azide	14.6
KK-24	X-ray	13.0
M25	X-ray	13.0
MM106	X-ray	13.1
FAM94-41	Natural	9.0
Others…		

Deletion or loss of function mutations in *SACPD-C* only raise stearic acid to 10-15% of seed oil

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PLOS | ONE

Mutations in SACPD-C Result in a Range of Elevated Stearic Acid Concentration in Soybean Seed

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1 Crop Production and Pest Control Research Unit, Agricultural Research Service (ARS), United States Department of Agriculture (USDA), West Lafayette, Indiana, United States of America, **2** Department of Agronomy, Purdue University, West Lafayette, Indiana, United States of America

Abstract

Soybean oil has a wide variety of uses, and stearic acid, which is a relatively minor component of soybean oil is increasingly desired for both industrial and food applications. New soybean mutants containing high levels of the saturated fatty acid stearate in seeds were recently identified from a chemically mutagenized population. Six mutants ranged in stearate content from 6–14% stearic acid, which is 1.5 to 3 times the levels contained in wild-type seed of the Williams 82 cultivar. Candidate gene sequencing revealed that all of these lines carried amino acid substitutions in the gene encoding the delta-9-stearoyl-acyl-carrier protein desaturase enzyme (SACPD-C) required for the conversion of stearic acid to oleic acid. Five of these missense mutations were in highly conserved residues clustered around the predicted di-iron center of the SACPD-C enzyme. Co-segregation analysis demonstrated a positive association of the elevated stearate trait with the SACPD-C mutation for three populations. These missense mutations may provide additional alleles that may be used in the development of new soybean cultivars with increased levels of stearic acid.

Citation: Carrero-Colón M, Abshire N, Sweeney D, Gaskin E, Hudson K (2014) Mutations in SACPD-C Result in a Range of Elevated Stearic Acid Concentration in Soybean Seed. PLoS ONE 9(5): e97891. doi:10.1371/journal.pone.0097891

Carrero-Colón, M., Abshire, N., Sweeney,D., Gaskin,E., Hudson,K. PLoS One. 2014; **9(5):** e97891

Deletions of the *SACPD-C* locus elevate seed stearic acid levels but also result in fatty acid and morphological alterations in nitrogen fixing nodules

Jason D Gillman[1*†], Minviluz G Stacey[2†], Yaya Cui[2], Howard R Berg[3] and Gary Stacey[2]

Abstract

Background: Soybean (*Glycine max*) seeds are the primary source of edible oil in the United States. Despite its widespread utility, soybean oil is oxidatively unstable. Until recently, the majority of soybean oil underwent chemical hydrogenation, a process which also generates *trans* fats. An alternative to chemical hydrogenation is genetic modification of seed oil through identification and introgression of mutant alleles. One target for improvement is the elevation of a saturated fat with no negative cardiovascular impacts, stearic acid, which typically constitutes a minute portion of seed oil (~3%).

Results: We examined radiation induced soybean mutants with moderately increased stearic acid (10-15% of seed oil, ~3-5 X the levels in wild-type soybean seeds) via comparative whole genome hybridization and genetic analysis. The deletion of one *SACPD* isoform encoding gene (*SACPD-C*) was perfectly correlated with moderate elevation of seed stearic acid content. However, *SACPD-C* deletion lines were also found to have altered nodule fatty acid composition and grossly altered morphology. Despite these defects, overall nodule accumulation and nitrogen fixation were unaffected, at least under laboratory conditions.

Conclusions: Although no yield penalty has been reported for moderate elevated seed stearic acid content in soybean seeds, our results demonstrate that genetic alteration of seed traits can have unforeseen pleiotropic consequences. We have identified a role for fatty acid biosynthesis, and SACPD activity in particular, in the establishment and maintenance of symbiotic nitrogen fixation.

Keywords: Soybean (*Glycine max*), Stearic acid, Fatty acid composition, Radiation mutagenesis, Comparative genome hybridization, Nodulation

Gillman, J. D., M. G. Stacey, Y. Cui, H. R. Berg and G. Stacey (2014). " BMC Plant Biology **14(1): 143**

Stearic project overview

- **Gillman (largely completed)**
  - QTL mapping project A6 x 194D
  - comprehensive NIR fatty acid calibrations
- **Bilyeu (Ongoing/Completed)**
  - Remutagenesis of two moderate stearic lines (~11-13%) screening for elevated stearic acid (Ongoing/completed)
  - Combination of High Oleic and Moderate Stearic (Completed)
- **Hudson (Ongoing/Completed)**
  - Mutagenesis screening and QTL mapping (ongoing)
  - Combination of moderate stearic with other fatty acid traits (18:3↓, 18:1↑) (Completed)
- **Mian/Taliercio (Ongoing)**
  - Population containing 1) 2 FAD2 mutants (>80% oleic); 2) two *sacpd-c* mutants; 3) a new minor stearic QTL (in progress) Some preliminary lines have ~18% stearic and 62-70% oleic
  - Remutagenesis of a moderate stearic line (LLL05-14, 13-15% stearic)
- **Shannon (Completed)**
  - Germplasm screen and intercrossing of elevated stearic lines – not successful
  - yield selection of populations derived from crossing of two ~13% stearic lines (Miranda/Cardinal/Burton)
- **All:** Stability study of most promising preliminary lines in 2016 (Completed)

Development of a QTL mapping population (A6 x 194D, ~23% x ~11% stearic)

Table 3 Descriptive statistics of traits of the RIL populations and the parents

		RILs			Parents		
	Trait	Average[a]	Range	SD[b]	A6		194D
BR 2014	Height (cm)	49.31	22.50–87.50	11.13	46.25	*	64.17
	Total seed oil (%)	18.63	15.04–21.44	1.13	15.84		19.81
	Palmitic (% seed oil)	8.98	7.14–11.16	0.67	8.70		9.62
	Stearic (% seed oil)	15.12	7.83–26.11	3.44	23.88	*	9.97
	Oleic (% seed oil)	21	15.17–33.29	3.38	18.57		18.05
	Linoleic (% seed oil)	48.06	33.09–55.40	3.83	42.60	**	54.57
	Linolenic (% seed oil)	6.85	4.47–9.37	0.86	6.25	**	8.05
2014 SF	Height (cm)	63.82	31.67–86.67	11.01	40		68.75
	Total seed oil (%)	17.3	14.16–19.57	1.15	11.85		18.59
	Palmitic (% seed oil)	9.44	8.42–10.75	0.51	9.13		9.77
	Stearic (% seed oil)	14.34	9.66–20.99	2.87	22.32	*	11.77
	Oleic (% seed oil)	18	15.13–23.43	1.75	16.92		18.67
	Linoleic (% seed oil)	49.9	44.46–54.42	2.43	43.51	*	48.92
	Linolenic (% seed oil)	8.32	6.82–10.30	0.69	8.13		8.12
RB 2015	Height (cm)	56.8	38.00–91.11	9.42	60.75		78.94
	Total seed oil (%)	17.79	14.91–19.47	0.73	18.55		17.02
	Palmitic (% seed oil)	9.19	7.73–11.30	0.68	8.76		9.09
	Stearic (% seed oil)	15.91	6.03–24.76	3.35	20.85	**	12.67
	Oleic (% seed oil)	21.69	17.24–33.56	2.84	18.76	**	22.19
	Linoleic (% seed oil)	46.76	34.77–53.35	3.51	44.88	*	49.13
	Linolenic (% seed oil)	6.45	4.634–7.87	0.58	6.75		6.91

* Parent values are significantly different at $P < 0.05$; ** parent values are significantly different at $P < 0.01$. RIL, recombinant inbred line; BR, Bradford Research Center; SF, South Farm; RB, Hinkson Field.
[a] Full details on individual RILs are in Supplemental Materials.
[b] SD, standard deviation.

Heim and Gillman, *G3* January 2017 **7**:299-308

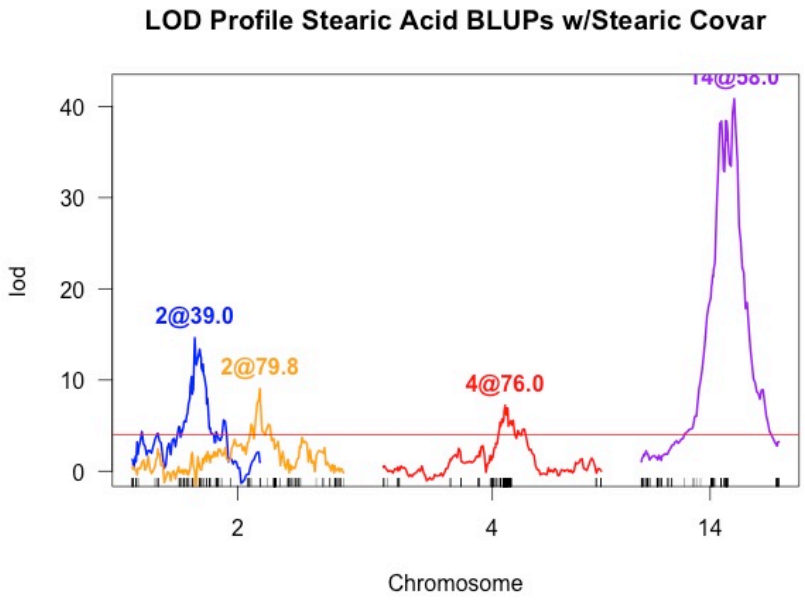
Gillman

A major effect QTL and 3 minor effect QTL were identified which control seed stearic acid

Table 4 SIM and SIM + covariate analysis for stearic acid-related QTL using BLUPs

Trait	Method	Chromosome	SNP Marker Nucleotide	Position	LOD	R^2	1.5 Interval
Stearic acid	SIM	2	Gm2:15552879	78.00	4.38	4.4	44–111
		4	Gm4:18312993	76.00	4.08	4.1	72–83
		14	Gm14:42206409	49.00	34.25	53.2	48–54
Stearic acid	SIM + Cov	2	Gm2:5946912	39.00	14.63	13.75	38–42
		2	Gm2:15552879	79.79	9.05	7.9	78–80
		4	Gm4:18312993	75.90	7.23	6.1	73–78
		14	c14.loc58	58.00	40.84	56.8	57–59

LOD, logarithm of the odds; SIM, SIM, standard interval mapping; Cov, covariate analysis.



Heim and Gillman, *G3* January 2017 **7**:299-308

By stacking three QTL, it is possible to achieve 20% seed stearic acid in three environments

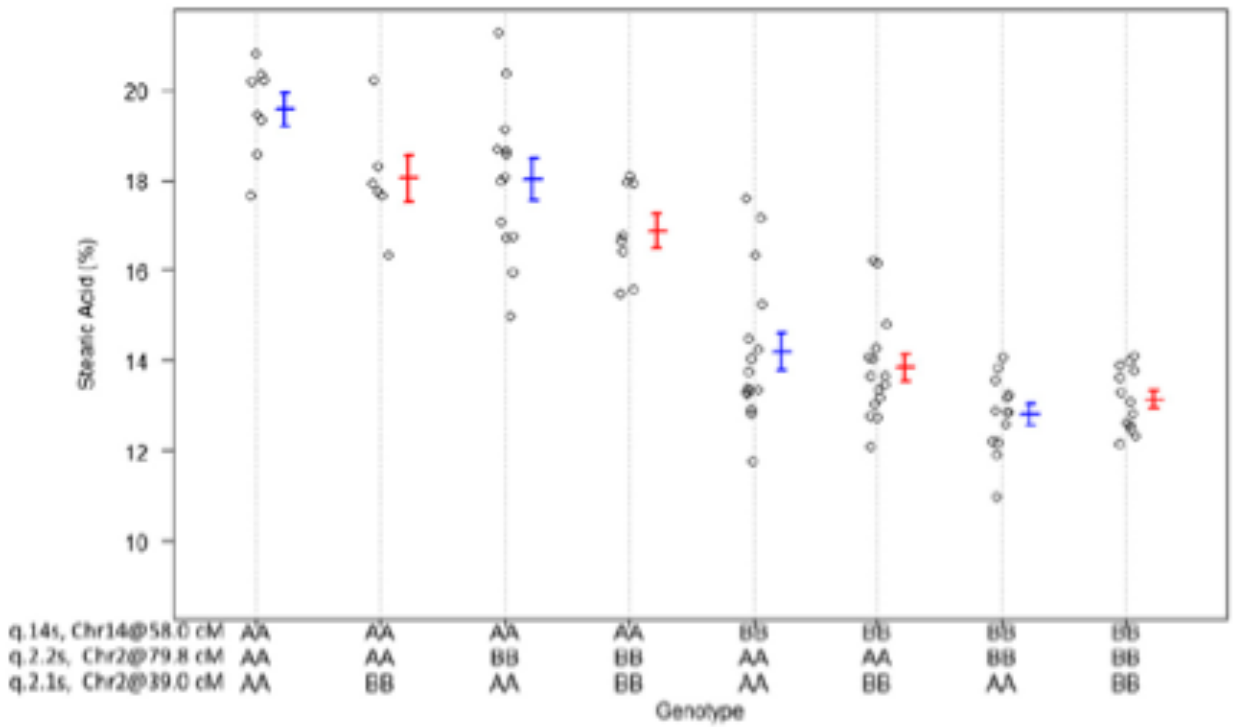


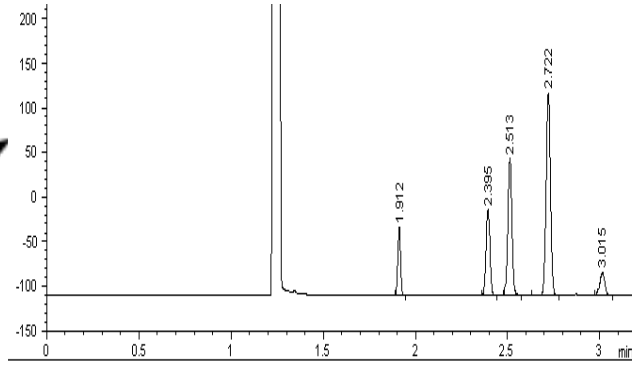
Figure 3 Interaction plot between *q.2.1s* *q.2.2s* and *q.14s*. Genotype "AA" indicates homozygosity for A6 alleles, allele "BB" indicates homozygosity for alleles from 194D. Middle bar indicates mean of genotypes, top and bottom bars indicate ± 1 SEM. Parental stearic acid contents averaged over all locations were: A6 = 22.4 ± 3.8%, 194D = 11.4 ± 1.6%. Chr, chromosome.

Substantial residual variation means values are shrunk towards to grand mean in LSM/BLUP analysis

Heim and Gillman, *G3* January 2017 **7**:299-308

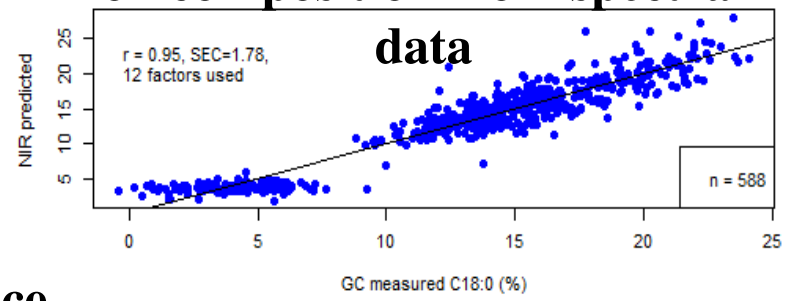
Gillman

Gas chromatography analysis
destructive assay
slow/non-automatable

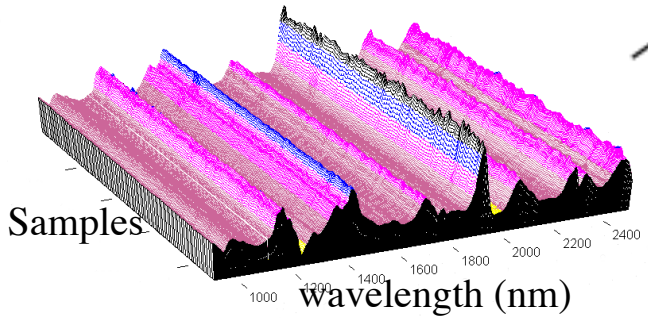


soybean seed

NIRS model accurately predicts oil composition from spectral data



Near Infrared Reflectance
non-destructive
rapid/automatable



A Karn, C. Heim, S. Flint-Garcia, K. Bilyeu, K.; J. Gillman, J., *JAOCS* ***(2017) 94, 69-76.***

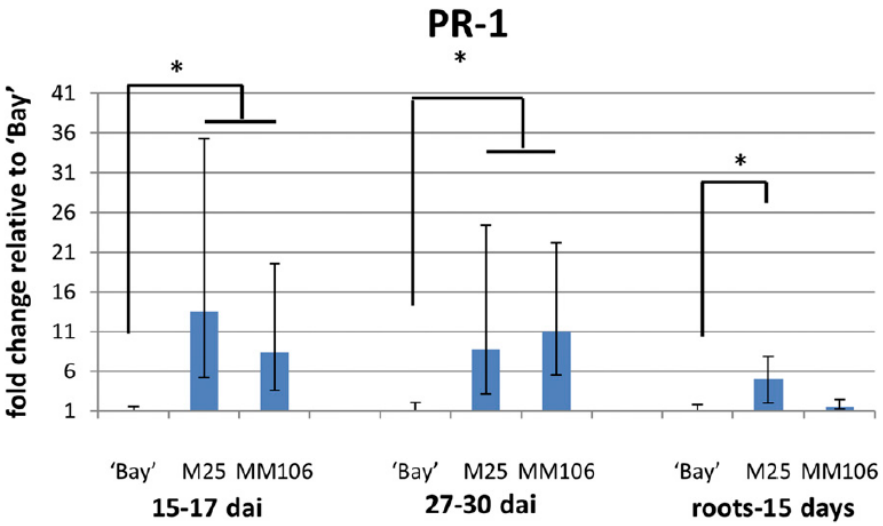
NIR calibration (FOSS 6500) is able to accurately predict

Fatty Acids	n	Spectral range	NIR Pretreatment	PLS Factors	SEC	SECV	RMSECV	r
C16:0	583	900 - 2500 nm	MSC; 1 Der	7	0.64	0.67	0.67	0.82
C18:0	588	900 - 2500 nm	MSC; 1 Der	12	1.78	2.17	2.17	0.95
C18:1	596	900 - 2500 nm	MSC; 1 Der	7	5.81	6.14	6.14	0.97
C18:2	591	900 - 2500 nm	MSC; 1 Der	6	3.61	3.73	3.73	0.98
C18:3	584	900 - 2500 nm	MSC; 1 Der	6	0.64	0.66	0.66	0.92

Number of samples (*n*); Standard error of cross-validation (SECV); Root mean square error for cross validation (RMSECV); coefficient of correlation (*r*)

A Karn, C. Heim, S. Flint-Garcia, K. Bilyeu, K.; J. Gillman, J., *JAOCS* ***(2017) 94, 69-76.***

SACPD-C also has a role in proper defense signaling and maintenance of nodules– yet mutants do not have a yield cost



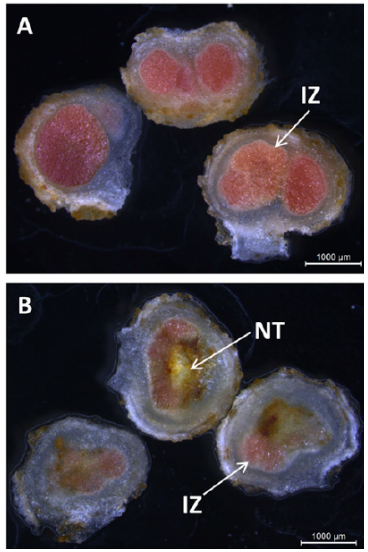


Table 4. Quantification of root phytohormones in nodules infected by *Bradyrhizobium japonicum* USDA110 (30 days after infection) and noninfected roots at 30 days after seed germination

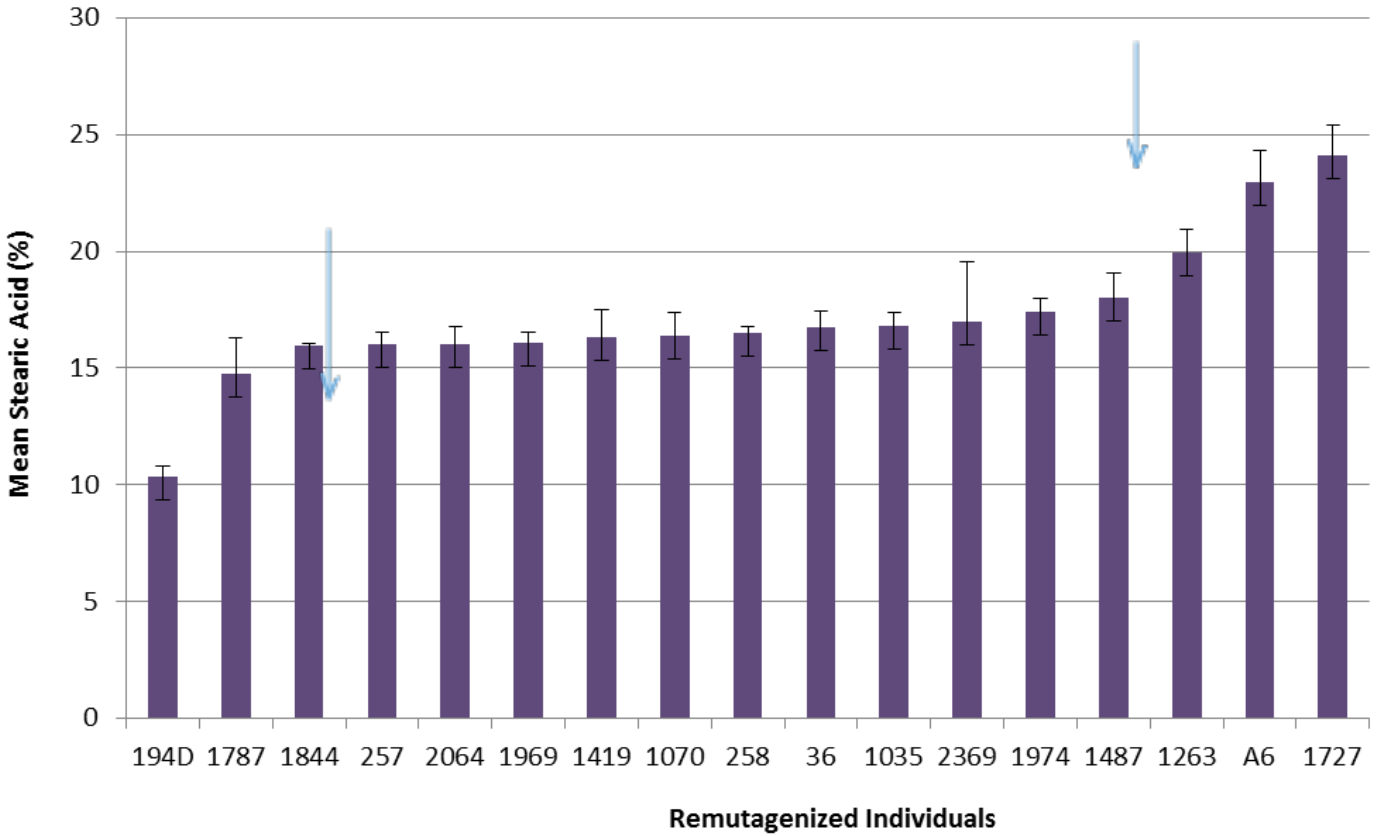
Tissue	Enzyme[x]	'Bay'[y]	M25[y]	MM106[y]	ANOVA[z]
Nodule	ABA	4.93 ± 0.86	5.57 ± 3.61	7.8 ± 0.10	0.3008 NS
	JA	419.77 ± 61.01 a	174 ± 18.10 b	327.63 ± 128.26 ab	0.0290*
	JA-Ile	2,010.43 ± 541.26 a	290.60 ± 45.25 b	1,006.43 ± 521.56 b	0.0083**
	OPDA	2,799.33 ± 384.73 a	9,892.93 ± 2,485.03 b	9,655.43 ± 1,789.29 b	0.0044**
	JA + OPDA	5,229.53 ± 836.89 a	10,357.50 ± 2,542.17 b	10,989.47 ± 2,436.37 b	0.0282*
	SA	71.10 ± 3.82	58.73 ± 0.90	73.83 ± 17.96	0.2562 NS
Root	ABA	199.65 ± 61.22	252.14 ± 68.44	222.54 ± 13.43	0.5800 NS
	JA	3,551.63 ± 434.51	2,921.71 ± 259.85	2,623.71 ± 73.12	0.0530 NS
	JA-Ile	35,165.84 ± 3,994.66 a	28,983.03 ± 1,365.67 b	23,880.81 ± 1,047.68 b	0.01550*
	OPDA	1,509.40 ± 556.04	1,452.15 ± 443.02	2,397.25 ± 1,249.30	0.3679 NS
	JA + OPDA	4,0226.88 ± 4,362.94	33,356.90 ± 1,524.40	20,893.46 ± 13,971.66	0.0790 NS
	SA	7,583.22 ± 1,529.25	13,848.81 ± 10,454.38	5,089.58 ± 3,340.75	0.3924 NS

[x] ABA = abscisic acid; JA = jasmonic acid; Ile = isoleucine; OPDA = 12-oxophytodienoic acid; and SA = salicylic acid.
[y] In nanograms per gram, $n = 3$.
[z] Analysis of variance (ANOVA); Prob > F. Single or double asterisks (* or **) indicate significance at the <0.05 or <0.01 level, respectively. Letters indicate the result of t test comparison for significant ANOVA results.

Krishnan, H. B., A. A. Alaswad, N. W. Oehrle and J. D. Gillman (2016). MPMI **29(11): 862-877.**

Gillman

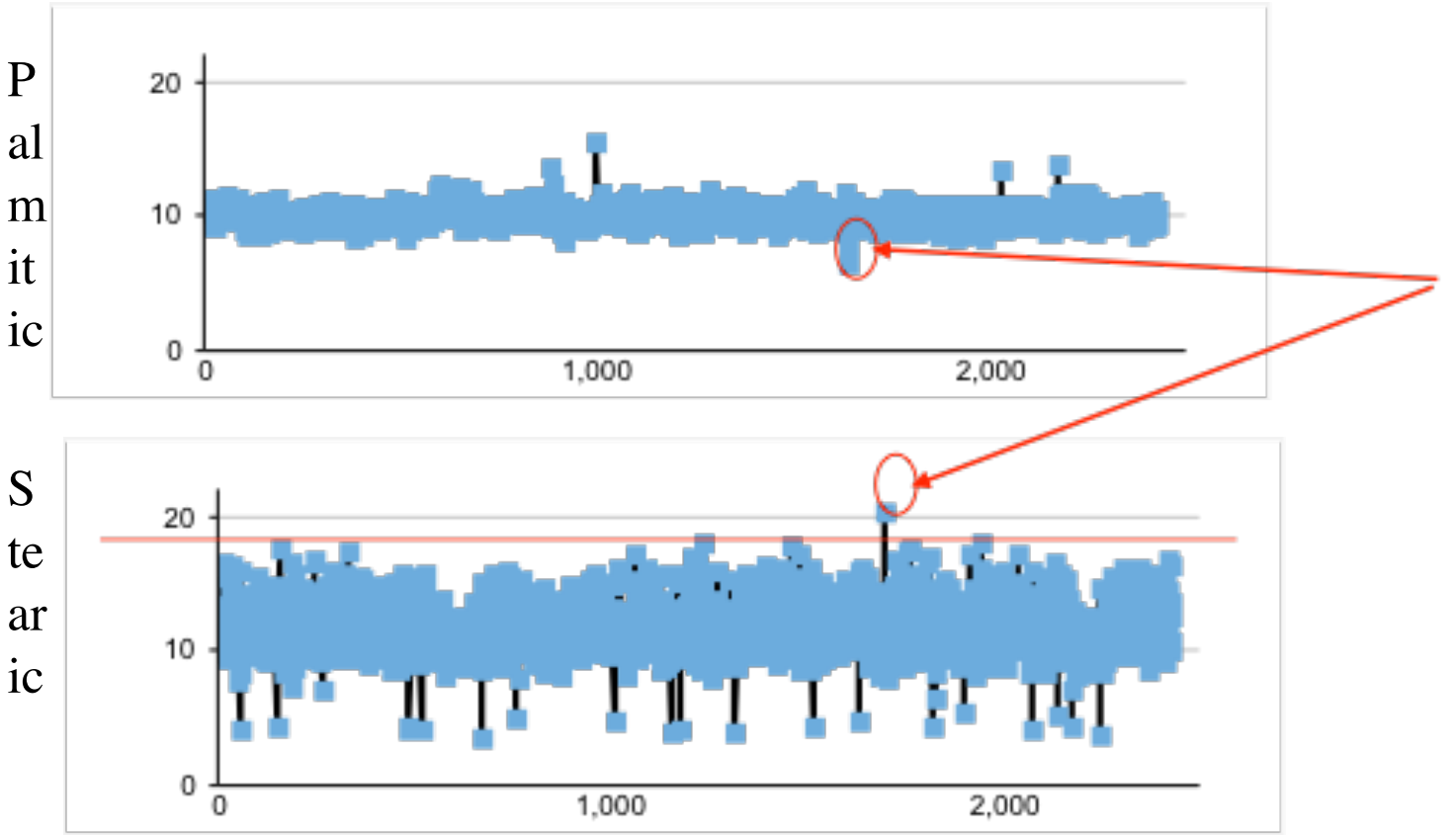
Characterization of two re-mutagenized populations identified several putative lines which could meet the goal



Unpublished data, 2015

Bilyeu lead

Unfortunately, none proved to be consistently above 16% threshold in subsequent years

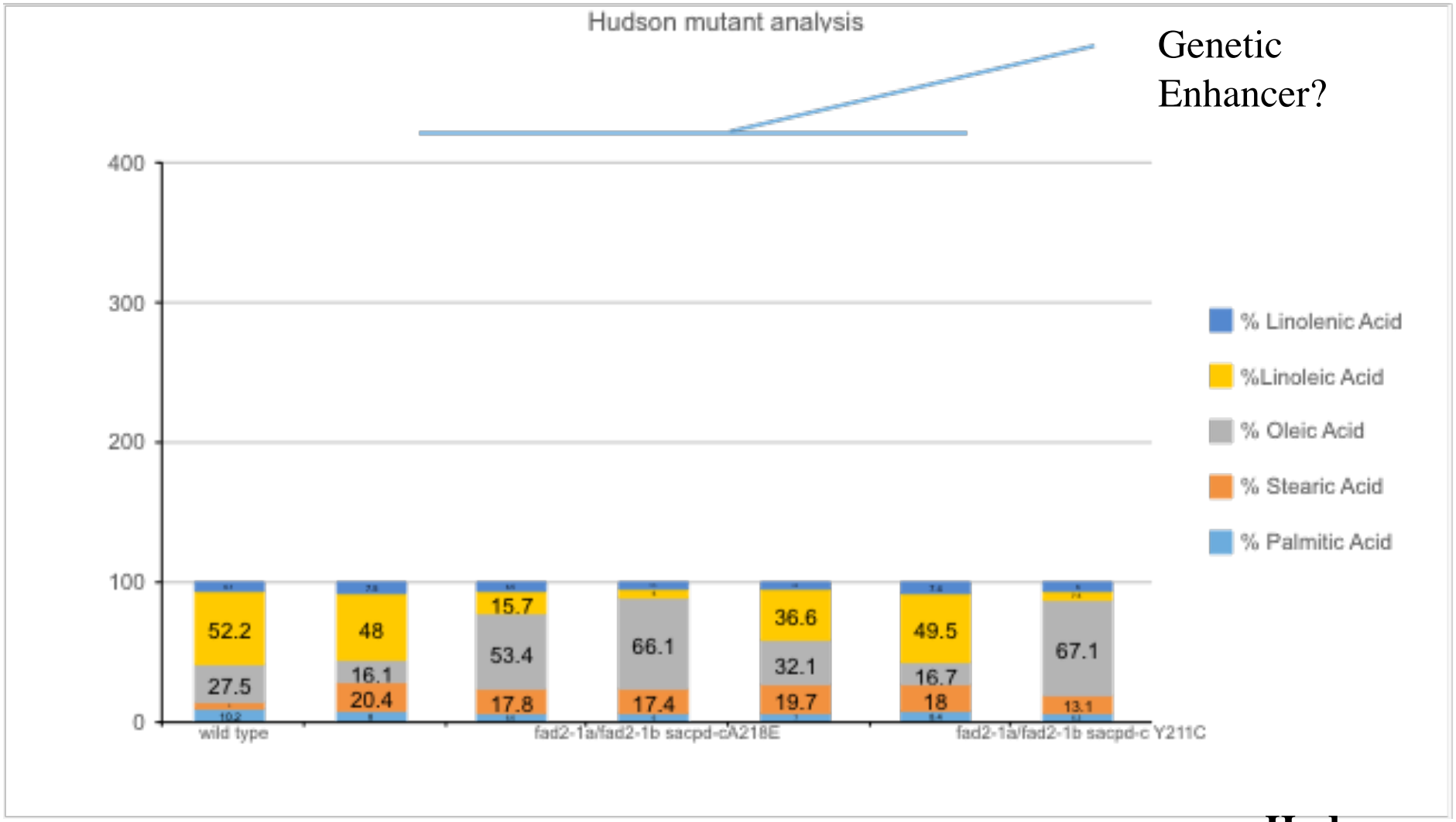


Several new mutants validated

But in subsequent studies, none are >16% C18:0

Bilyeu lead

Mutagenesis and combination of *SACPD-C* with high oleic and low linolenic genes



Hudson lead

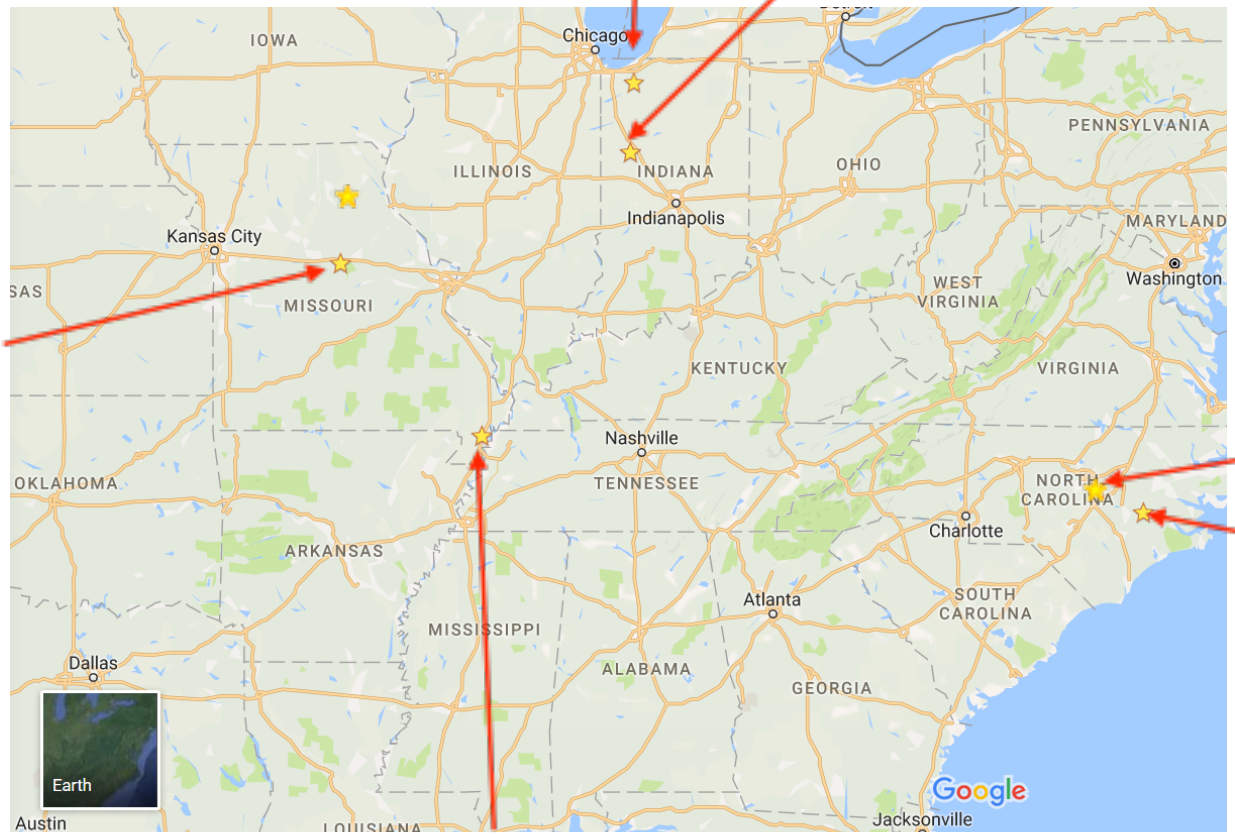
Stearic project overview

- **Gillman (largely completed)**
  - QTL mapping project A6 x 194D
  - comprehensive NIR fatty acid calibrations
- **Bilyeu (Ongoing/Completed)**
  - Remutagenesis of two moderate stearic lines (~11-13%) screening for elevated stearic acid (Ongoing/completed)
  - Combination of High Oleic and Moderate Stearic (Completed)
- **Hudson (Ongoing/Completed)**
  - Mutagenesis screening and QTL mapping (ongoing)
  - Combination of moderate stearic with other fatty acid traits (18:3↓, 18:0↑) (Completed)
- **Mian/Taliercio (Ongoing)**
  - Population containing 1) mid-oleic acid ; 2) two *sacpd-c* mutants; 3) a new minor stearic QTL (in progress) Some preliminary lines have ~18% stearic and 62-70% oleic
  - Remutagenesis of a moderate stearic line (LLL05-14, 13-15% stearic)
- **Shannon (Completed)**
  - Germplasm screen and intercrossing of elevated stearic lines – not successful
  - yield selection of populations derived from crossing of two ~13% stearic lines (Miranda/Cardinal/Burton)
- **All: Stability study of most promising preliminary lines in 2015 (Completed)**

7 locations
2 planting dates in Columbia, MO
3 plot replicates RCBD

5/24/16

5/22/16



5/20/16

5/31/16

6/8/16

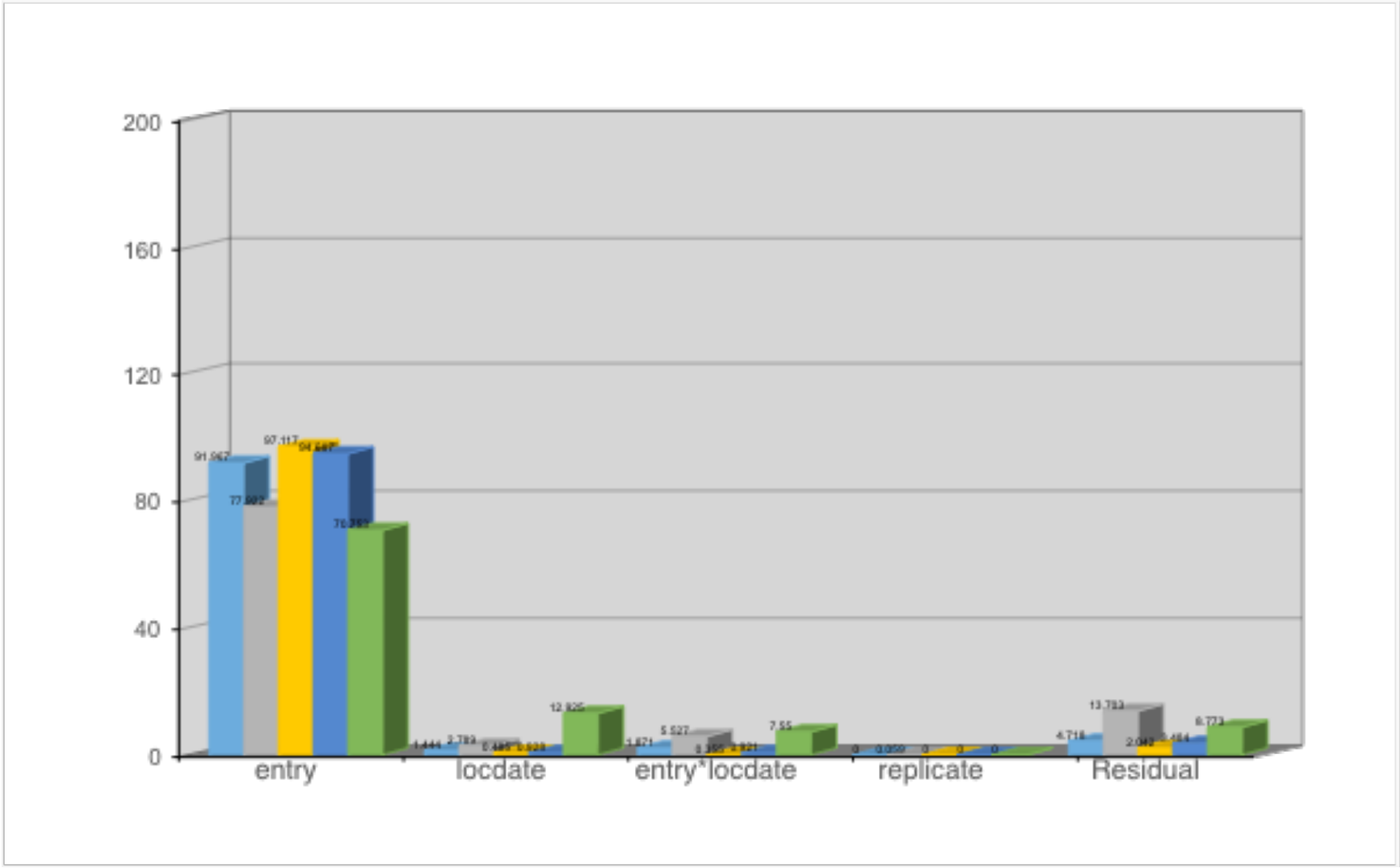
6/30/16

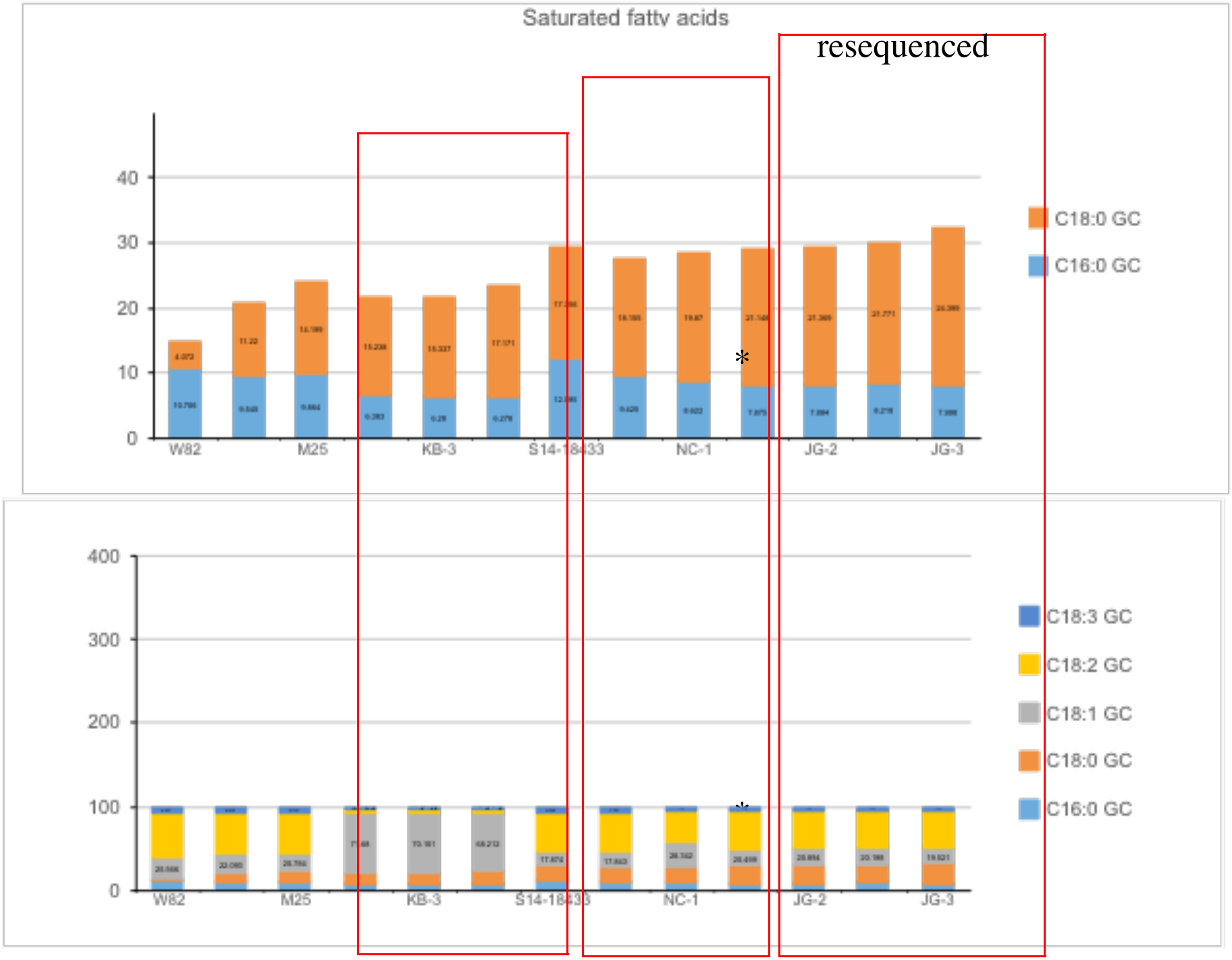
6/13/2016

9 experimental lines
(3 Gillman, 3 Bilyeu, 1 Shannon, 1 Hudson, 1 Mian/Taliercio)
plus three control lines
(A6, W82, M25; ~23%, ~4% and ~12.5% seed stearic acid respectively)

Gillman lead/cooperative

Both G, E and GxE affect fatty acid composition
G has an overwhelming role over other factors

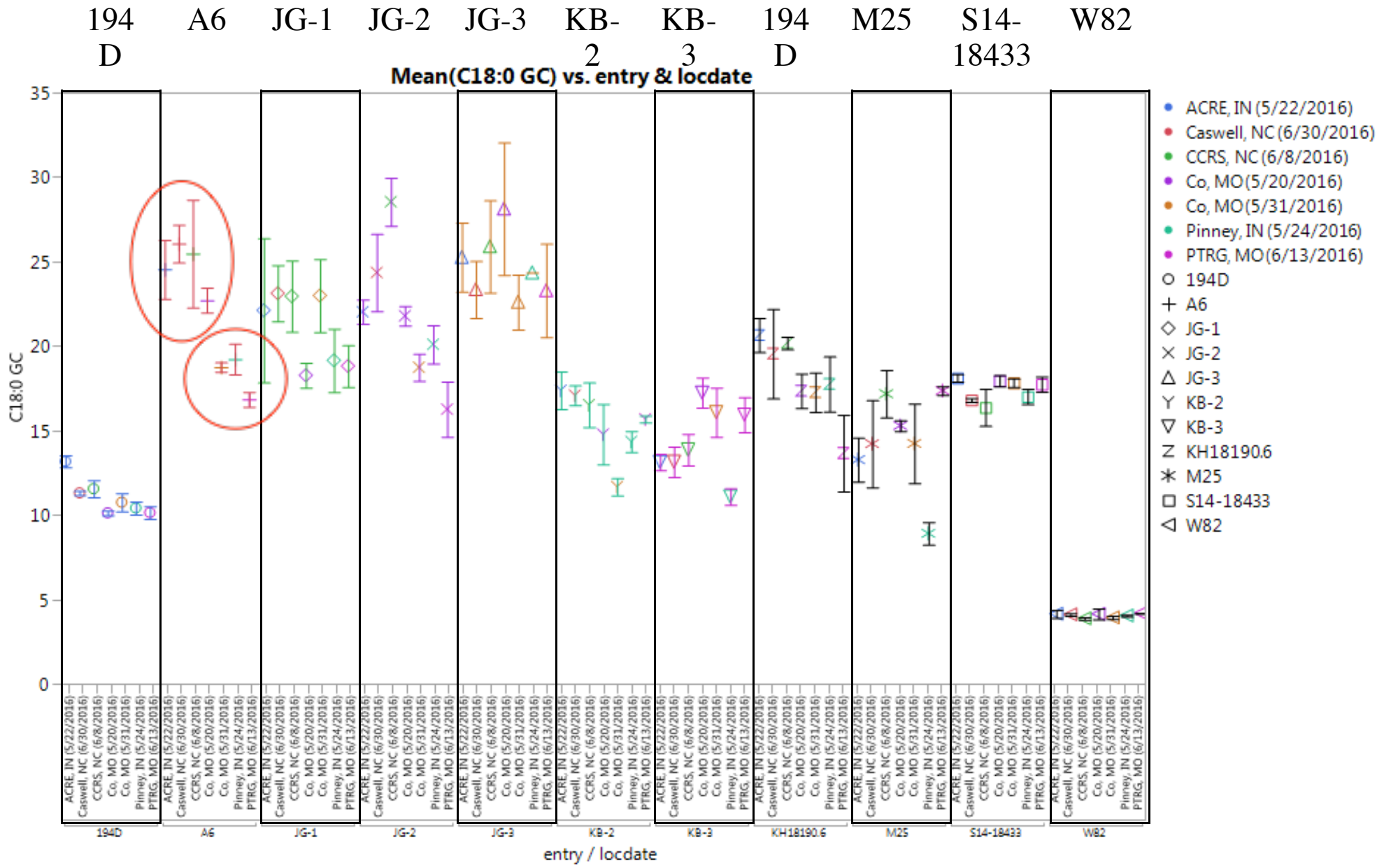




* NC-1 only in three environments due to maturity

Gillman

Certain genotypes showed variation between locations
Reasons unclear, but looking at environmental maturity data further



Each error bar is constructed using 1 standard error from the mean.

Report on seed yield in moderate stearic line S14-18433 in 2013 and 2014

MG-VI Southern Regional Prelim tests in 2013 and 2014

2013- it yielded about **93%** of the average of the checks (48.0 bu/ac)

2014- it yielded about **88%** of the average of the checks (57.7 bu/ac)

S14-18433 =

LLL-05-1 (FAM94-41-3 X N98-4445A)

x

TCJWB03-806-7-19 (B)

elevated stearic acid from holladay gamma irradiated (deletion)

FAM94-41-3 pantalone selection from ~9% stearic acid (snp 229)

N98-4445A Burton mid oleic line

Shannon lead

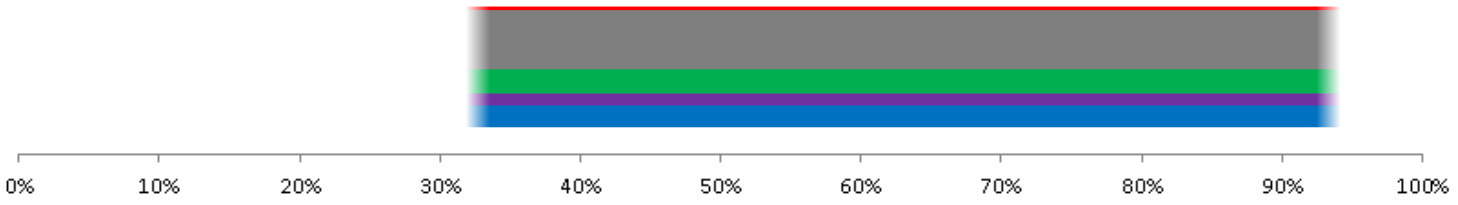
Grover Shannon report on yield study with a elevated palmitic/elevated stearic acid line

Yield of MO high stearic line S14-18433 vs RR2 checks, 2015

Line	BU/A	Maturity	Palmitic	Stearic
S14-18433*	56.5	60	10.4	17.7
AG4632R2	60.7	64	-	-
AG5335R2	58.9	70	-	-
AG4835R2	56.1	68	-	-

S14-18433 is from LL05-14 X TCJWB03-806-7-19

fap2 allele from C1727 and two *sacpd-c* mutations (and an enhancer mutation?) was in the background of this cross



Shannon

High Stearic project original projected timeline

Year 1: Assessment, discovery, initial characterization
Year 2: Phenotype confirmation, characterization, population development (discovery continues)
Year 3: Gene characterization, genetic mapping, deployment, germplasm initiation
Year 4: Marker assisted selection (MAS), yield impact, environmental stability
Year 5: Select best germplasm, pre-breeding
Year 6: Population development with MAS
Year 7: Broad Yield testing