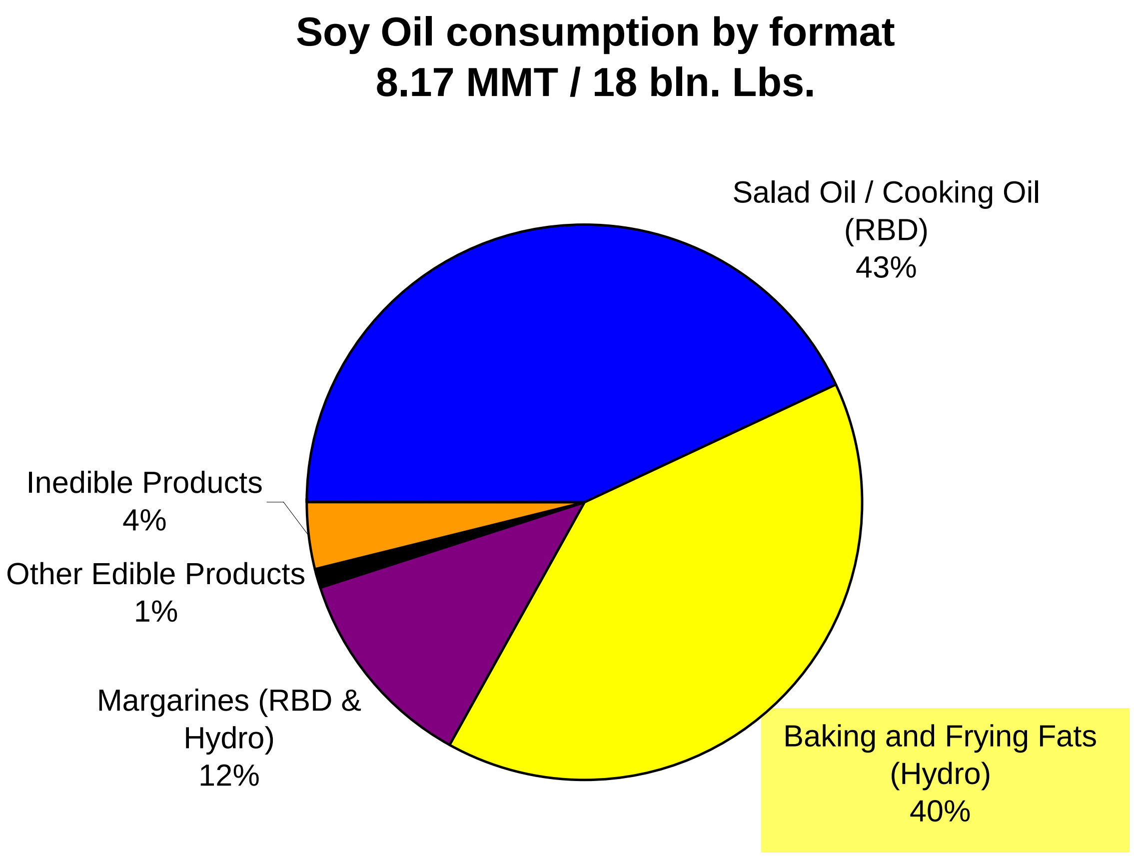
METABOLIC ENGINEERING

Manipulation of Lipid Metabolism in Plants to Produce Healthier Food Oils

Tony Kinney

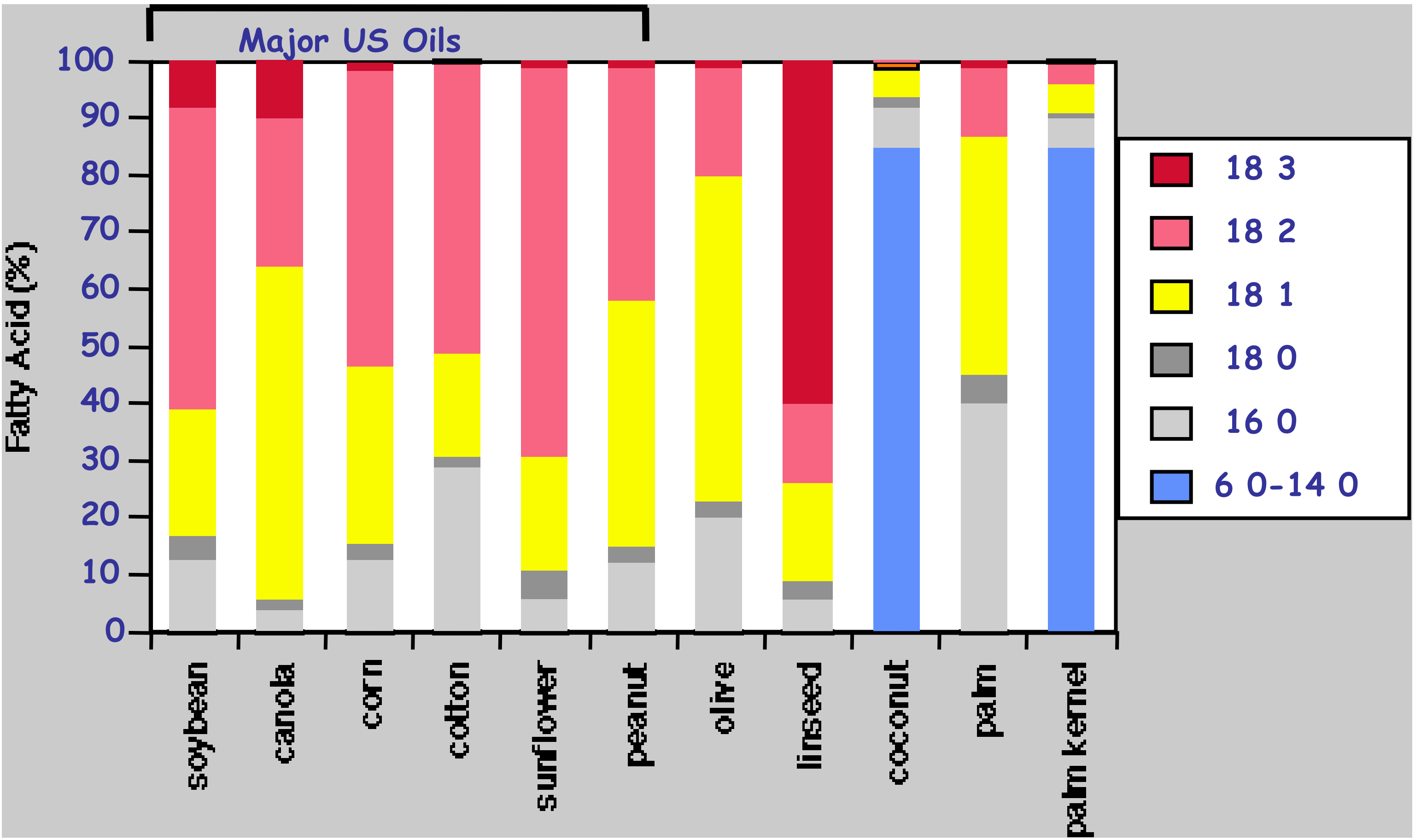
DuPont Experimental Station

US Soybean Oil Consumption 2004

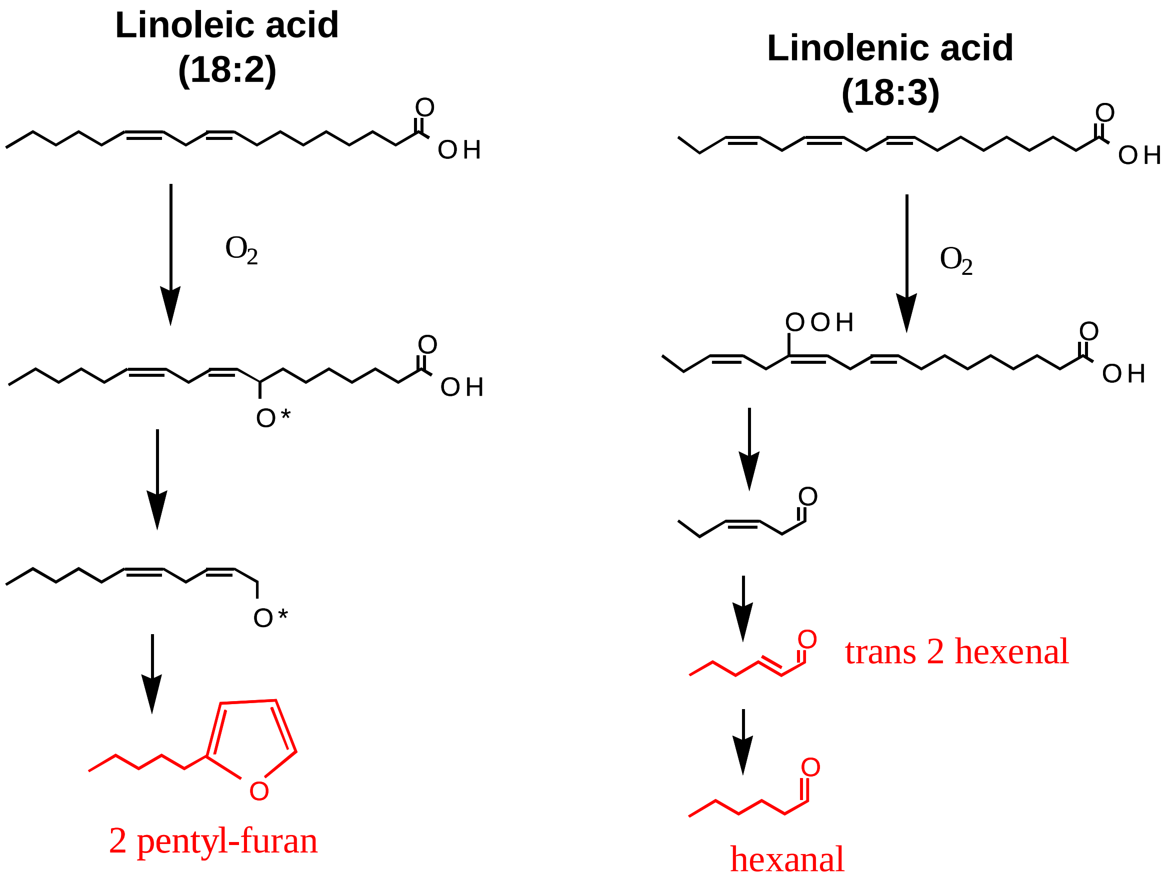


40% of soy is partially hydrogenated

Major food oils, including soybean, are rich in polyunsaturated fatty acids (18:2 & 18:3)

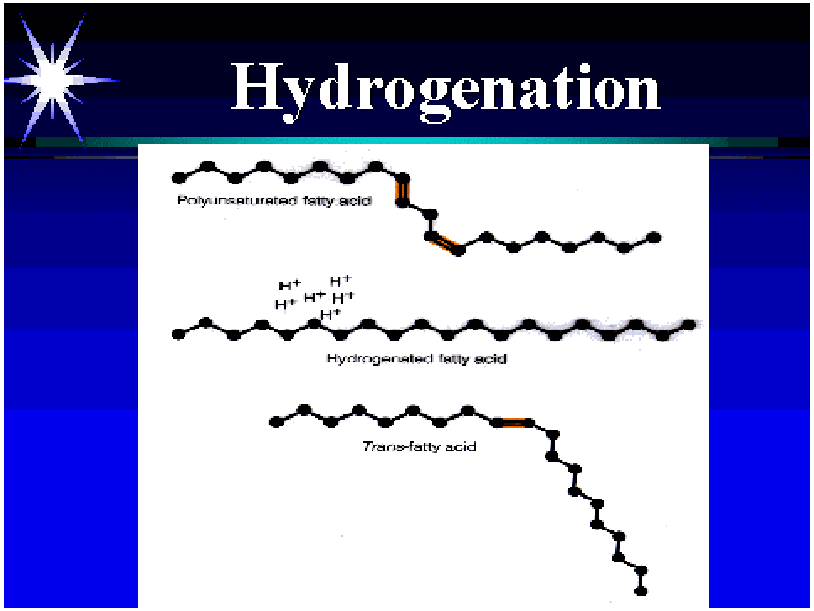


Soybean is unstable during storage and cooking and undergoes to "flavor reversion"

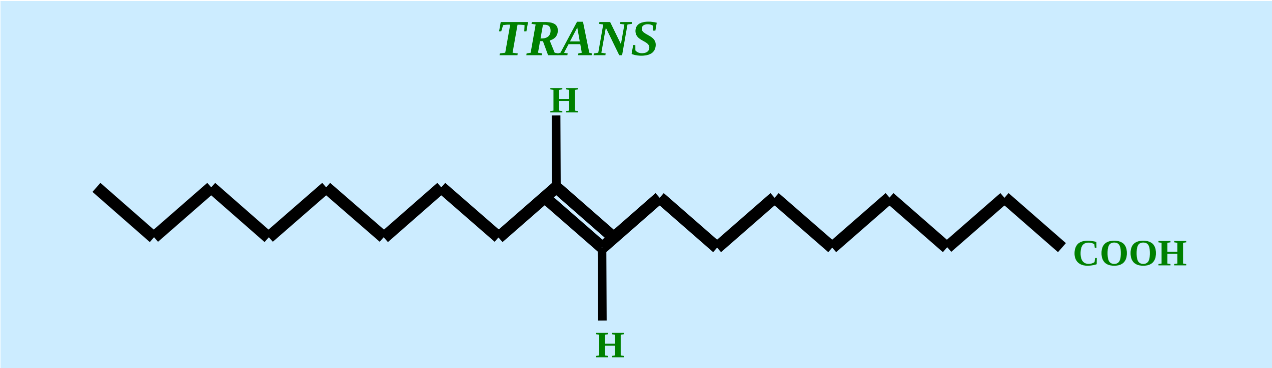


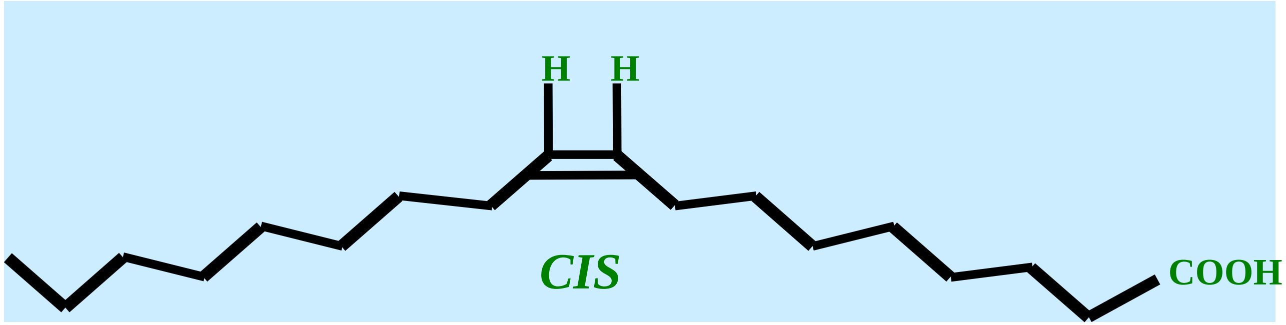
Predominant volatile compounds causing flavor reversion are result of polyunsaturated fatty acid oxidation

For this reason soybean oil is partially hydrogenated to increase its oxidative stability and other functional properties



Hydrogenation results in *trans* fatty acid formation



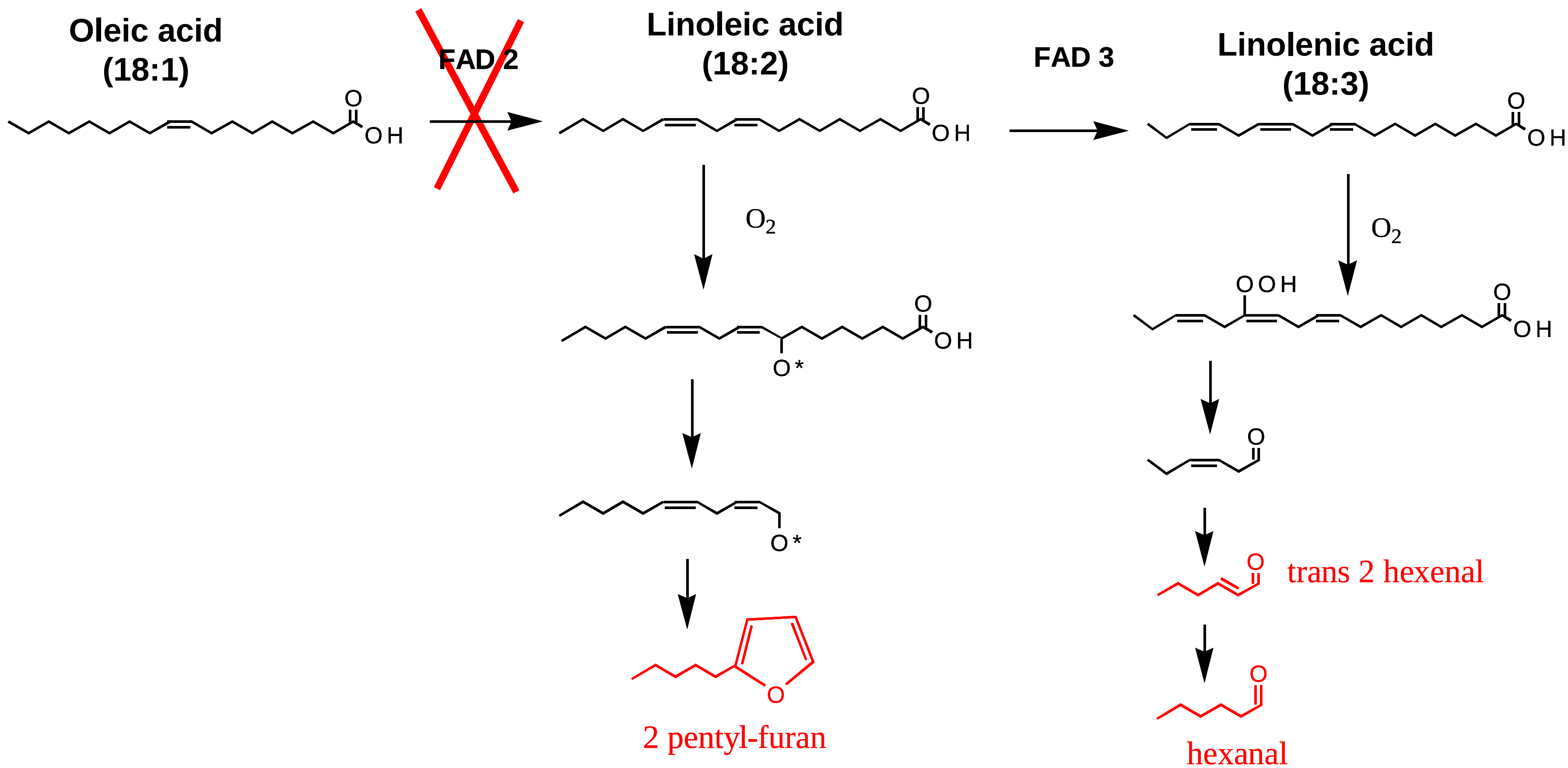


Consumption of *trans* fatty acids is linked to CHD

The FDA has estimated that 2,100 to 5,600 lives are lost each year, and 6,300 to 17,100 cases of fatal and non-fatal coronary heart disease occur each year, because people don't realize they are eating this stuff

Federal Register, Vol. 64, No. 221, November 17, 1999 at page 62772

Polyunsaturated fatty acids are the result of the activity of fatty acid desaturases (Fads) in the seed: Fad 2 is first step in this process



Blocking this step in the bean will prevent polyunsaturate formation and result in oleic acid accumulation in seed oil

Fad 2 gene from soy cloned in 1992 by reverse genetics

The Plant Cell, Vol. 6, 147–158, January 1994 © 1994 American Society of Plant Physiologists

Arabidopsis *FAD2* Gene Encodes the Enzyme That Is Essential for Polyunsaturated Lipid Synthesis

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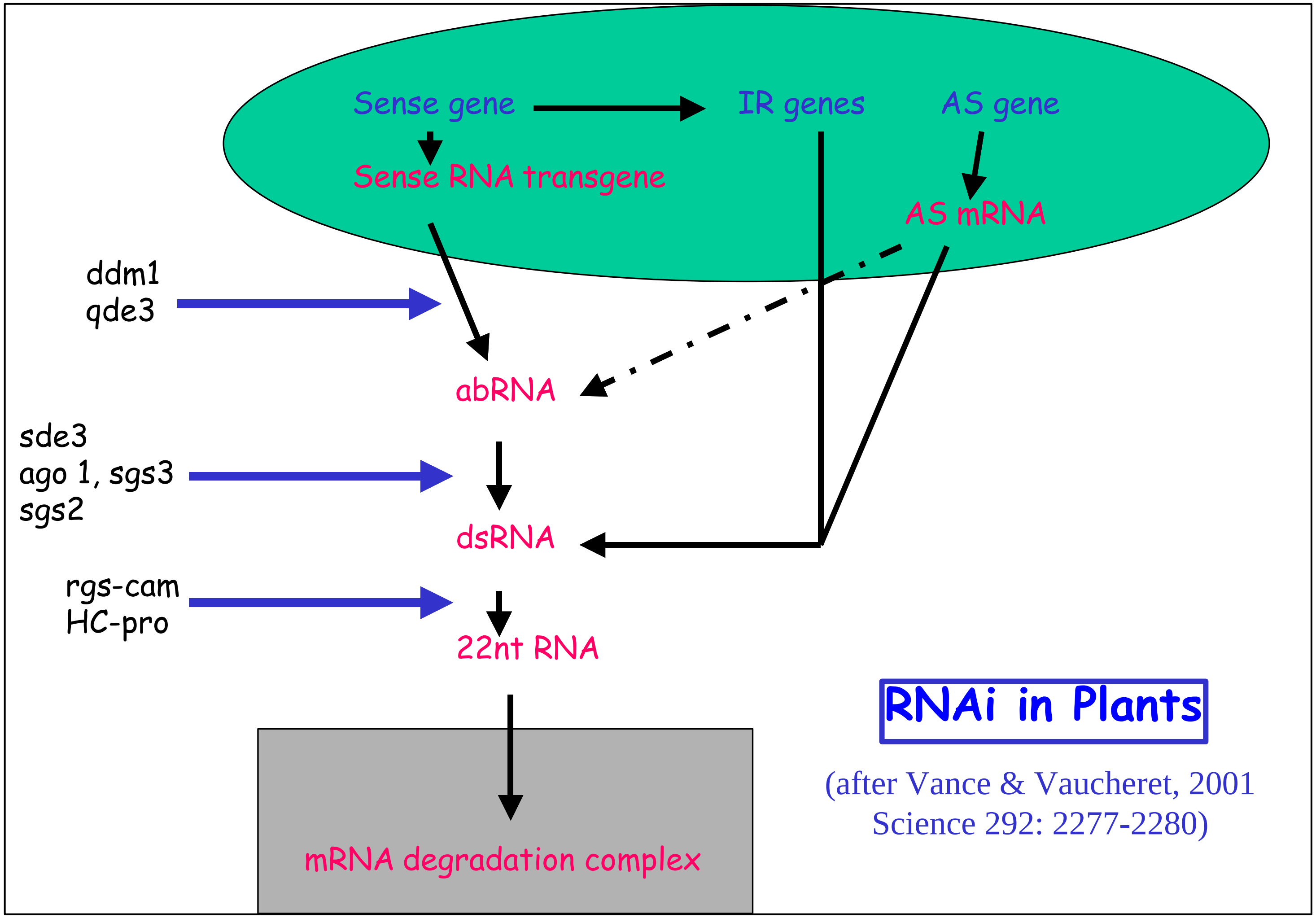
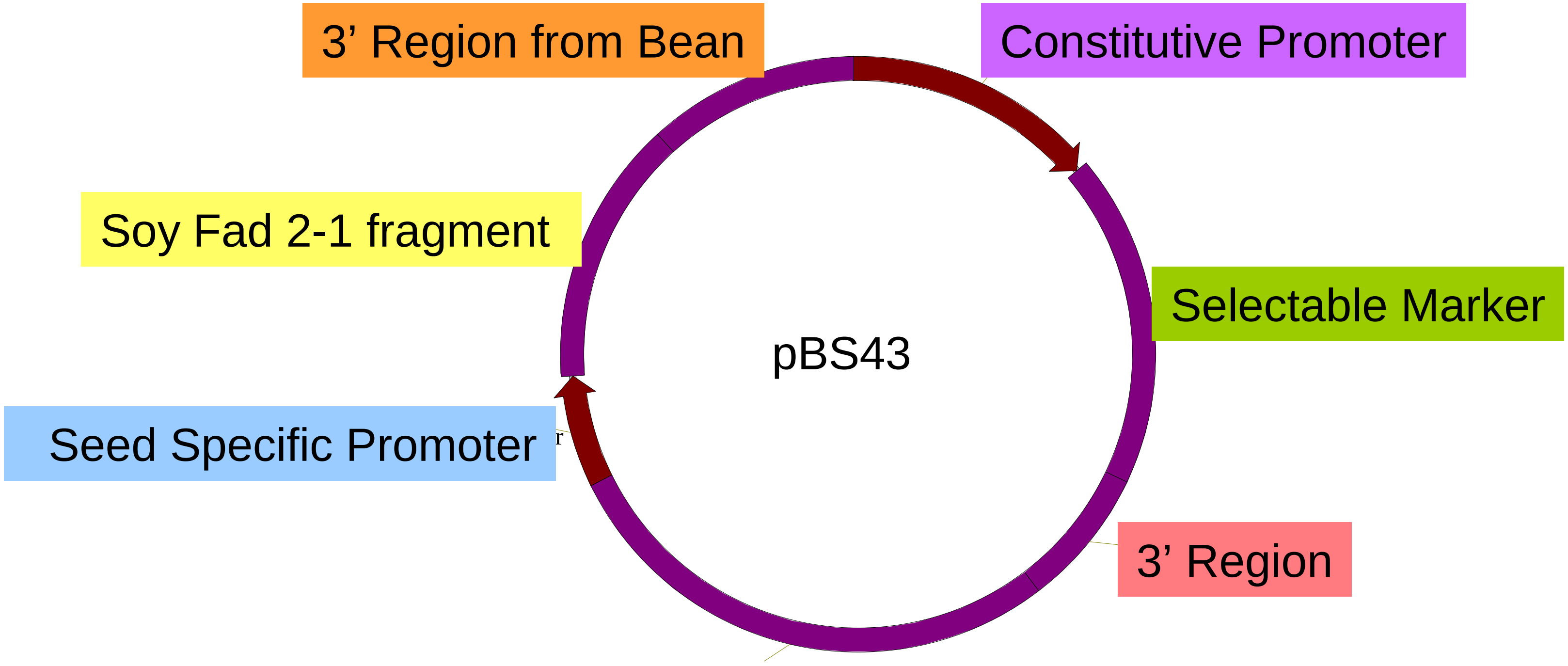
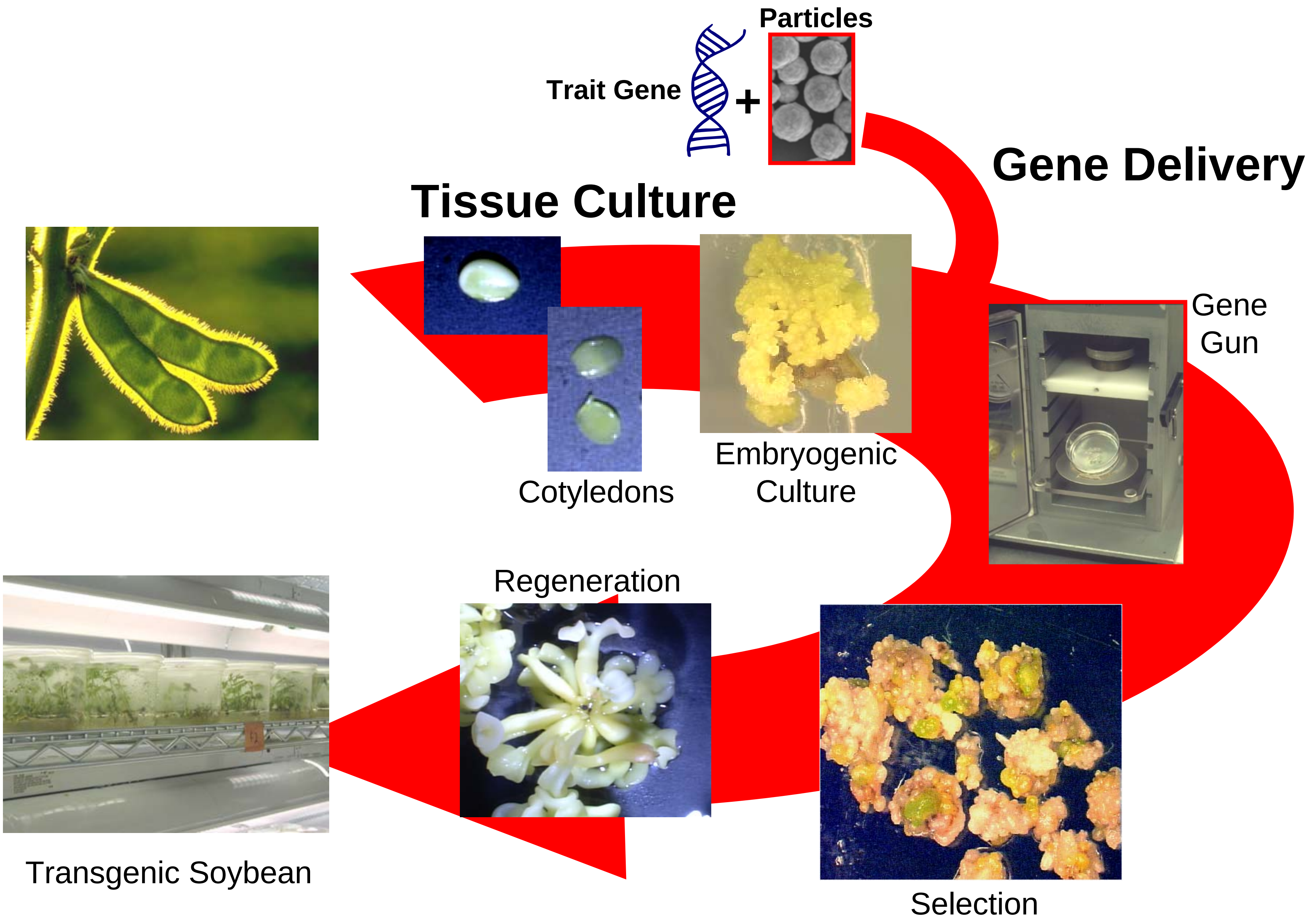
The polyunsaturated fatty acids linoleate and α-linolenate are important membrane components and are the essential fatty acids of human nutrition. The major enzyme responsible for the synthesis of these compounds is the plant oleate desaturase of the endoplasmic reticulum, and its activity is controlled in Arabidopsis by the *fatty acid desaturation 2* (*fad2*) locus. A *fad2* allele was identified in a population of Arabidopsis in which mutations had been created by T-DNA insertions. Genomic DNA flanking the T-DNA was cloned by plasmid rescue and used to isolate cDNA and genomic clones of *FAD2*. A cDNA containing the entire *FAD2* coding sequence was expressed in *fad2* mutant plants and shown to complement the mutant fatty acid phenotype. The deduced amino acid sequence from the cDNA showed homology to other plant desaturases, and this confirmed that *FAD2* is the structural gene for the desaturase. Gel blot analyses of *FAD2* mRNA levels showed that the gene is expressed throughout the plant and suggest that transcript levels are in excess of the amount needed to account for oleate desaturation. Sequence analysis identified histidine-rich motifs that could contribute to an iron binding site in the cytoplasmic domain of the protein. Such a position would facilitate interaction between the desaturase and cytochrome b_5, which is the direct source of electrons for the desaturation reaction, but would limit interaction of the active site with the fatty acyl substrate.

Using transgenes to silence endogenous genes in plants

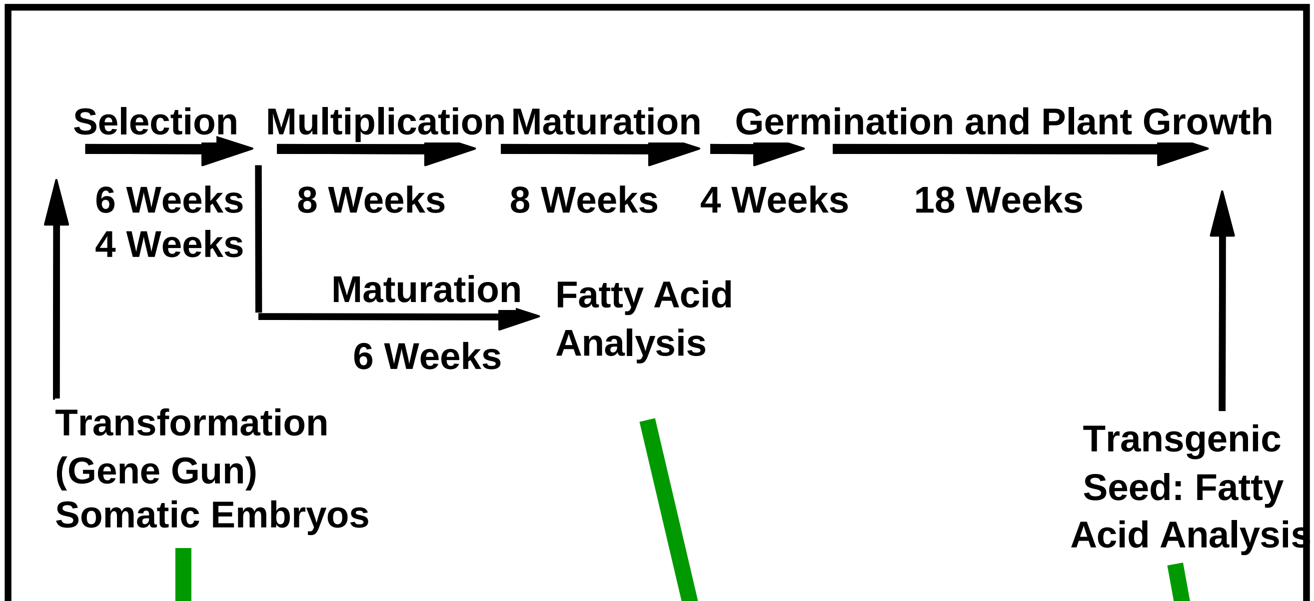


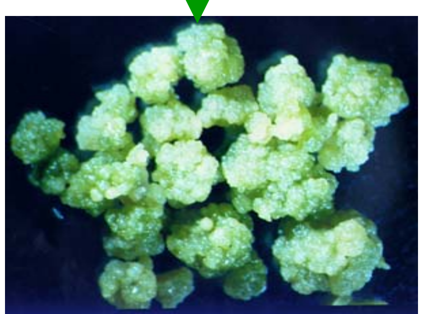
Using transgenes to silence endogenous genes in plants: seed specific Fad 2-1 silencing




Particles
Trait Gene
+
Gene Delivery
Tissue Culture
Gene
Gun
Embryogenic
Culture
Cotyledons
Regeneration
Transgenic Soybean
Selection

Putting genes into soy: somatic embryos and the particle gun

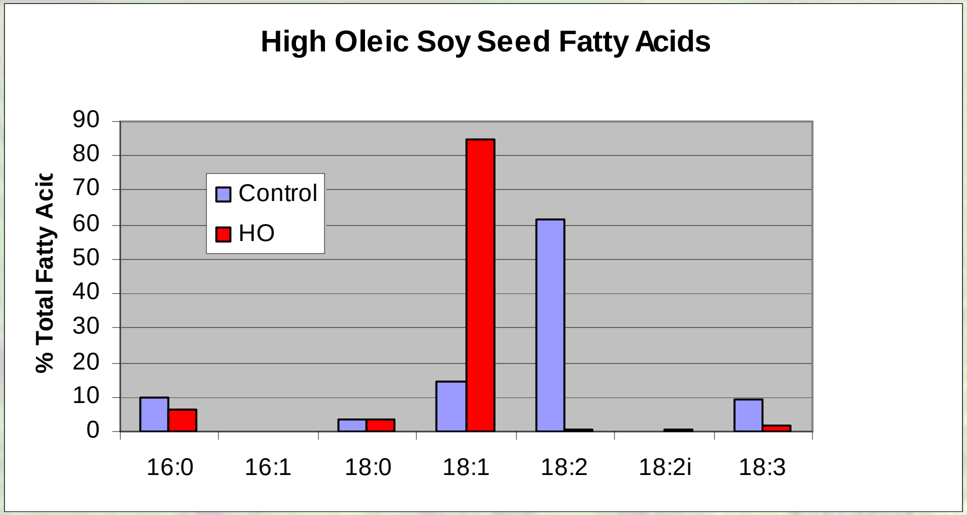




First GMO Soybean Developed for Consumer Benefit

Silencing of Fad 2-1 Gene in Soy Seeds

acetyl CoA C2 → palmitic acid C16:0 → stearic acid C18:0 → oleic acid C18:1 ┼→ linoleic acid C18:2 → linolenic acid C18:3

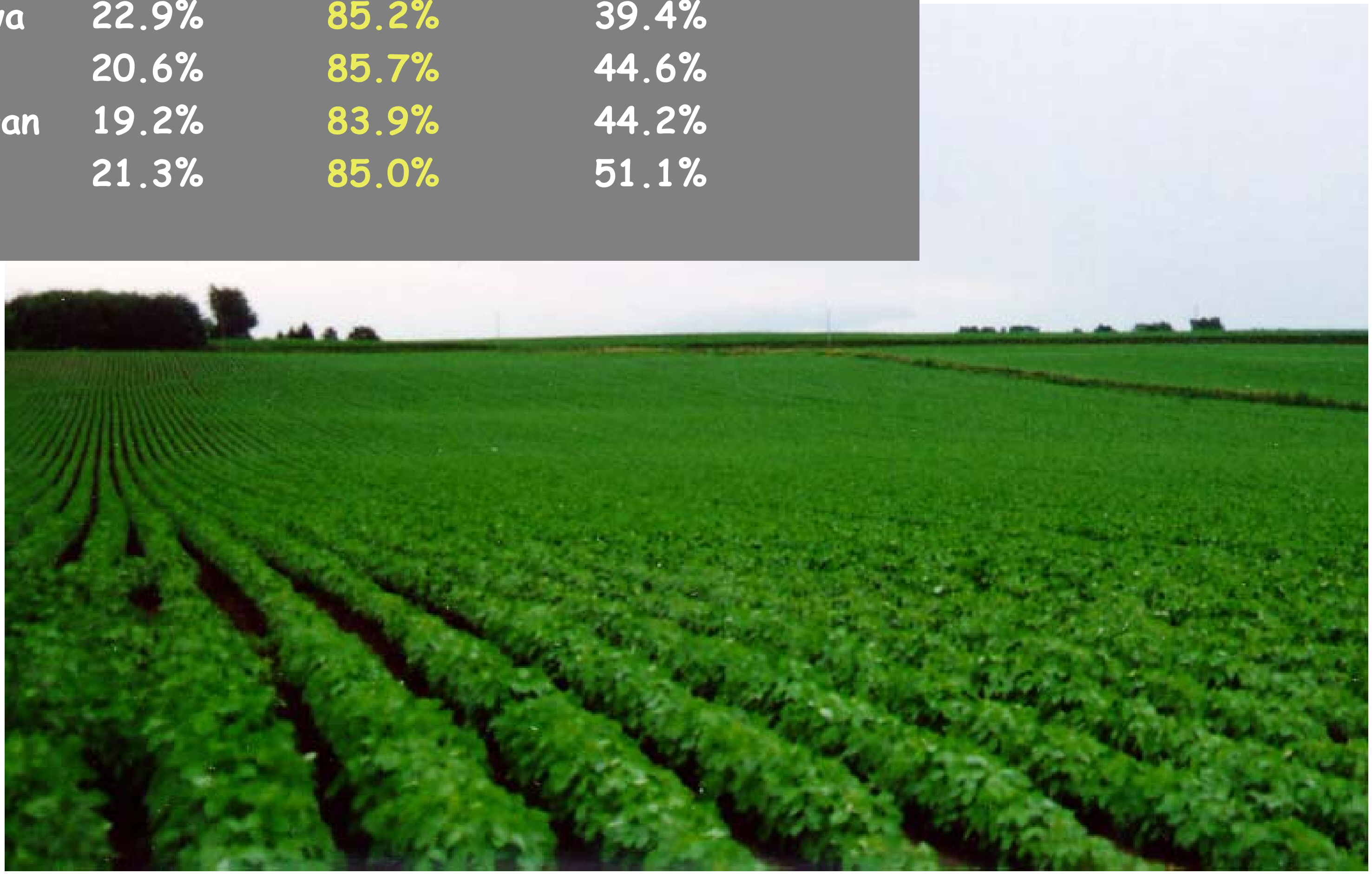


Clear liquid oil

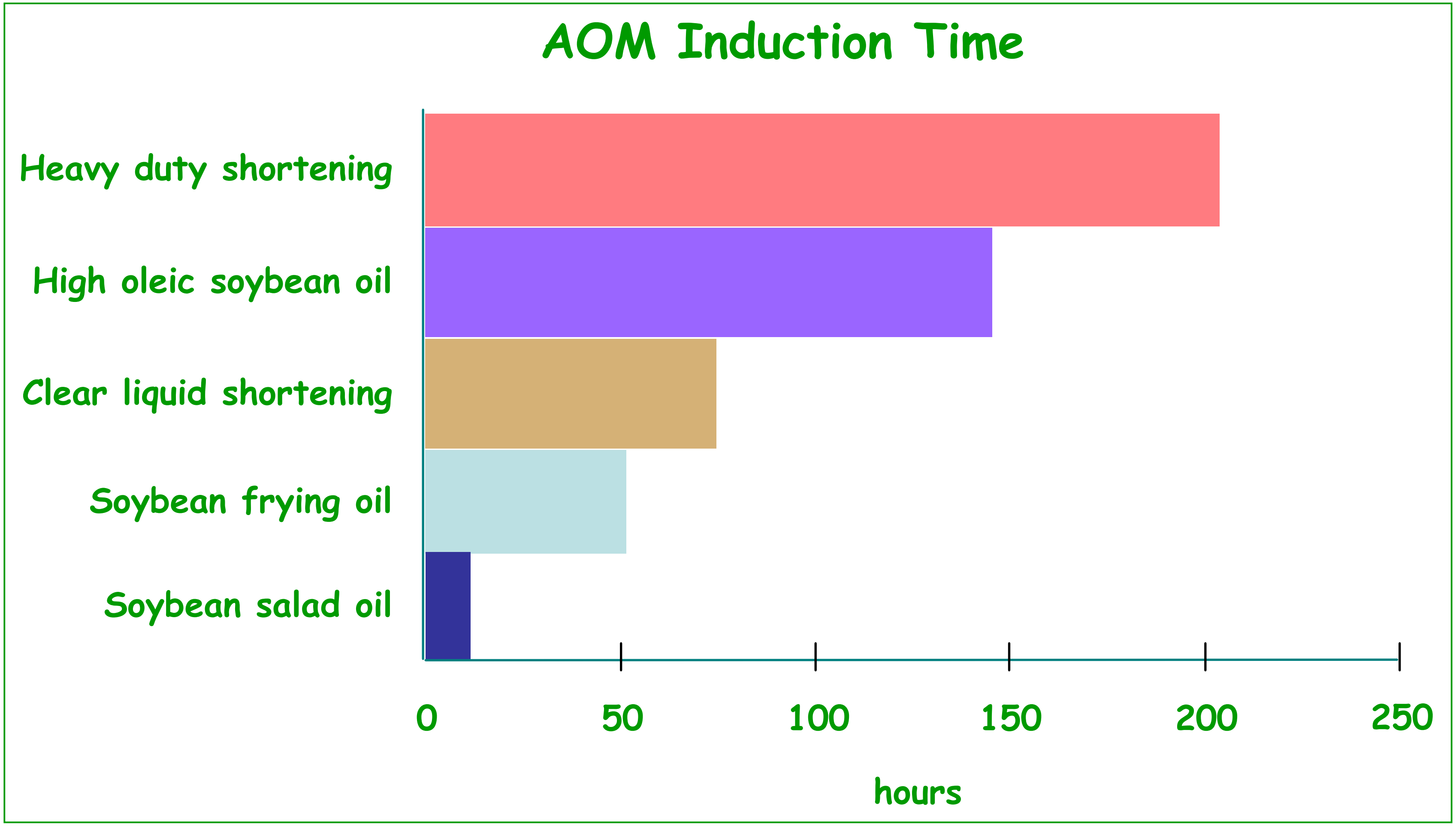
Polyunsaturates reduced from >70% to less than 5%
Oleic acid increased from 15% to 85%
Total saturates reduced from 14% to less than 10%

High Oleic Acid Trait is Environmentally Stable

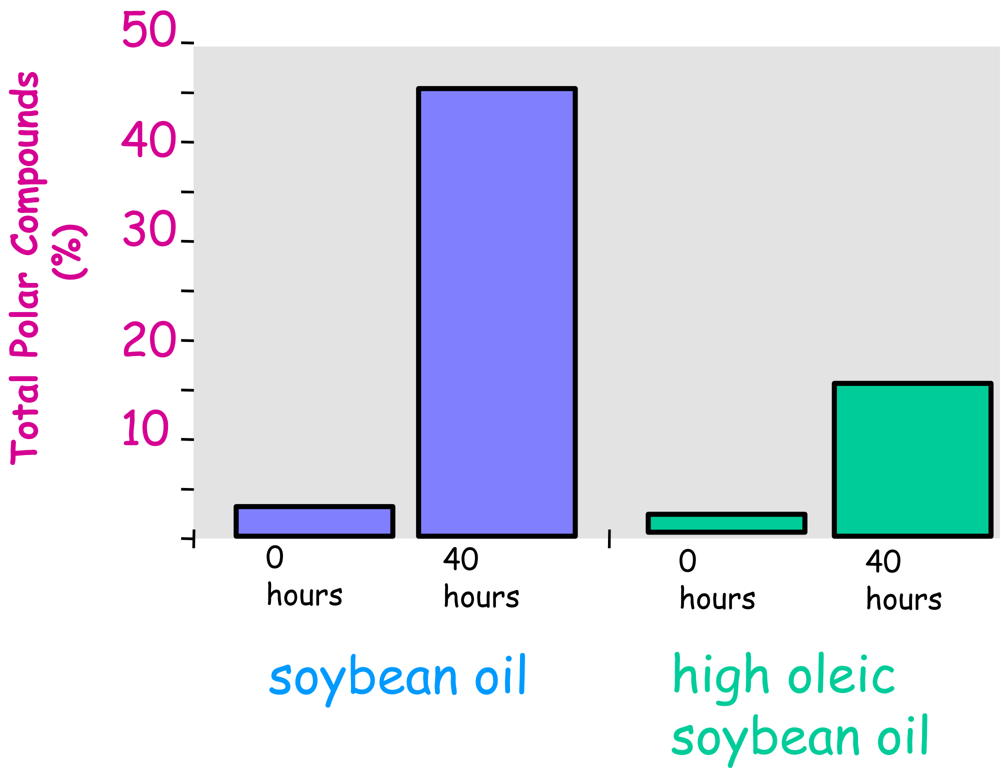
Location	Avg. Oleic Acid Content		
	Elite	*G168-12*	*L2494*HO7-9*
Ames, Iowa	20.3%	86.7%	56.0%
Cedar Rapids, Iowa	22.9%	85.2%	39.4%
Iowa City, Iowa	20.6%	85.7%	44.6%
Kalamazoo, Michigan	19.2%	83.9%	44.2%
Stine, Delaware	21.3%	85.0%	51.1%



High Oleic Acid Oil is Oxidatively Stable



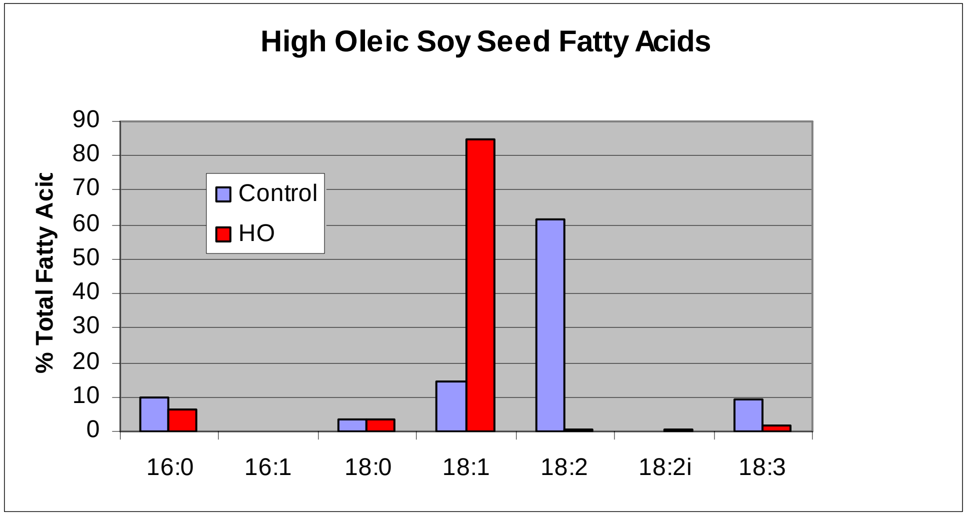
High Oleic Soybean Oil Superior for French Fries



French Fries, 40 hrs, 190 C

Cooking performance similar to partially hydrogenated oils

High Oleic Soy:
first GMO Soybean Developed for Consumer Benefit



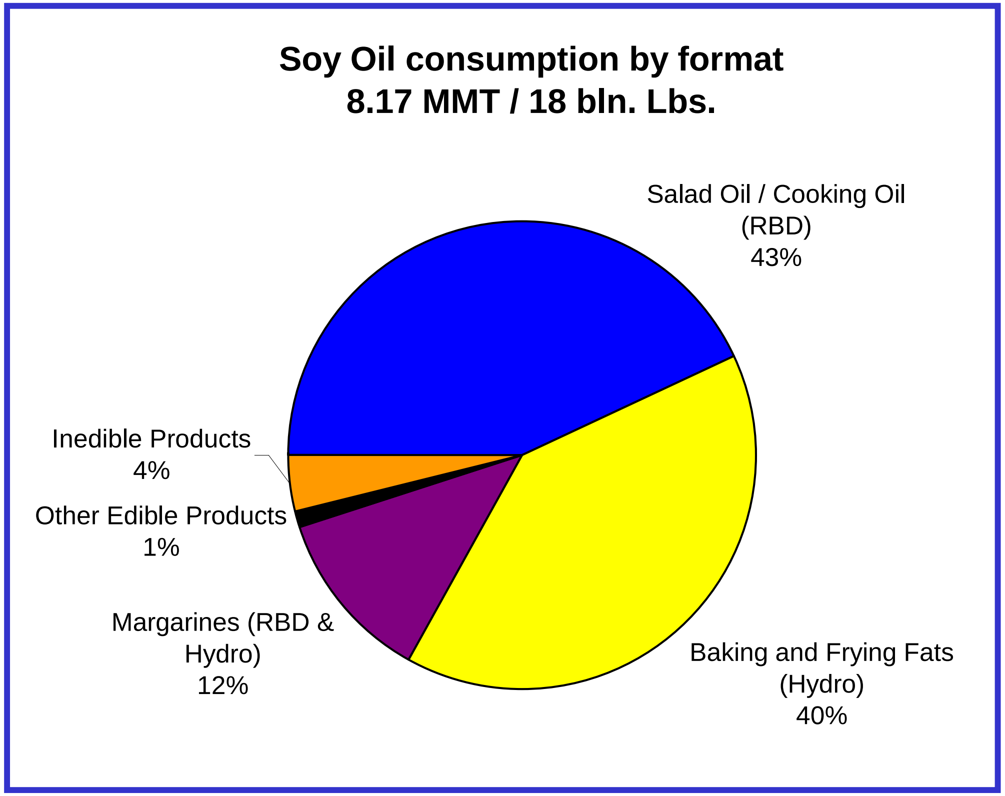
Clear liquid oil

Polyunsaturates reduced from >70% to less than 5%
Oleic acid increased from 15% to 85%
Total saturates reduced from 14% to less than 10%

- US regulatory package (FDA) completed 1996
- USDA-APHIS deregulated in 1996
- Commercialization on hold until 2003

US Soybean Oil Production 2005

Hydro Soy in Baking and Frying Fat applications (8 billion lbs) is likely to be replaced substantially with alternatives



At what value?

Cost of hydrogenation: $0.01-0.05/lb

DuPont Agriculture and Nutrition 2005

Pioneer:
Seed, Seed treatments & Traits

Solae Company:
Food Ingredients

Eighth Continent:
Soy food products

Input Providers → **Growers** → **Grain Processors** → **Food Mfg.** → **Food Sales**

Crop Protection:
Crop Protection Solutions

Qualicon, LiquiBox:
Food safety, packaging

DuPont-Bunge Alliance:
Edible oils, animal feed

BUNGE

In July 2003 the FDA announced that it will require mandatory *trans* fat labeling effective January 2006

Nutrition Facts

Serving Size 1 cup (228g)
Servings Per Container 2

Amount Per Serving

Calories 260	Calories from Fat 120
	% Daily Value*
Total Fat 13g	**20%**
Saturated Fat 5g	**25%**
Trans Fat 2g	
Cholesterol 30mg	**10%**
Sodium 660mg	**28%**
Total Carbohydrate 31g	**10%**
Dietary Fiber 0g	**0%**
Sugars 5g	
Protein 5g	

Vitamin A 4%	•	Vitamin C 2%	
Calcium 15%	•	Iron 4%	

* 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
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

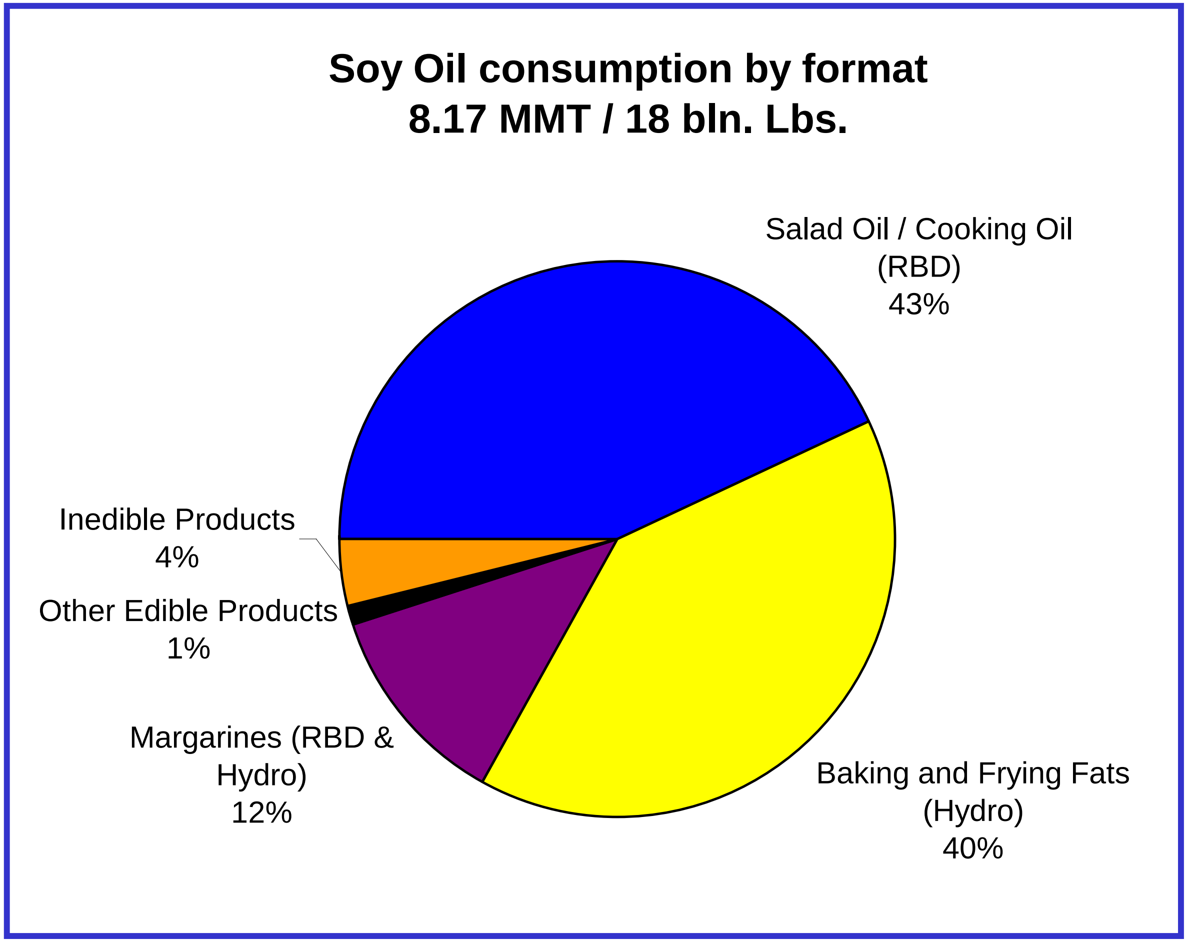
Calories per gram:
Fat 9 • Carbohydrate 4 • Protein 4

***Trans* Fat 2g**

Low linolenic (18:3) soy varieties will begin to replace hydrogenated soy oil in 2005

NUTRIUM™

Baking and Frying Fats:
High Oleic Soy will replace 10-20% of hydrogenated oil by 2012



High Oleic Soy Summary:

- Technically straightforward: one gene
- Low added value to grain, very large volume
- Required complex marketing infrastructure and stars to be in right place to move to market

......and now for something completely different

Cardiovascular Effects of Fatty Acid Consumption: State of the Heart?

Most saturated fats	Bad	Low sat soy
Some saturated fats (stearic)	Neutral/Good	HO-HS soy
Most monounsaturated fats	Good	High oleic soy
Monounsaturated *trans* fats	Bad	High oleic soy
Some *trans* fats (CLA)	Good	CLnA soy

Most beneficial fats mediate their positive health effects through blood lipids (HDL/LDL)

Beneficial Effects of Long Chain omega-3 PUFAS: *All Unrelated to Blood Lipid Content*

- Cardiovascular disease (*prevent arrhythmias*)
- Anti-inflammatory (*transcriptional regulators*)
- Anti-thrombotic (*platelet aggregation*)
- Stimulates NO (*vascular control*)
- Mental/CNS (*depression, schizophrenia, AHD, cognitive development*)

Strongest clinical data relate to heart disease

GISSI study (1999) 48 months

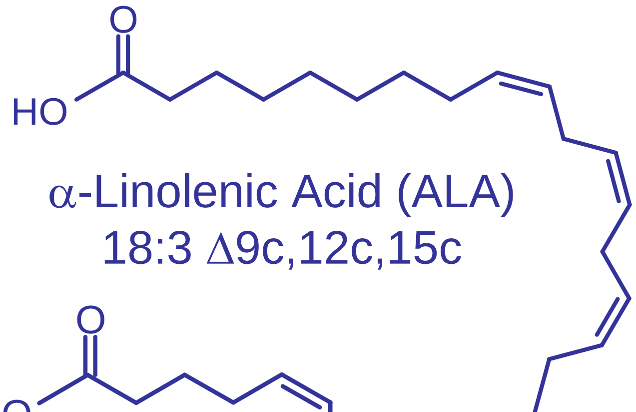
11324 post-MI

EPA/DHA capsules (850mg/day)	26% reduction in mortality/ non fatal MI/CHD

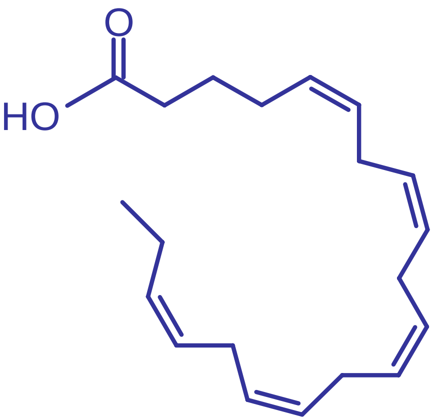
- comparable to drugs in mortality reduction
- reduced ventricular arrhythmia
- synergistic effect to lowering blood lipids

What are these LCPUFAs?

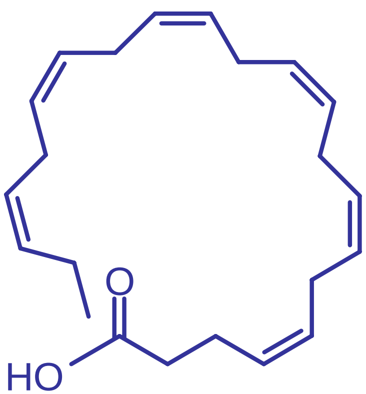
omega-3 PUFAs



α-Linolenic Acid (ALA)
18:3 Δ9c,12c,15c

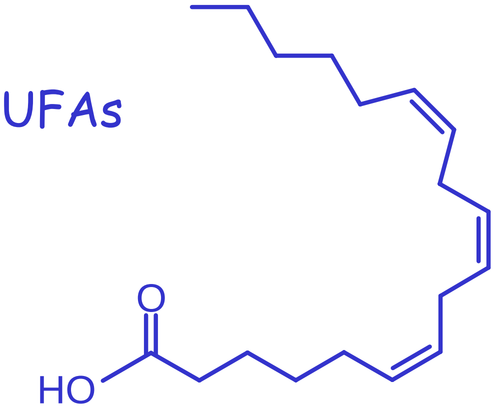


Eicosapentaenoic Acid (EPA)
20:5 Δ5c,8c,11c,14c,17c

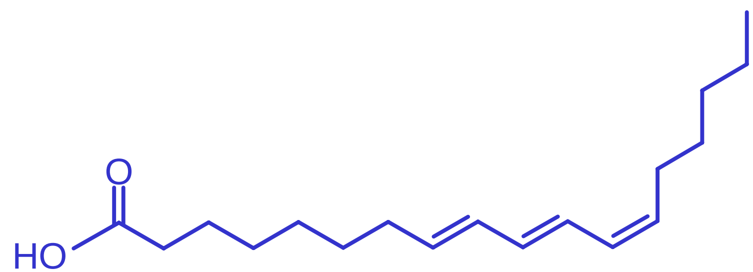


Docosahexaenoic Acid (DHA)
22:6 Δ4c,7c,10c,13c,16c,19c

omega-6 PUFAs

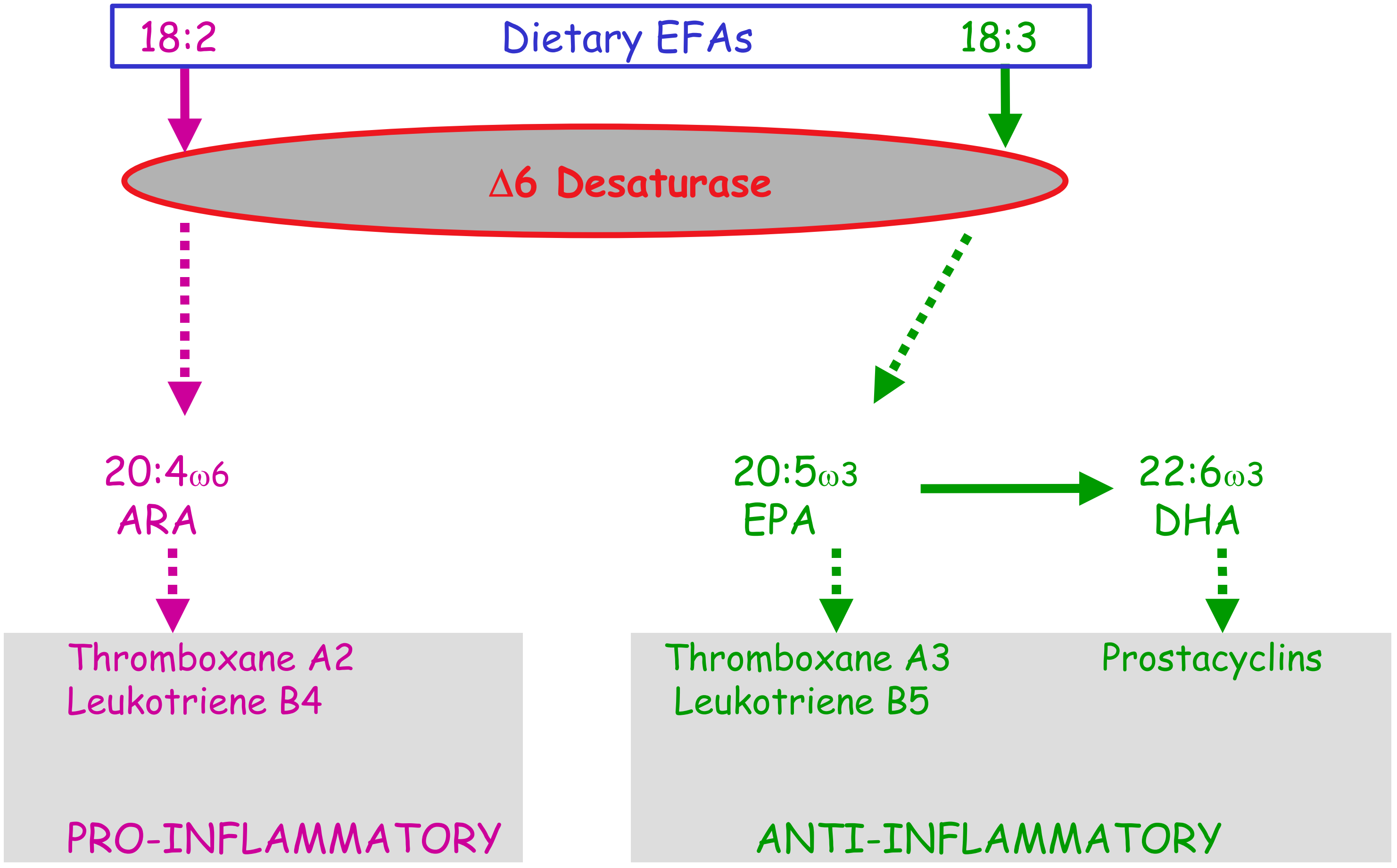


γ-Linolenic Acid (GLA)
18:3 Δ6c,9c,12c



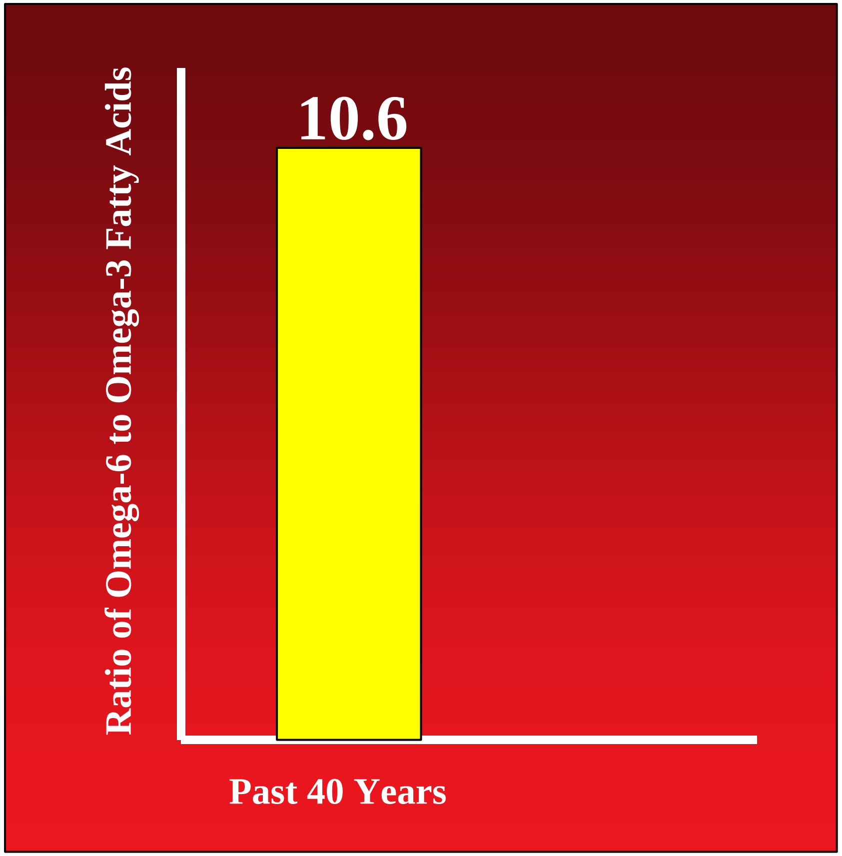
Calendic Acid (CLnA)
18:3 Δ8t,10t,12c

Pathways in humans



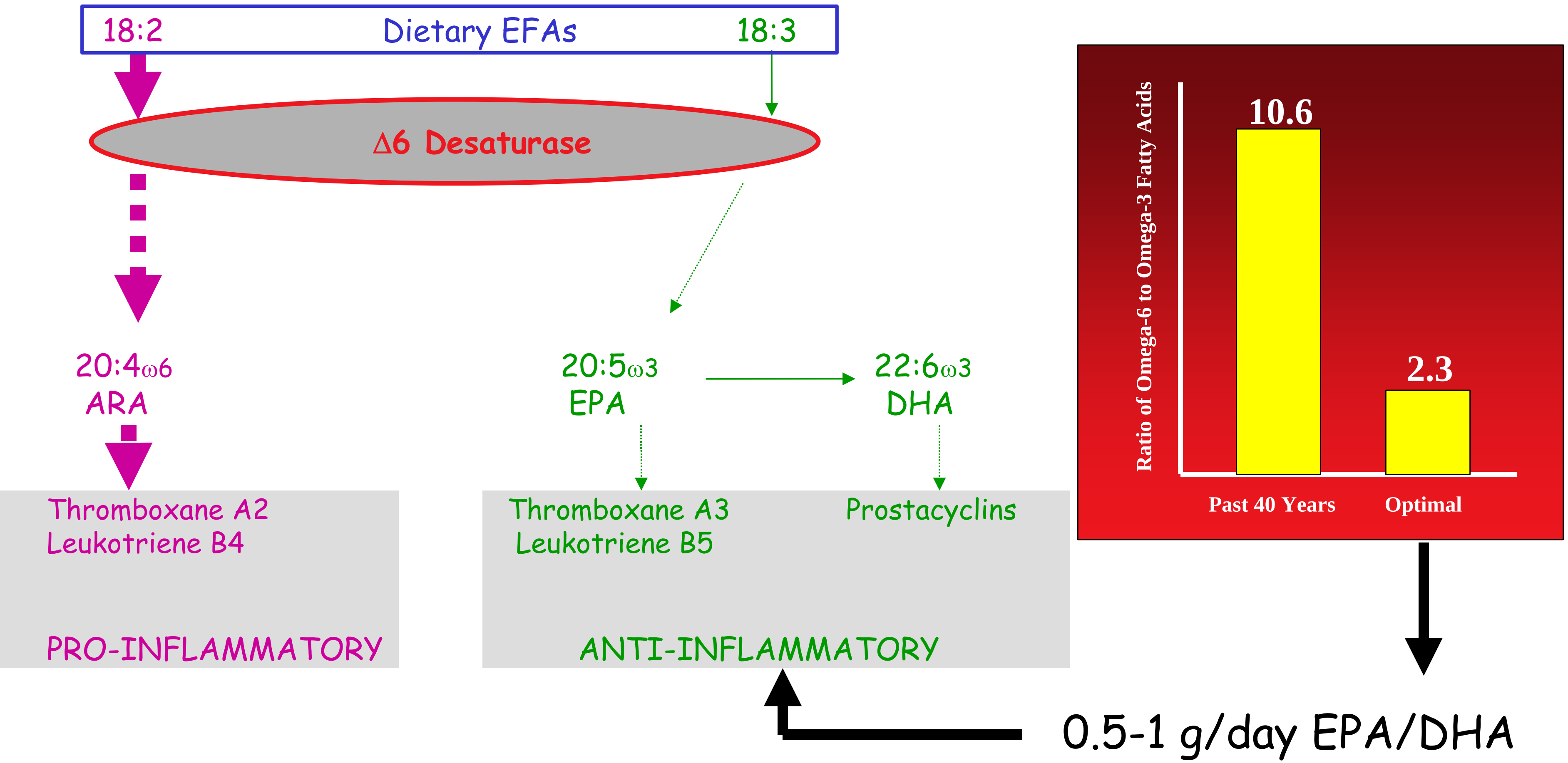
If our body makes omega-3s why do we need to eat them?

The ratio of omega-6 to omega-3 in our diet is important

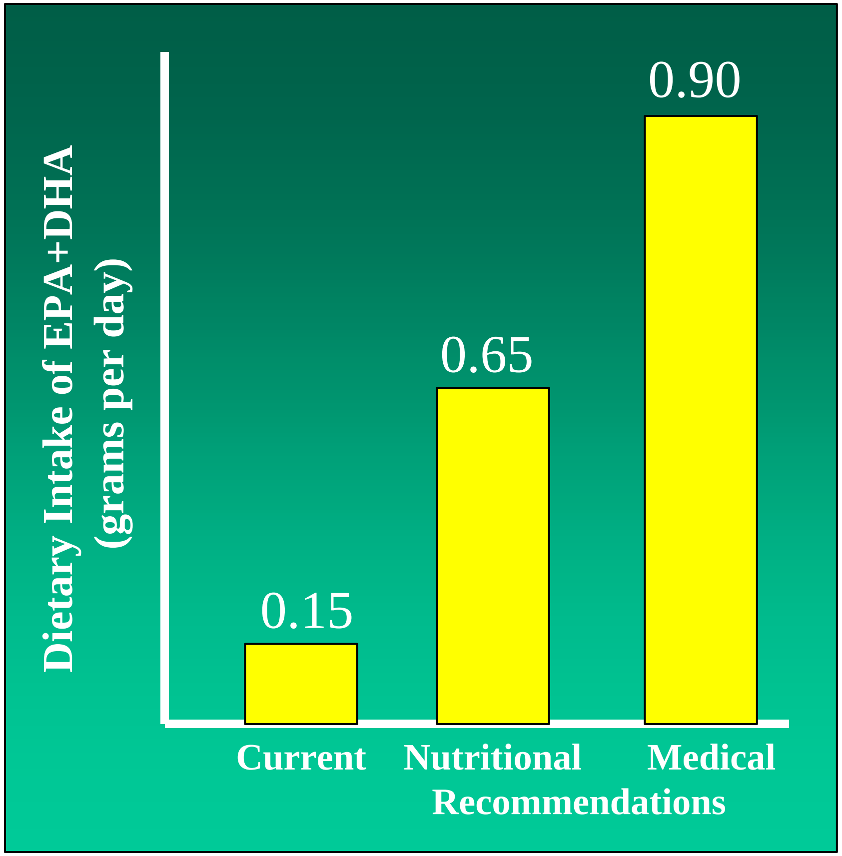


The ratio of omega-6 to omega-3 fatty acids has been relatively constant in the US food supply for the past 40 years

The ratio of omega-6 to omega-3 in our diet is important



An increase in omega-3 consumption to 0.5-1.0 g/day is recommended by experts



- **Kris-Etherton etal (2000)**
  Am. J. Clin. Nutr. 71:179S
- **ISSFAL Committee (1999)**
  Ann. Nutr. Metab. 43:127
- **American Heart (2000)**
  Circulation102:2284

Where do the LCPUFAS in our diet come from?

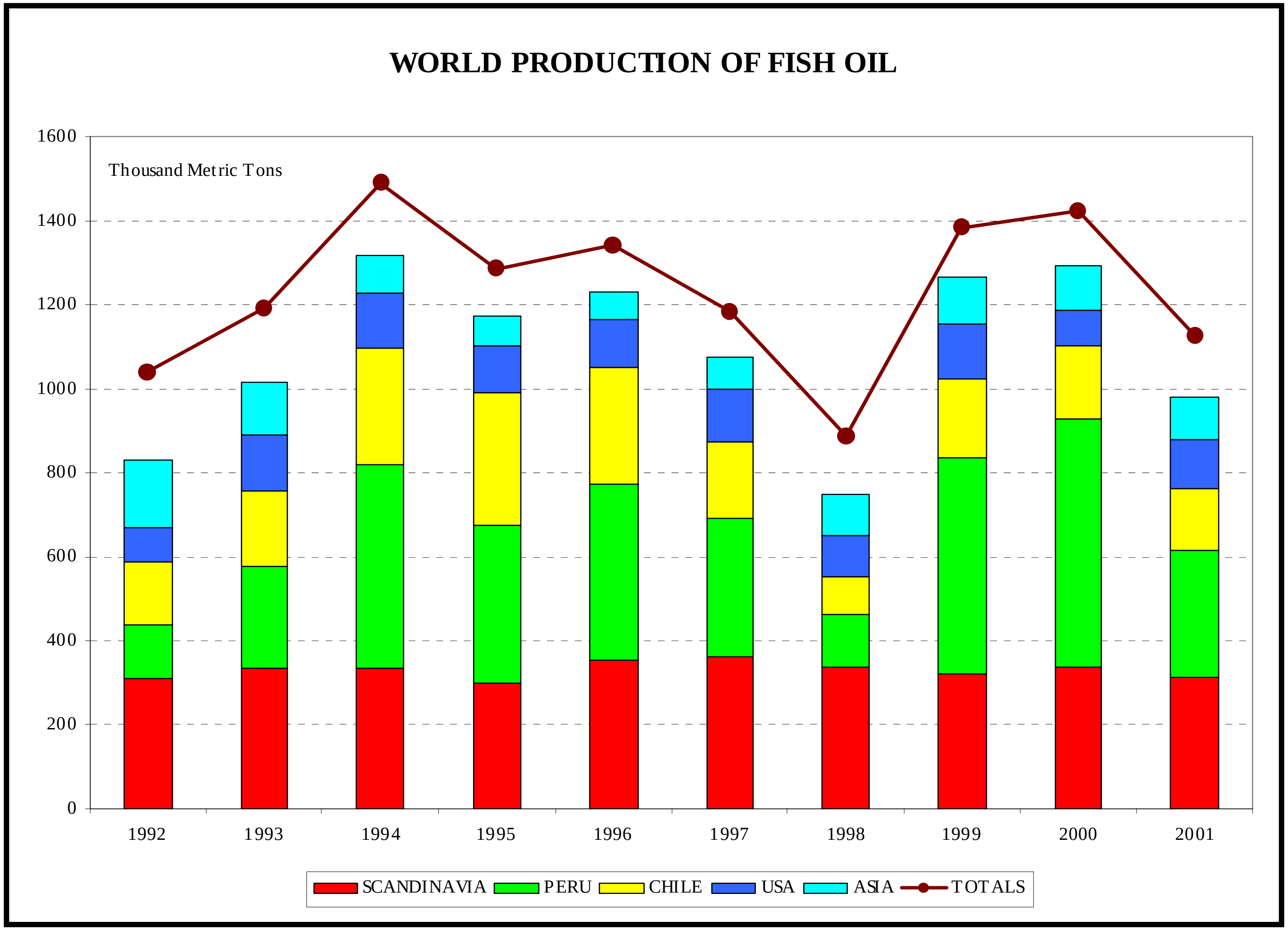
Consumer awareness and market demand high

% Consumer Recognition

Soy Products		Omega 3	
Good Source of Protein	19	Lowers risk of Heart Disease	41
General Good Health	17	Lowers Cholesterol	38
Relieves MenopausalSym.	15	Good for the Skin	14
Lower Cholesterol	15	Lowers Risk of Cancer	11
Prevents Osteoporosis	14	General Good Health	9
Lowers risk of Heart Disease	11	Improves Mental Function	9
Lowers Risk of Cancer	9	Relieves ADD	3
None	25	None	13

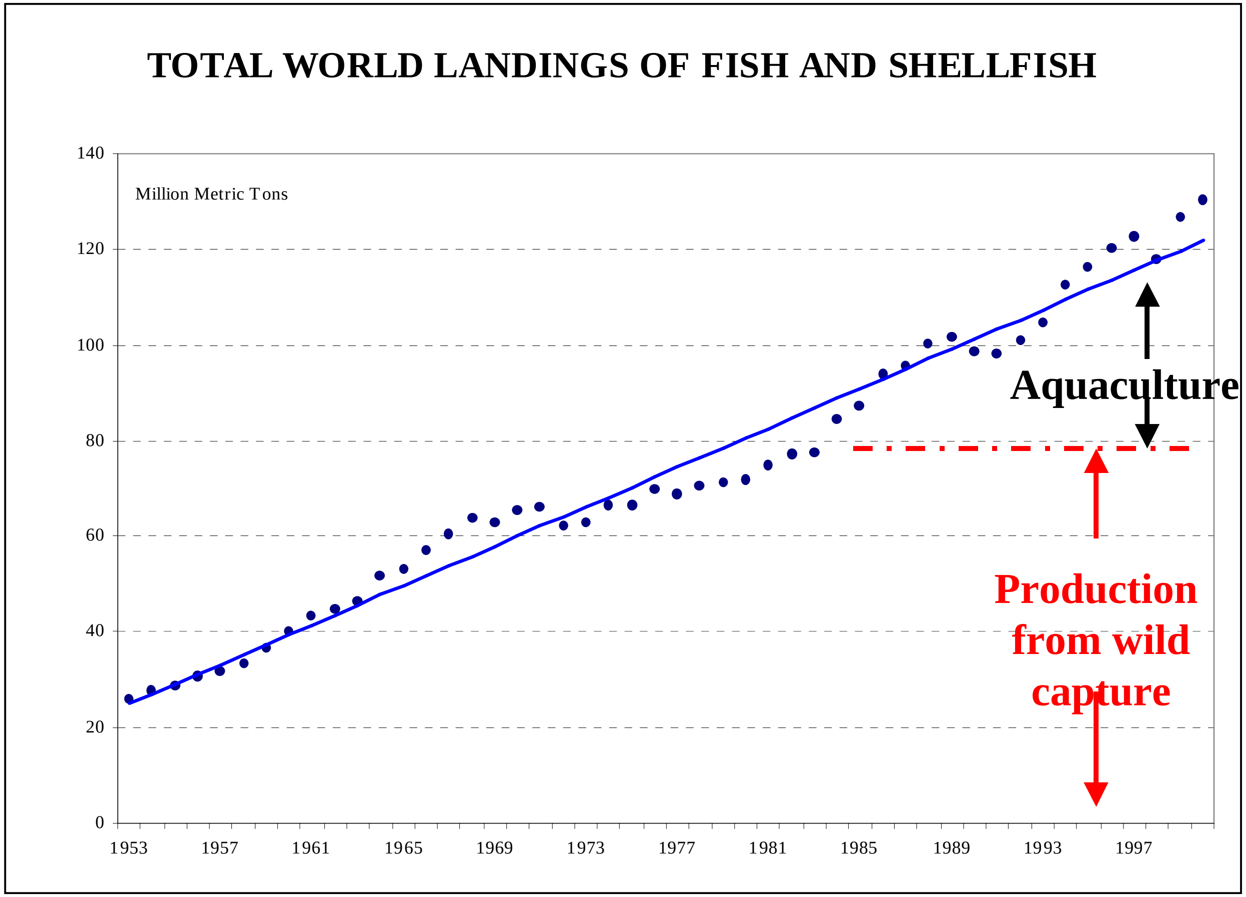
2001 Gallup Poll

There is increasing consumer demand for LCPUFAS



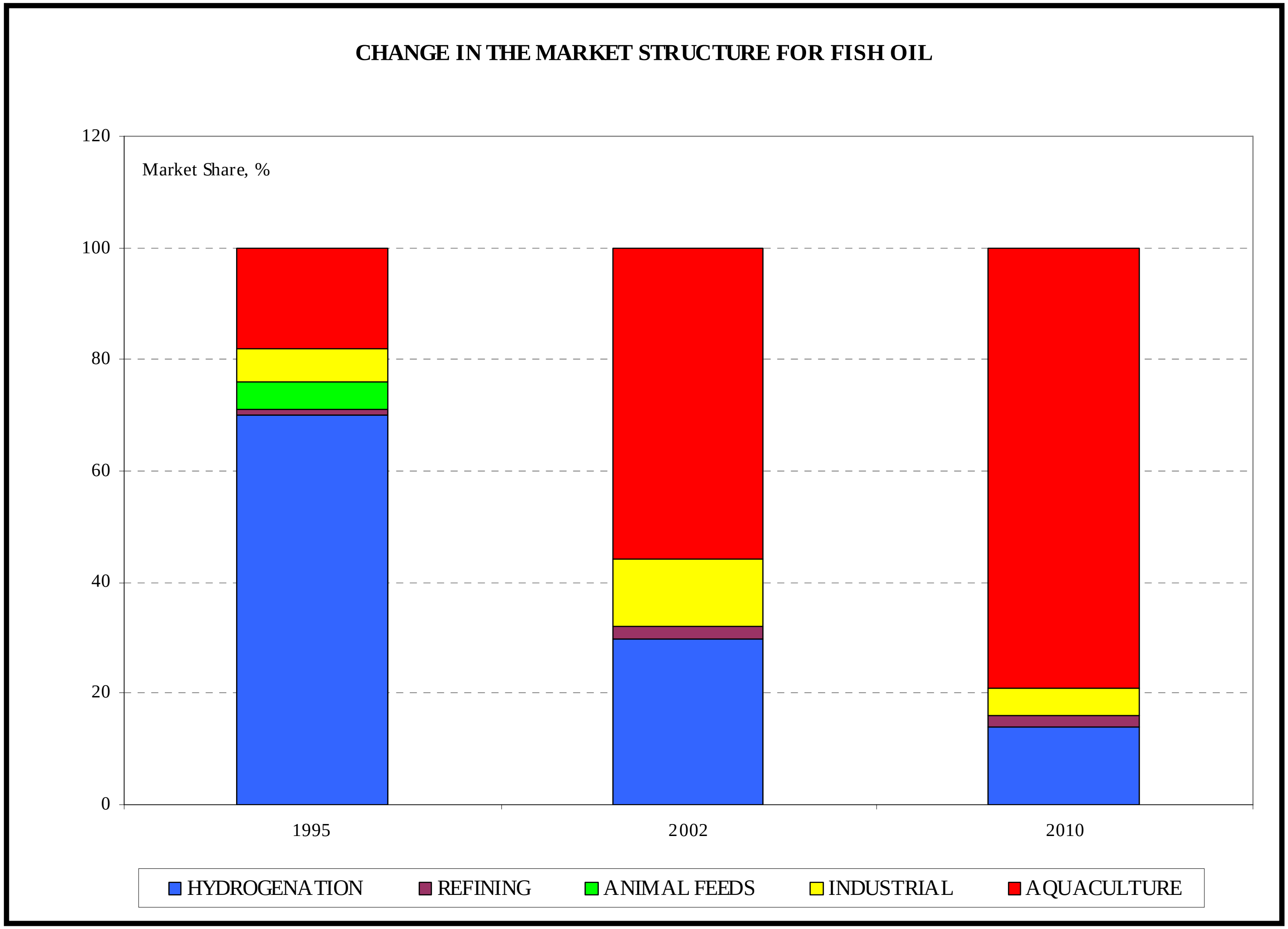
Current omega-3 fatty acid supply

There is increasing consumer demand for LCPUFAS

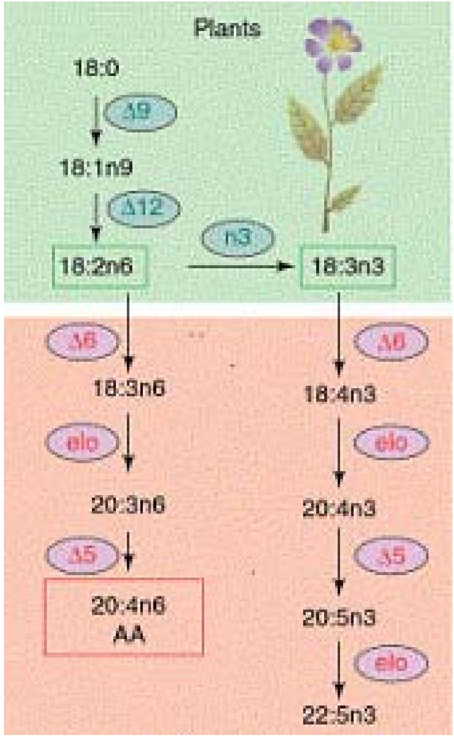


Use of omega-3 fatty acids for aquaculture is increasing

Use omega-3 fatty acids for aquaculture is increasing

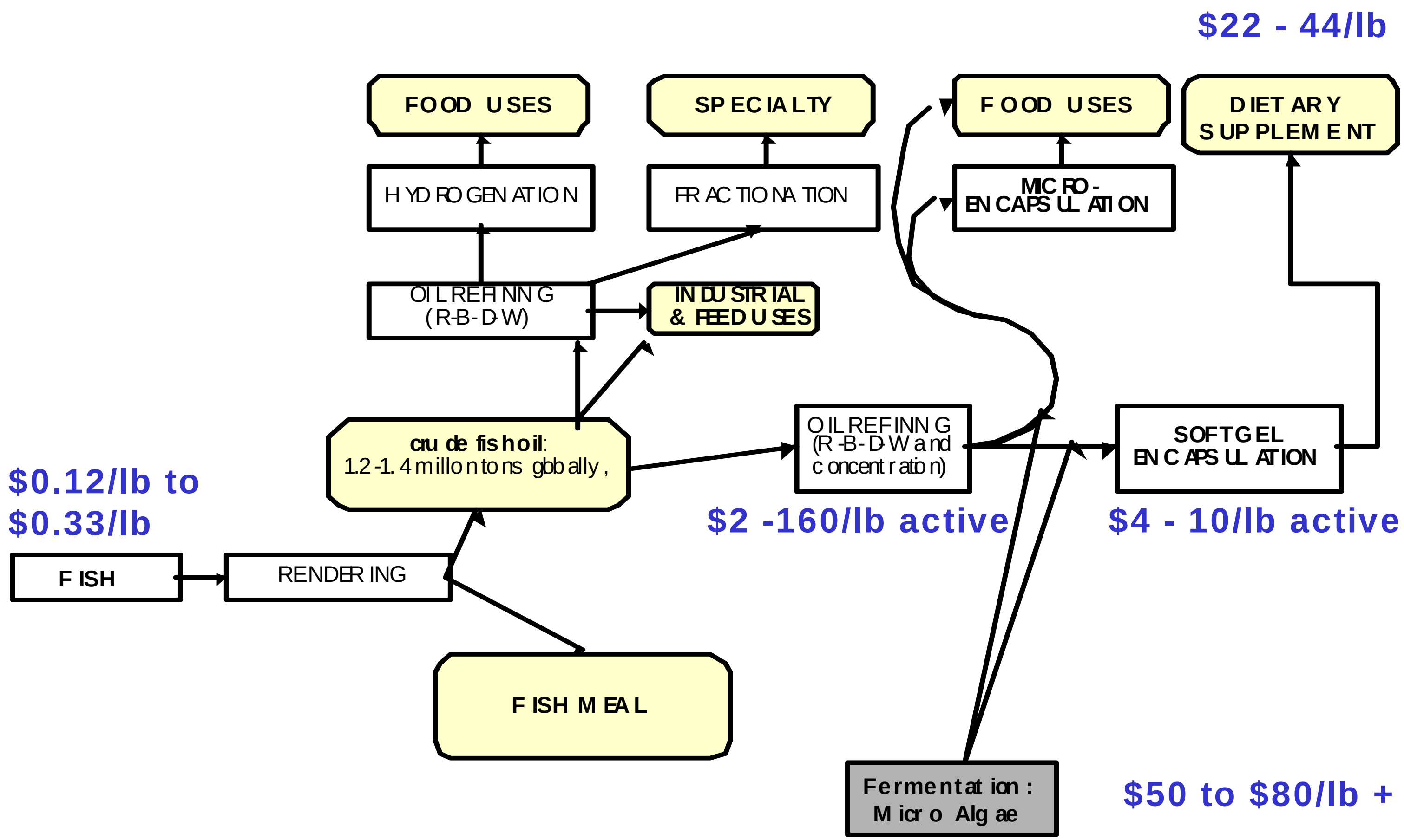


Providing safe, inexpensive, high quality and renewable source of long chain omega-3 for the human diet



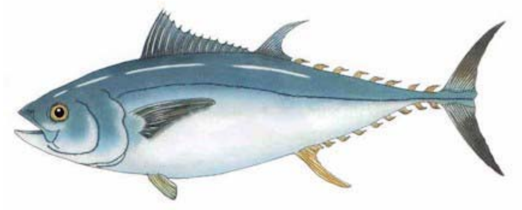
Adding an omega-3 lipid pathway pathway to soy: a technical challenge

Food Ingredient Market Requires Good Quality Oil



Food Ingredient Market is Small But High Value

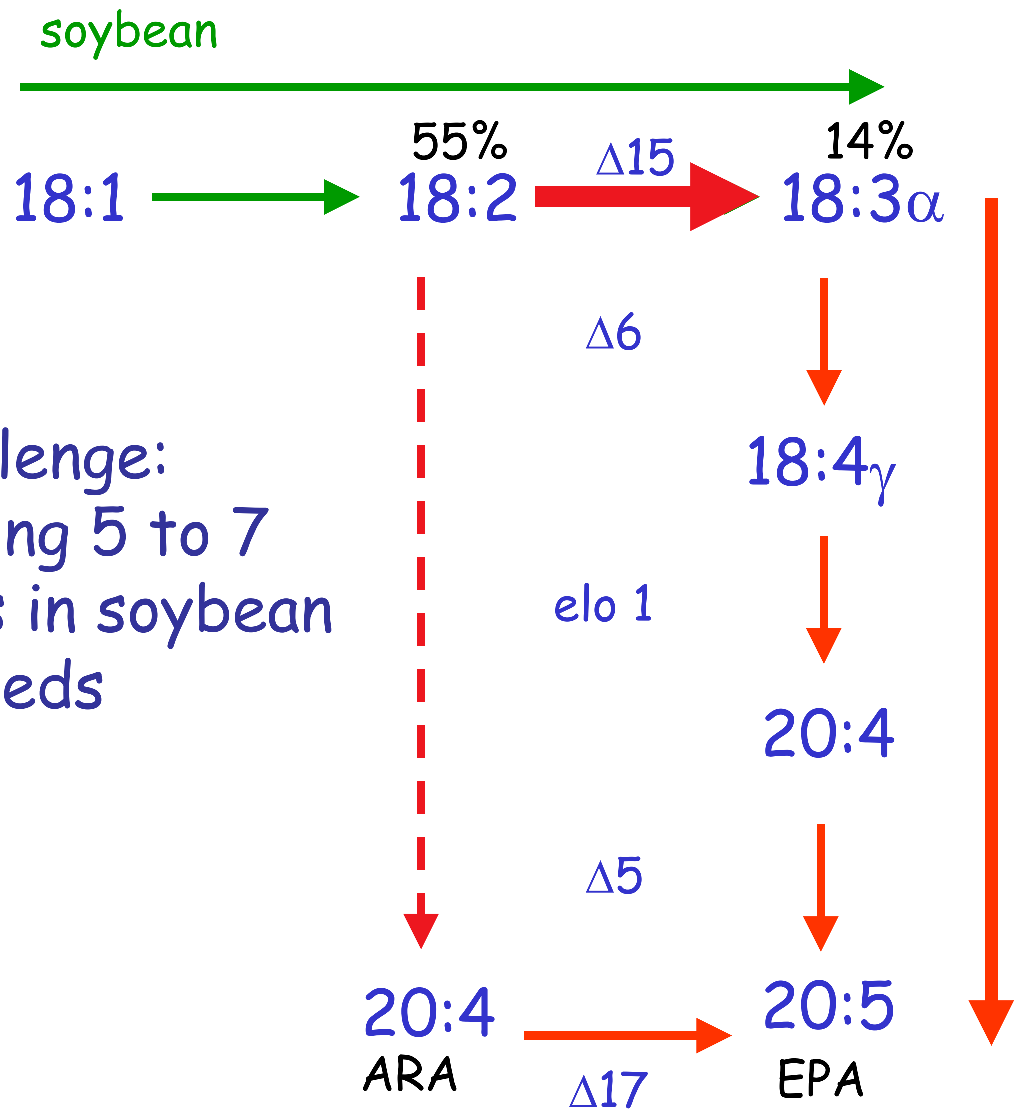
Where do the LCPUFAS in our diet really come from?



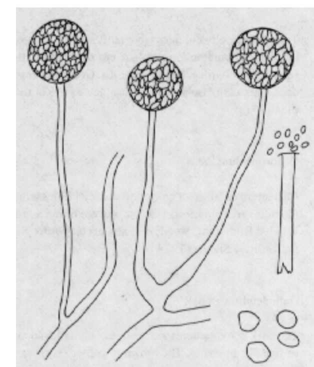
Mortierella alpina
35% LCPUFA
(ARA/EPA)

Saprolegnia diclina
30% LCPUFA
(ARA/EPA)

Adding an omega-3 lipid pathway pathway to soy



Challenge:
expressing 5 to 7
new genes in soybean
seeds



Introducing a multi-gene metabolic pathway into soybean seeds

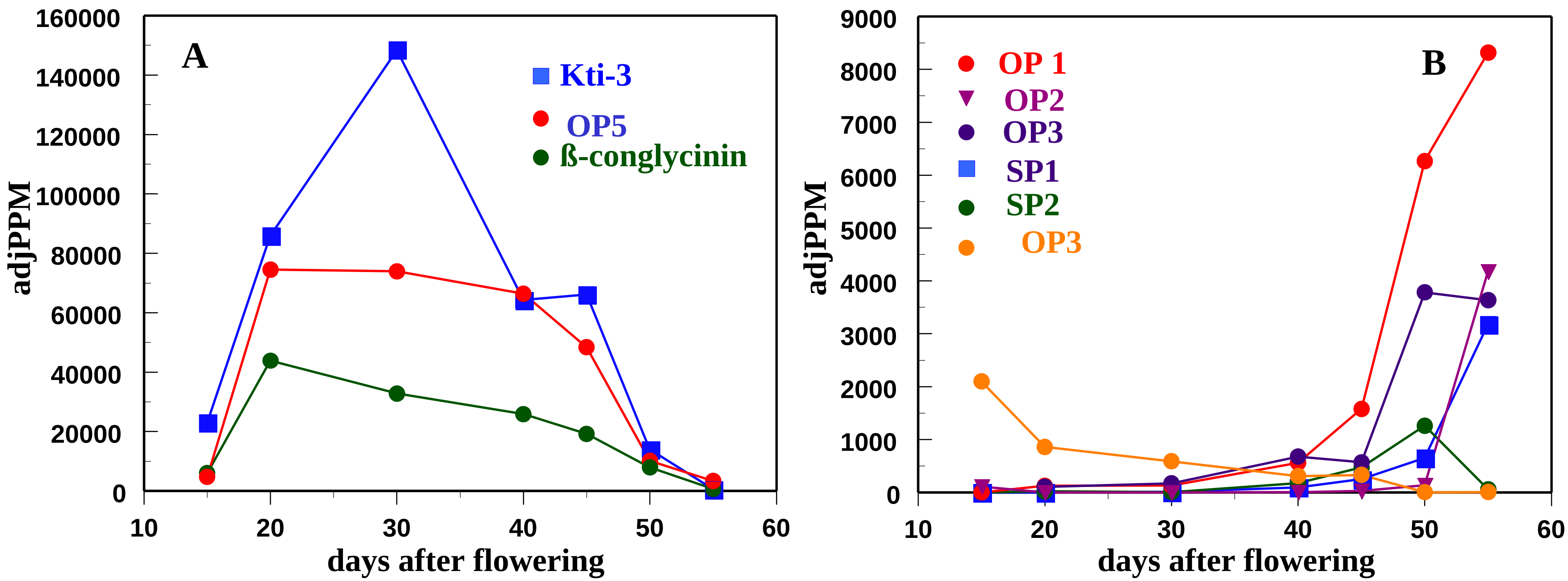
Key factors for success:

Multiple promoters needed
Model system that measures product
Promoters tested in metabolic/developmental context
Gene source for each enzyme in pathway
Construct design for expressing multiple genes
Minimum complexity of inserts

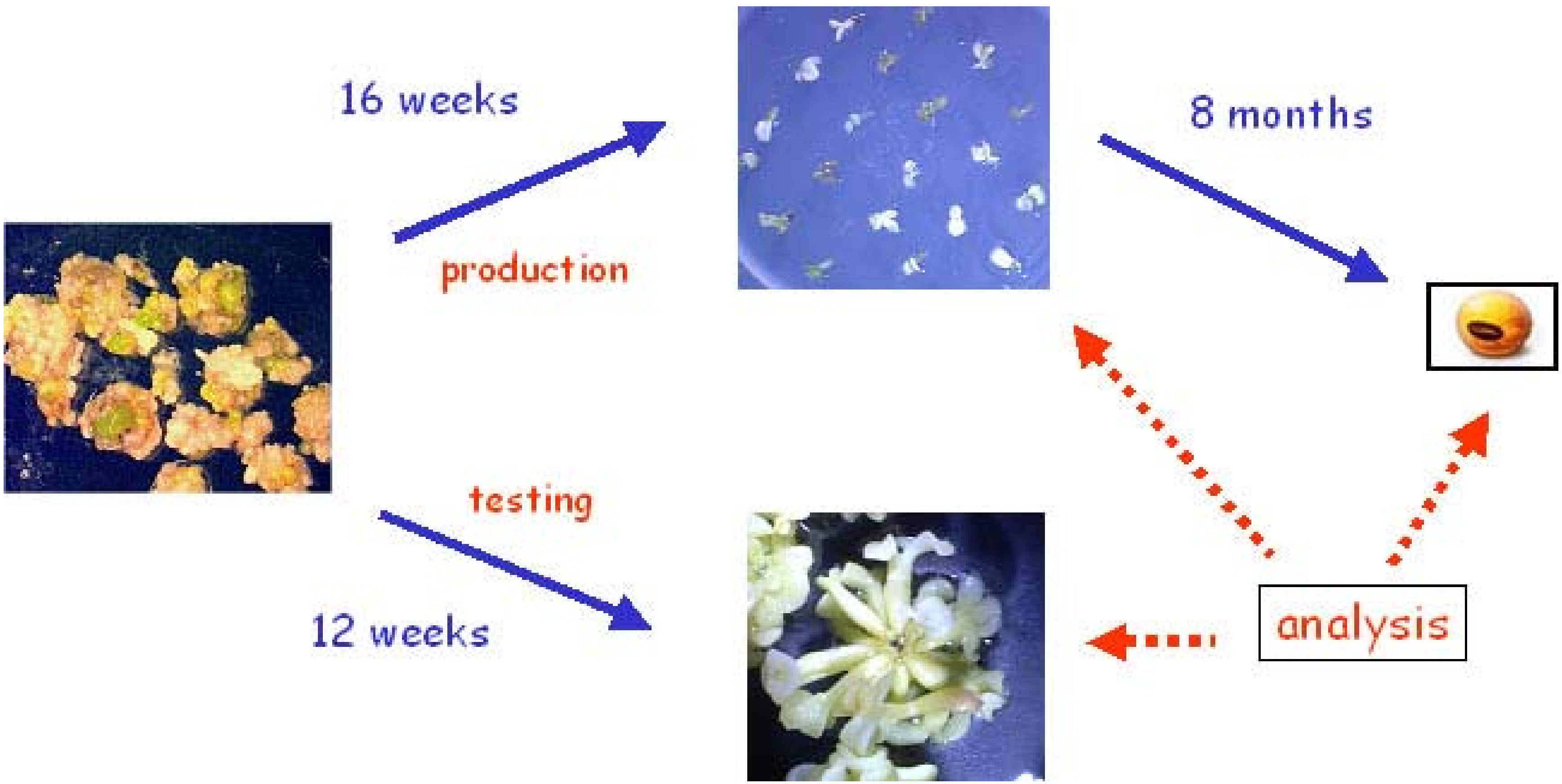
Multiple promoters needed
Promoter issues for expressing multiple genes in soy

- Initially only 2 fully tested soy embryo promoters effective for lipid modification
- Cosuppression of duplicate β-conglycinin α-subunit promoters
- Known recombination issues of KTi3 promoter on single fragment

MPSS Expression and promoter discovery

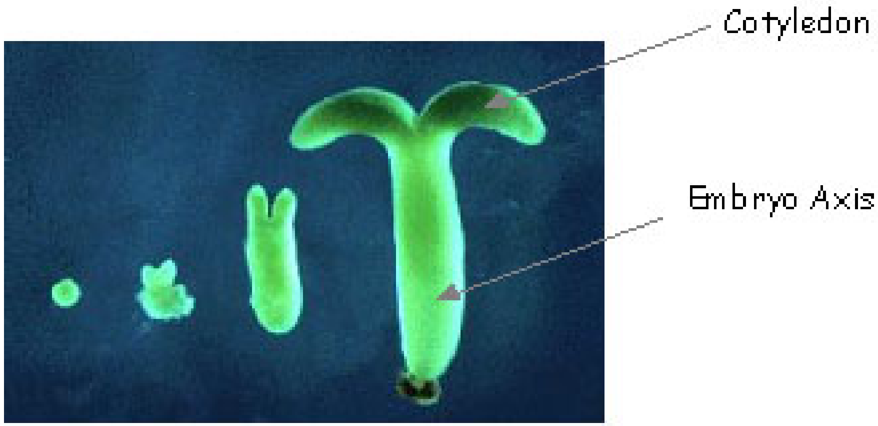


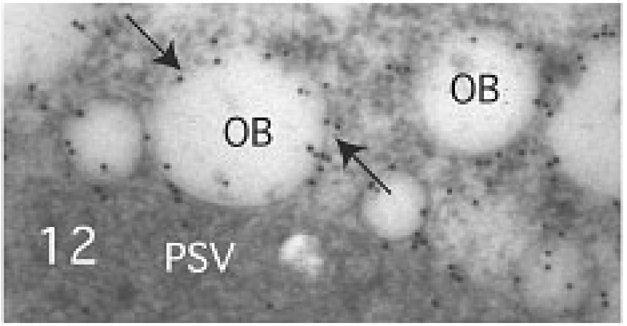
Model system that measures product



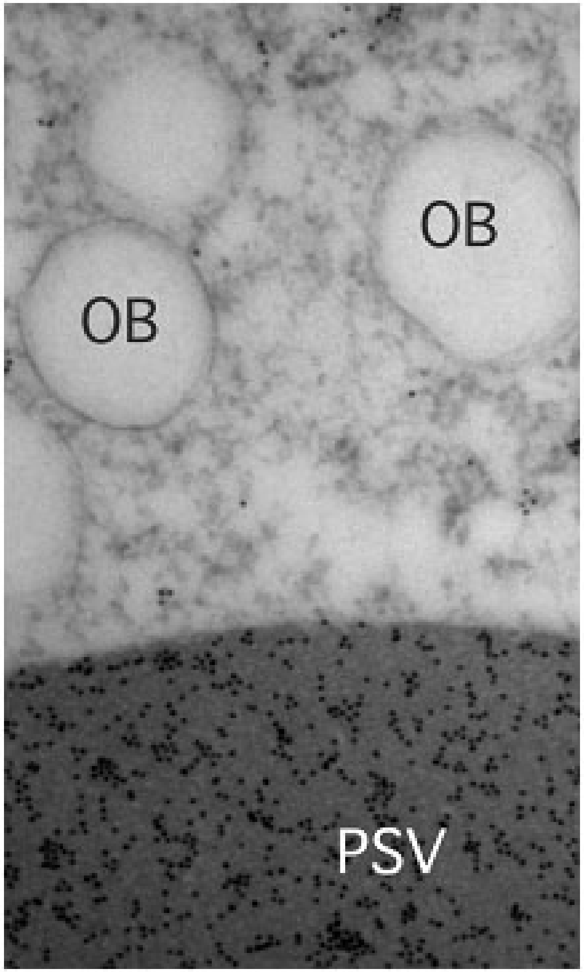
Time from transformation event to phenotype

Soybean transformation *via* somatic embryos: somatic embryos similar to zygotic embryos





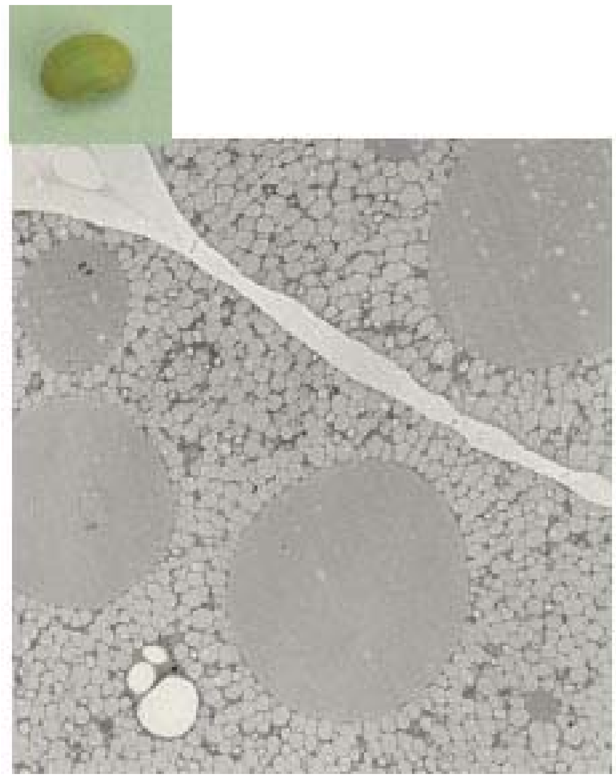
Anti-oleosin

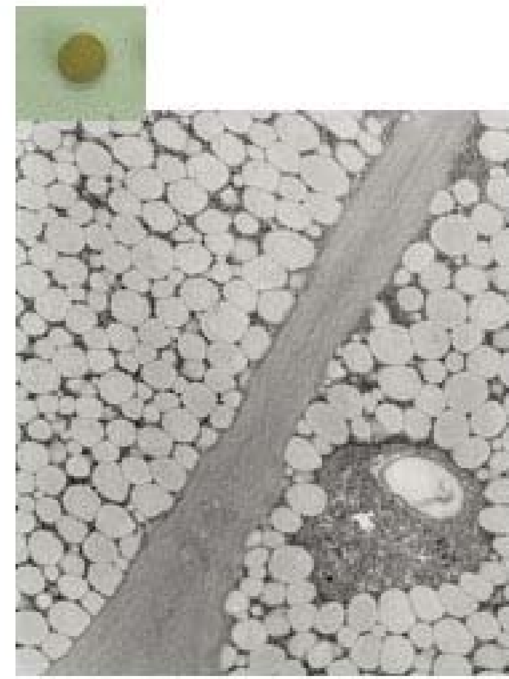


Anti-glycinin

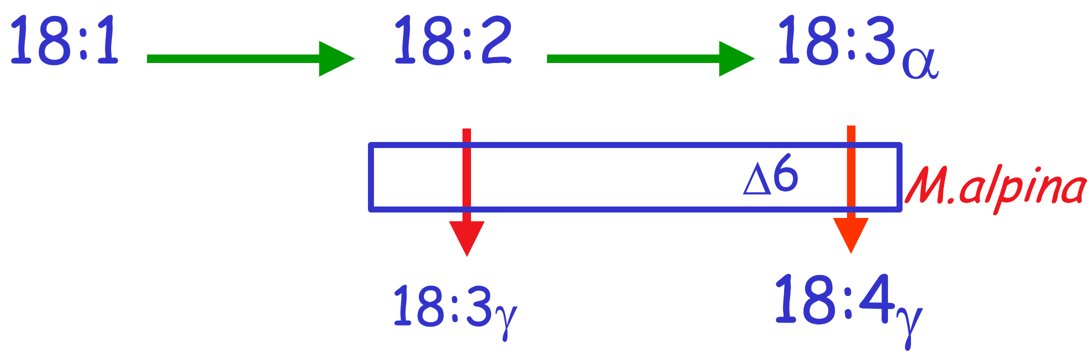
Model system that measures product:
TAG is formed over time and is not modified once made

15 DAF → 25 DAF





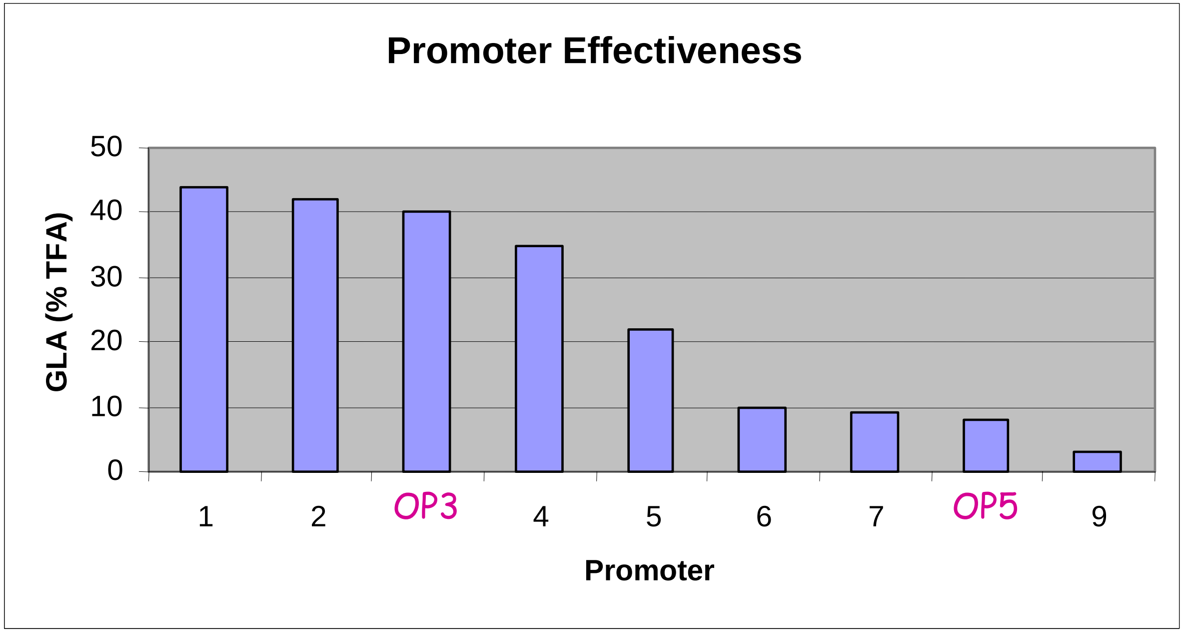
M. alpina delta-6 desaturase model for lipid expression

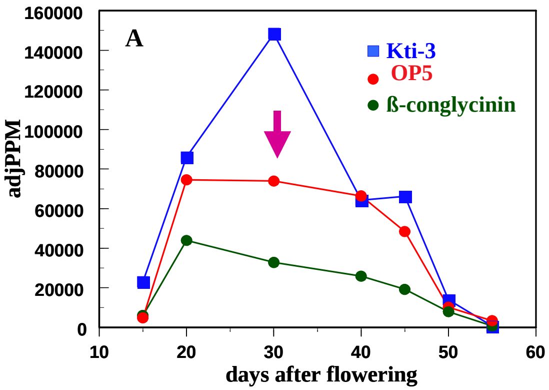


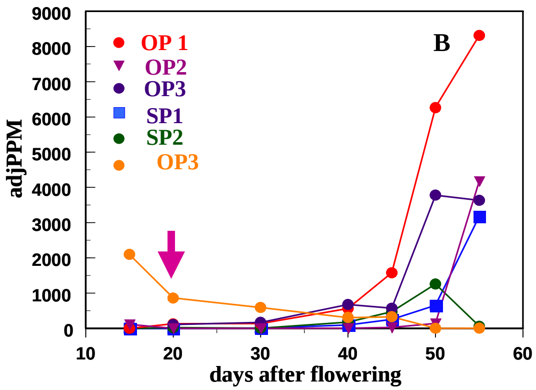
M.alpina		Borage		Jack
13%	16:0	13.4%	16:0	12.1%
2.5%	18:0	3.4%	18:0	3.7%
14%	18:1	7.9%	18:1	15.8%
9%	18:2	13.5%	18:2	54.7%
49%	18:3γ	48.5%	18:3γ	nd
4.2%	18:3α	5.6%	18:3α	13.6%
4.9%	18:4	7.7%	18:4	nd

Ranking of promoter effectiveness by GLA content of TAG

M.alpina Δ6 desaturase

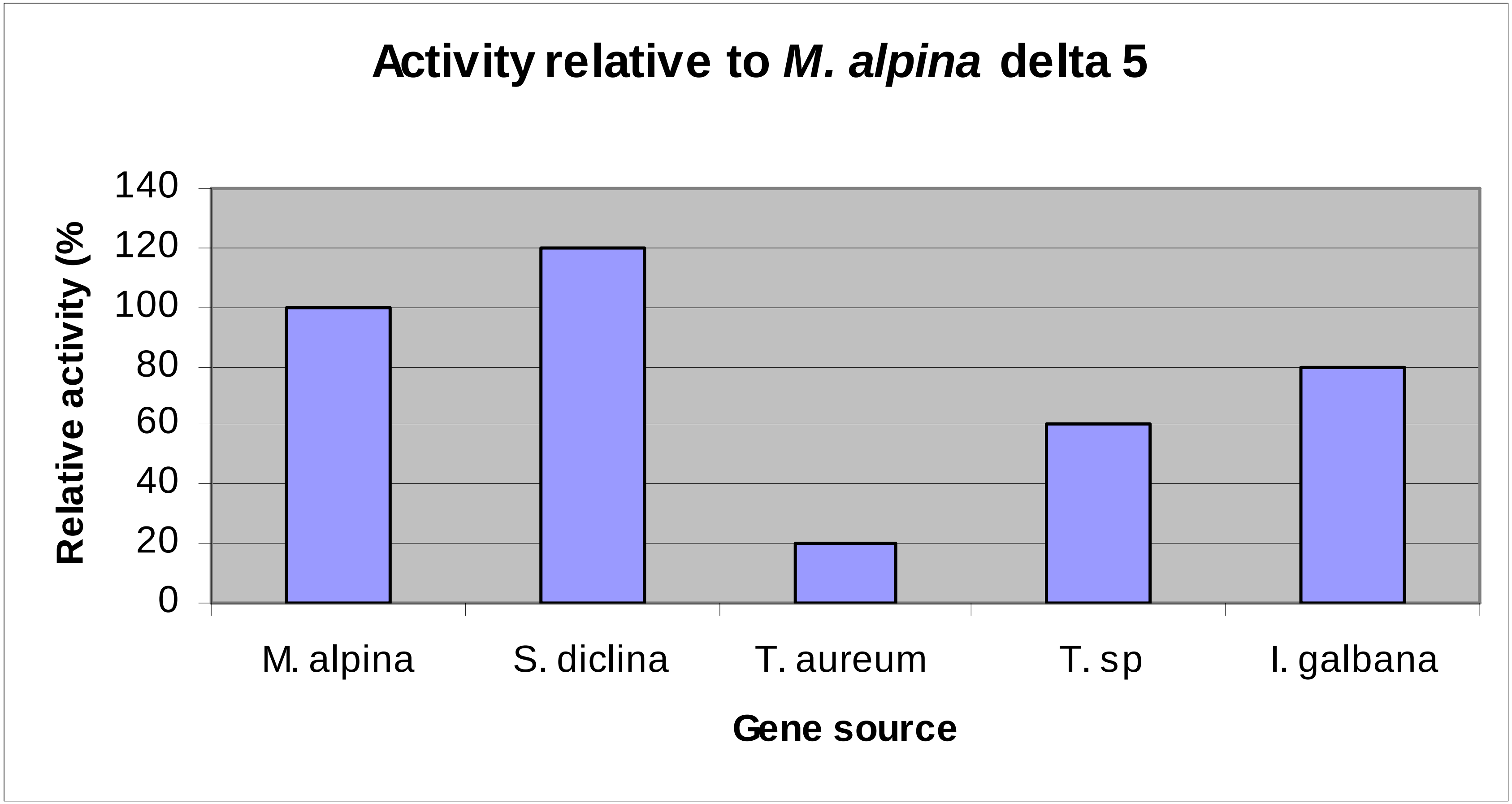




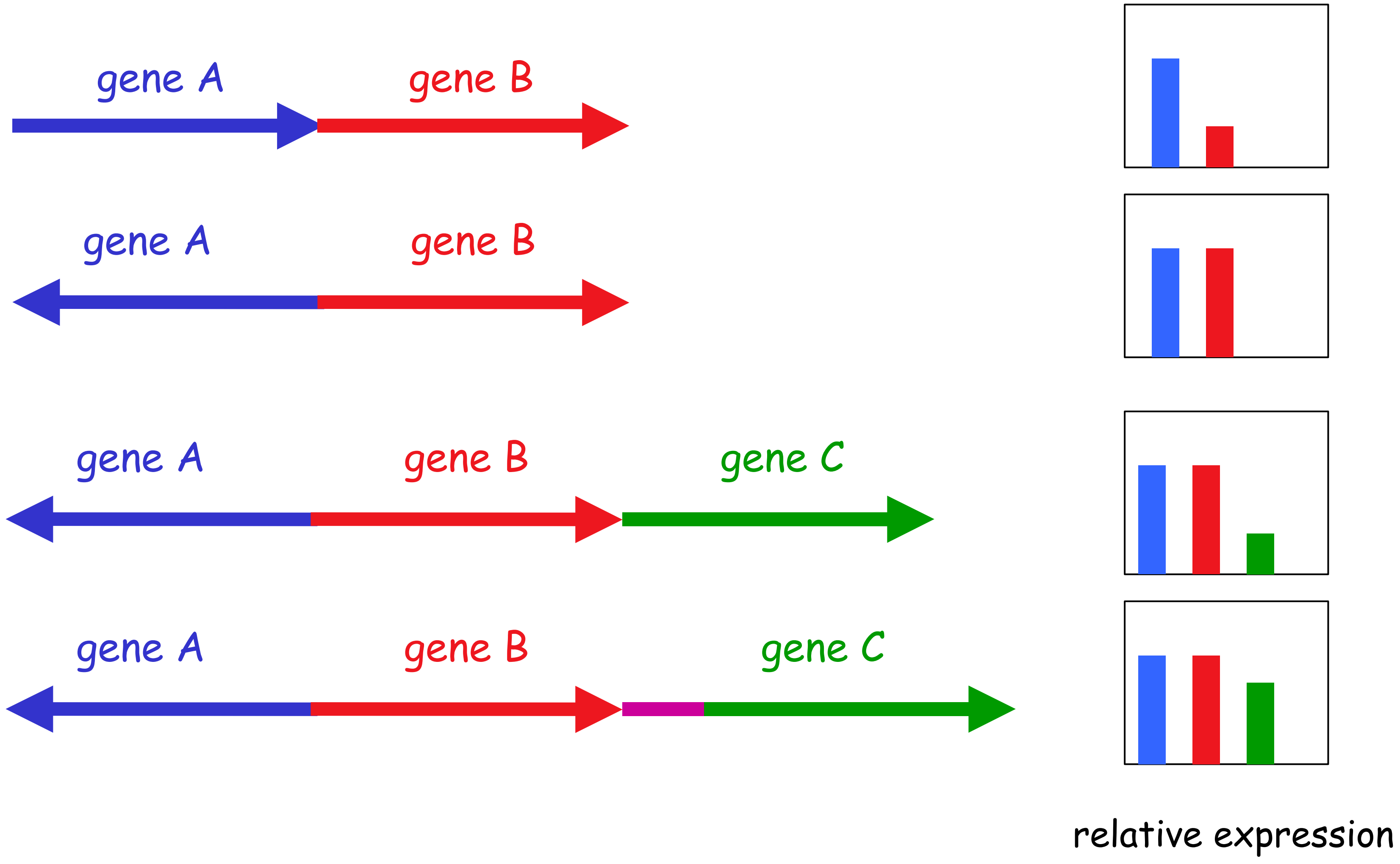


Gene source for each enzyme in pathway

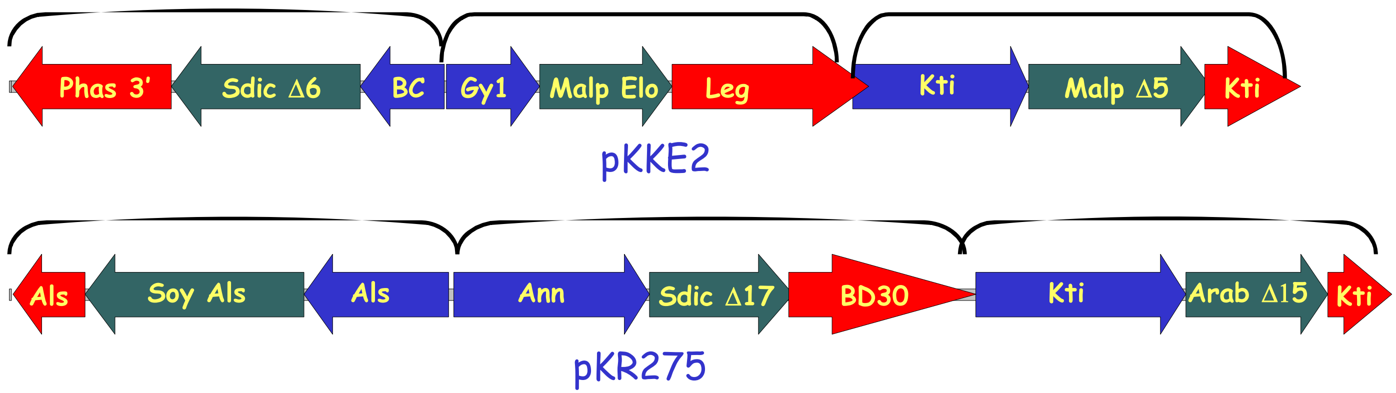
Δ5 desaturases and KTi3 promoter

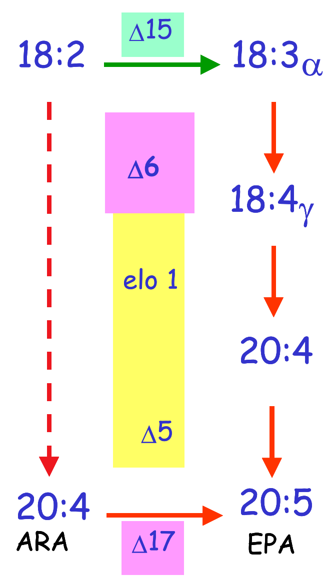


Construct design for multiple genes



The state-of-the-art multi-gene shot: 6-gene set (2 fragments)

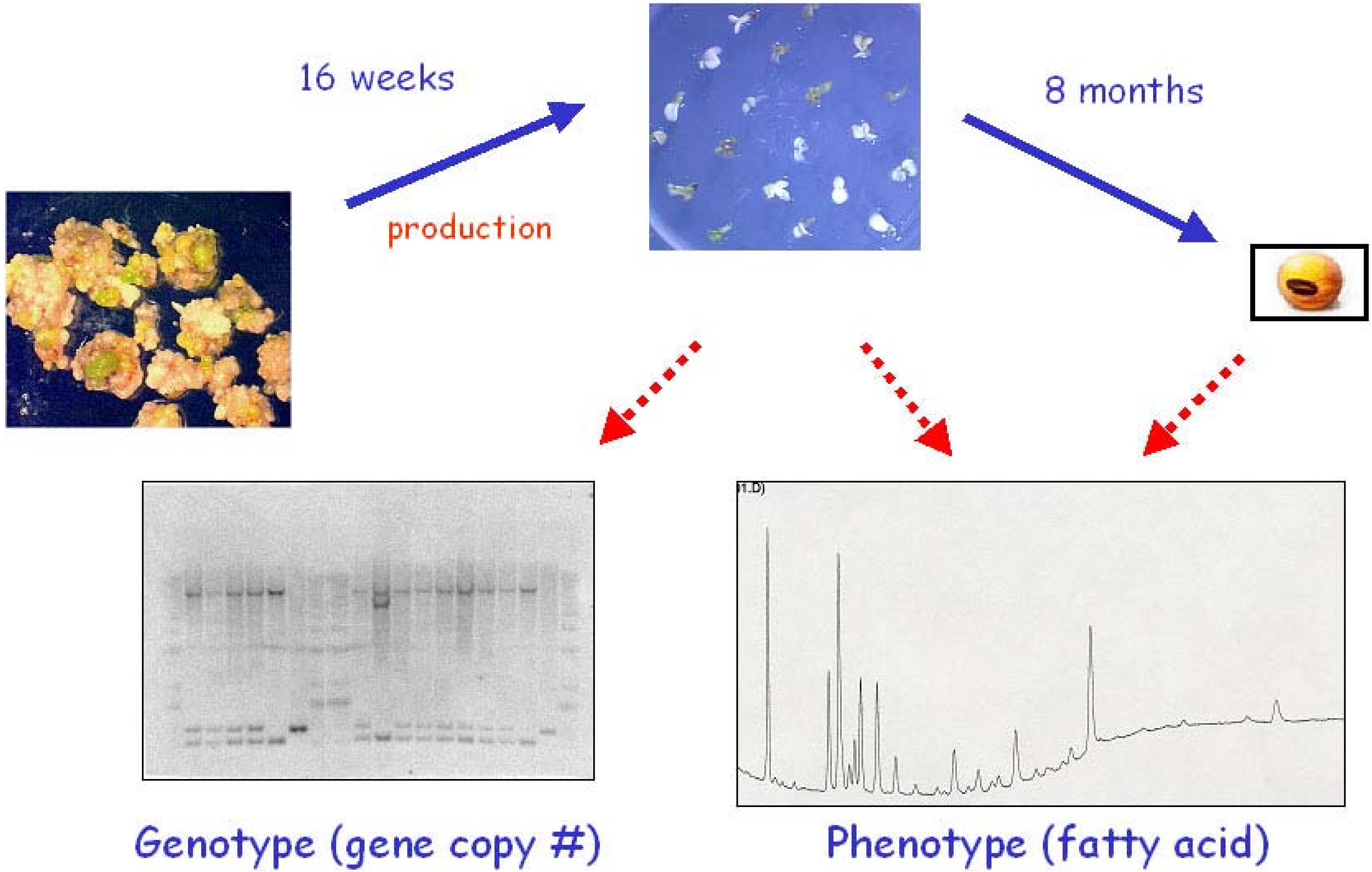




Screening the transgene insertion locus

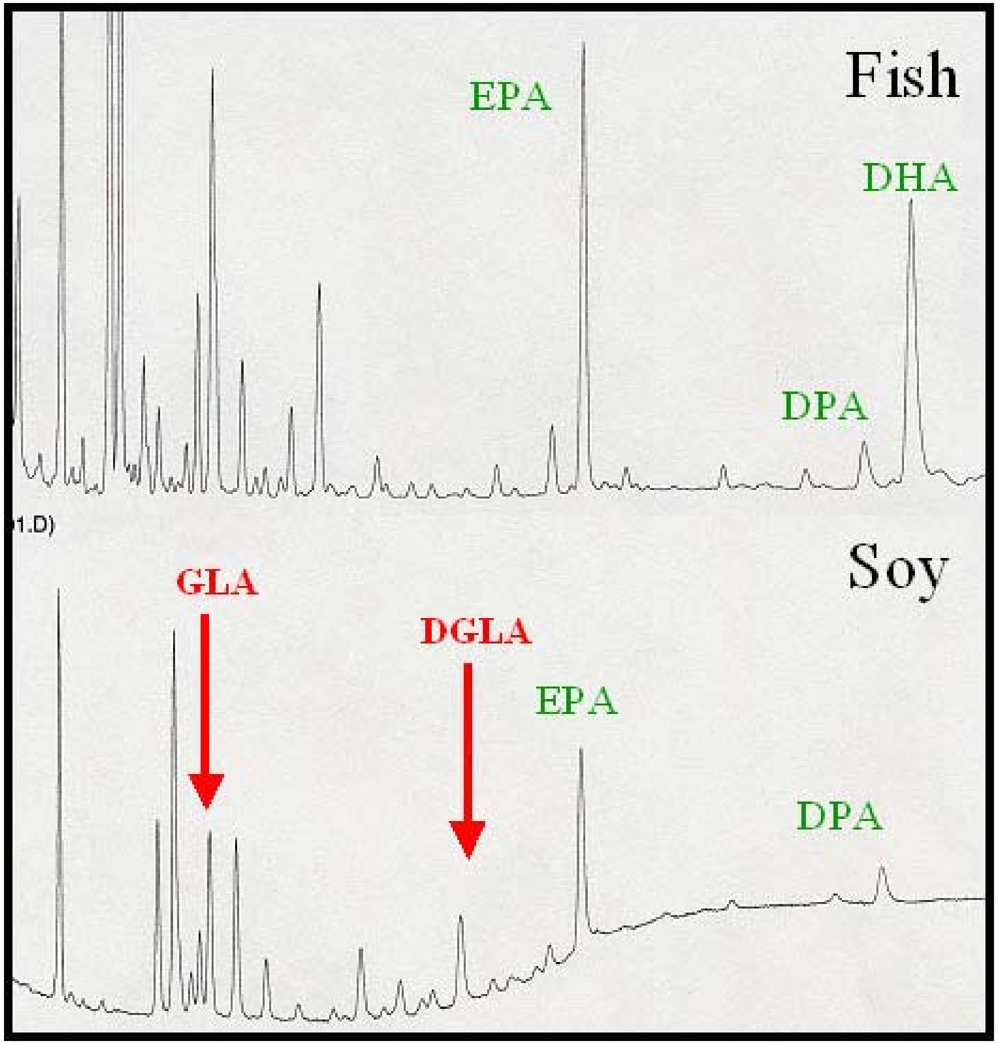
- Multiple copies or fragments of homologous genes leads to partial or complete gene silencing in seeds
- Complex insertion loci correlated with reduced transgene activity or trait instability
- Screen for "simple" inserts: single copy of each transgene at single locus

Co-bombarded fragments at single locus: single copy inserts selected

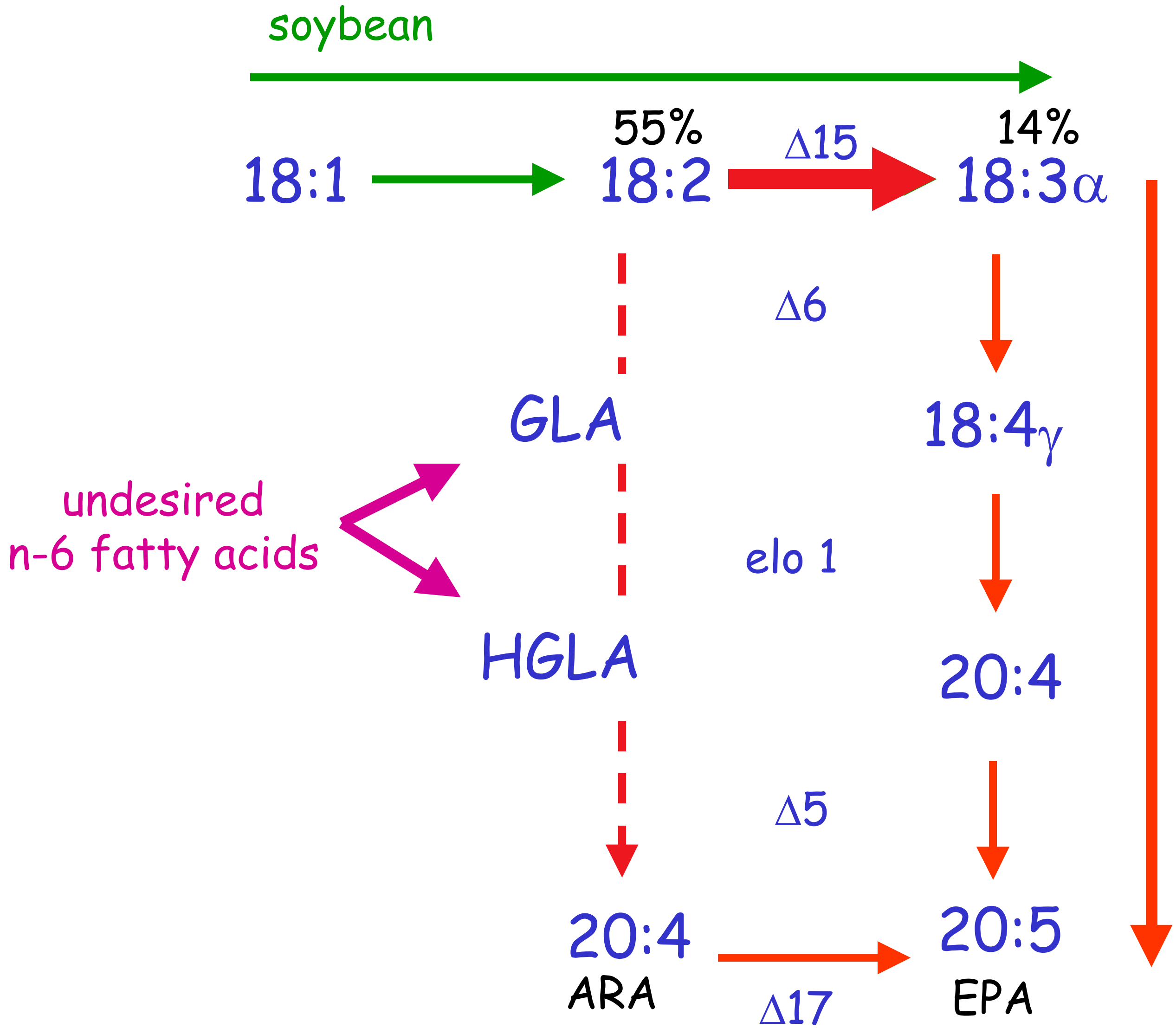


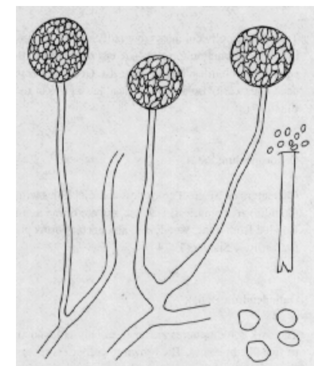
LC-FA profile similar to fish except higher omega-6 content (and lower in saturated fat)

Seed EPA +DPA = 24%
Total LCPUFA = 40%
Less than 0.5% ARA

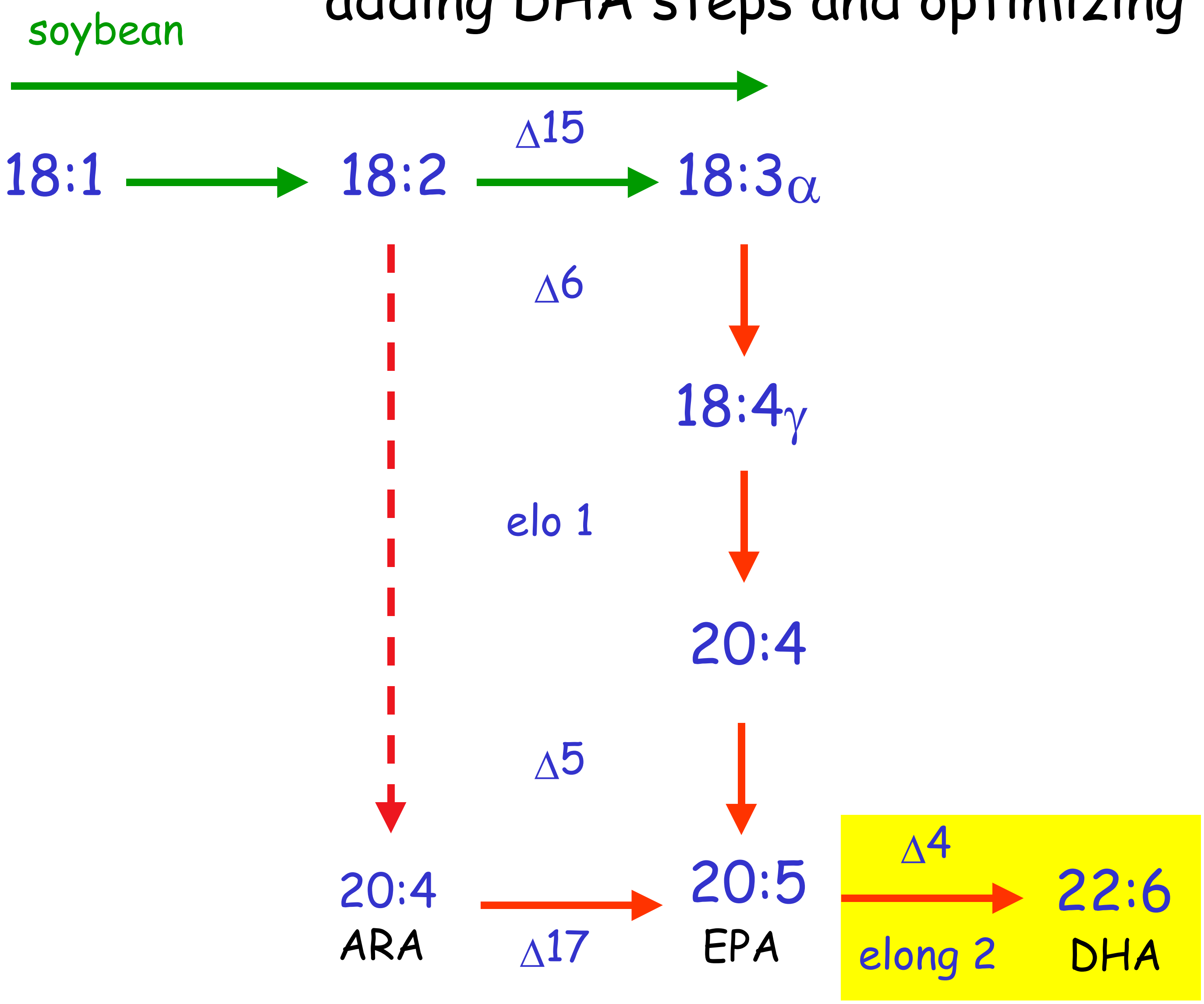


Fine tuning and tweaking: increasing flux into n-3 products





Fine tuning and tweaking: adding DHA steps and optimizing content



Providing safe, inexpensive, high quality and renewable source of long chain omega-3 for the human diet

cloning genes

↓

assembling pathway

↓

optimizing pathway

↓

target fatty acid profile

↓

agronomics

↓

commercial product

MANIPULATION OF LIPID METABOLISM IN CROP PLANTS TO PRODUCE HEALTHIER OILS

Credits:

Ed Cahoon, Howard Damude, Lennie Farrall, Kevin Ripp, Bruce Schweiger, Kevin Stecca, Naren Yadav, Bill Hitz

Crop Genetics Research and Development

DuPont Experimental Station, DE

Cheryl Caster and group

Zhongsen-Li and group

Soybean Transformation

Newark, DE

Suzette Pereira, Amanda Leonard, Pradip Mukerji

Ross Products Division

Abbott Laboratories, OH

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