Plant breeding, engineering, and compositional analysis

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Plant breeding, engineering, and compositional analysis

- **Crop Variability**
  - Domestication
  - Background variability present
  - Breeding & engineering
- **Composition analysis for safety assessment**
- **Sources of compositional variability**
  - Genetics
  - Agronomic Practices
  - Environment
- **Using variability to provide context in compositional assessments**

Mary of Guise
Scotland, Stirling Castle, 1540

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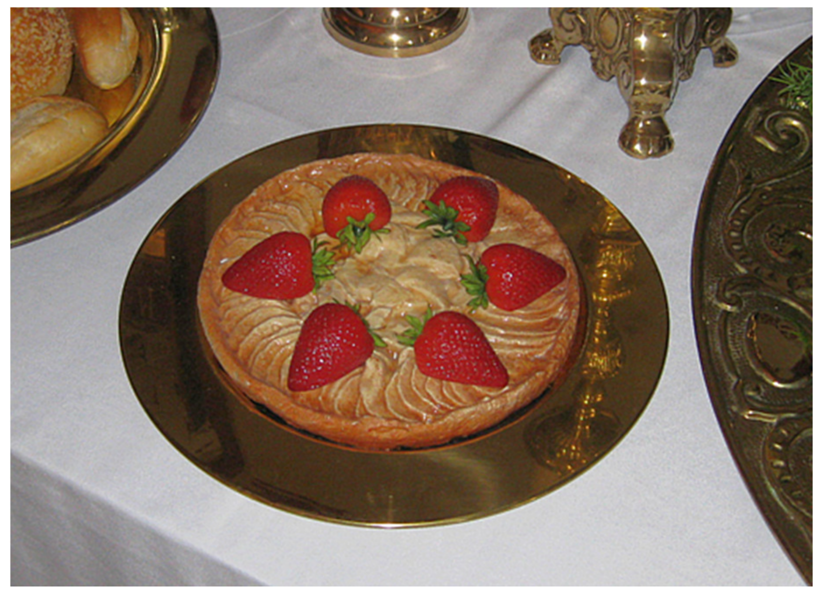

Modern strawberries never existed in nature

Fragaria chiloensis
Chile

Fragaria virginiana
Eastern North America

X

Fragaria ananassa
Europe, 1740's

The starting misconception

The Myth of Natural Food

Also has genes from wild species

Wild tomato

Cultivated (modified) tomato

Photos: USDA & Frary et al., 2002. *Science* 289: 85-88

Changes in tomato gene expression by domestication

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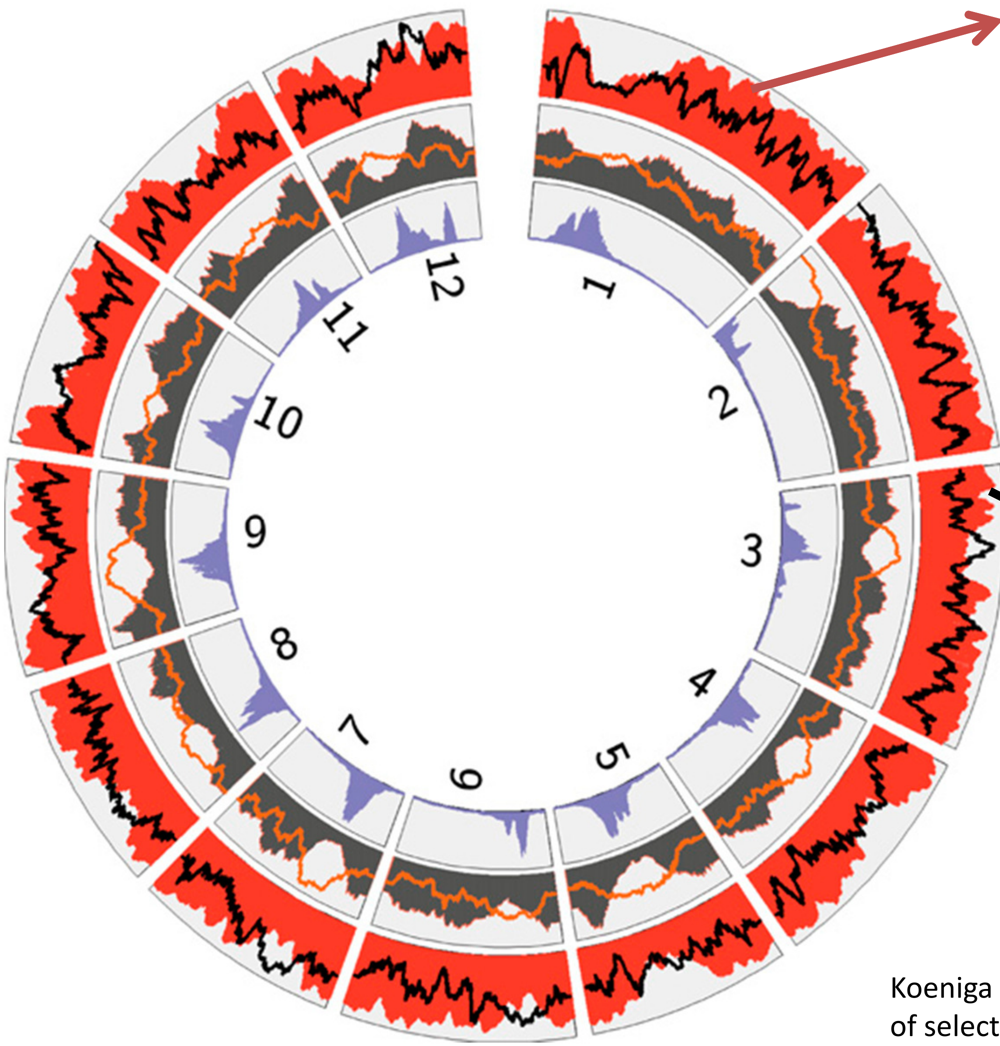
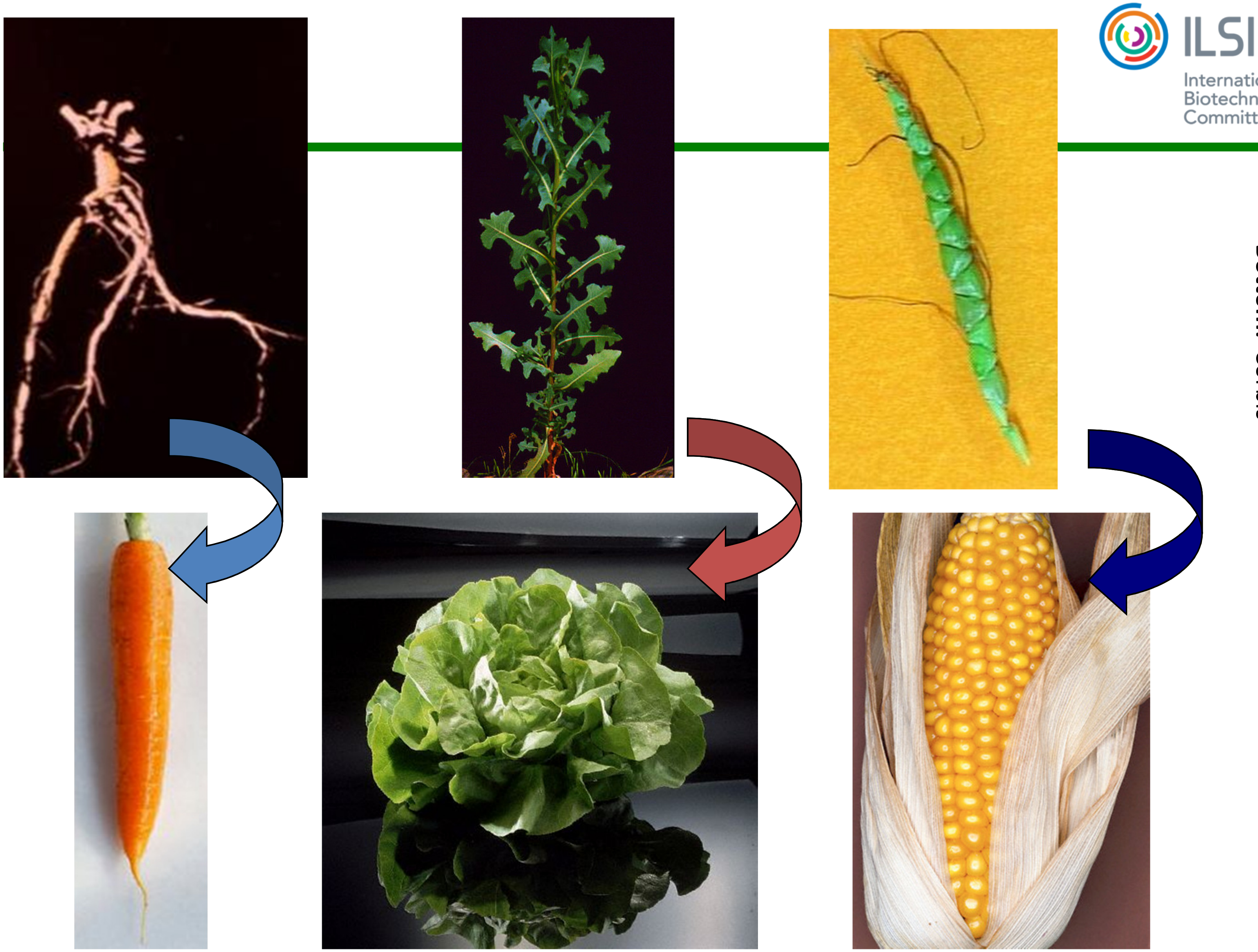


Gene expression in cultivated tomato

Gene expression in ancestral tomato

Differences in gene expression means differences in composition must be present

Koeniga et al. 2013 Comparative transcriptomics reveals patterns of selection in domesticated and wild tomato. Proc Natl Acad Sci.



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Top: Peggy Lemaux, John Meade, Raúl Coronado
Bottom: Corbis

Crop genomes are variable

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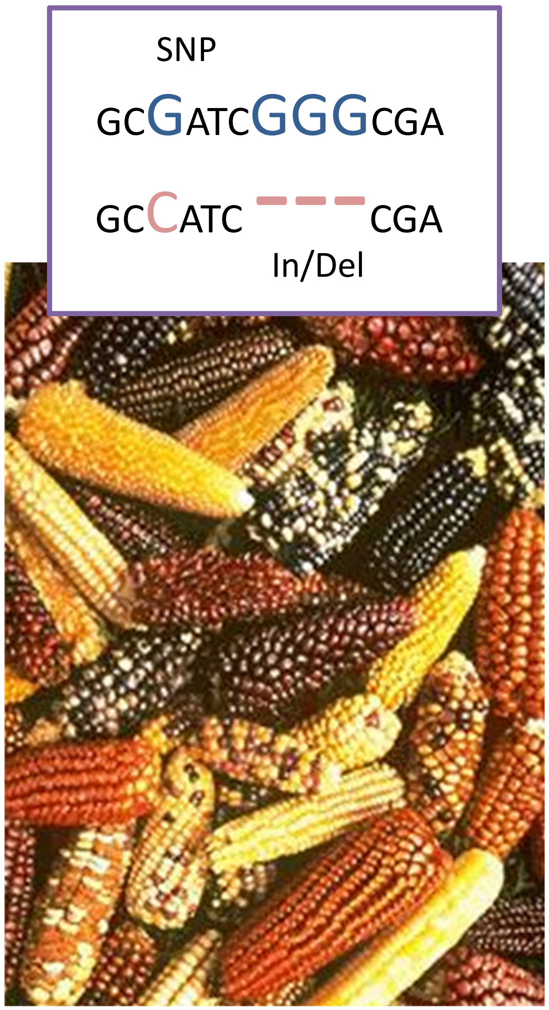
From S. Flint-Garcia, ILSI CCW, 2012



- Maize HapMap.v1 – gene-rich regions
  - 27 inbred lines (NAM founders)
  - 1.6 million SNP/indels
- Maize HapMap.v2 – whole genome
  - 103 lines
    - 60 inbreds, 25 landraces, 19 wild relatives
  - 55 million SNPs

Gore, et al. (2009) *Science*

This wide variability has **never** produced toxic maize

How much DNA diversity is possible?

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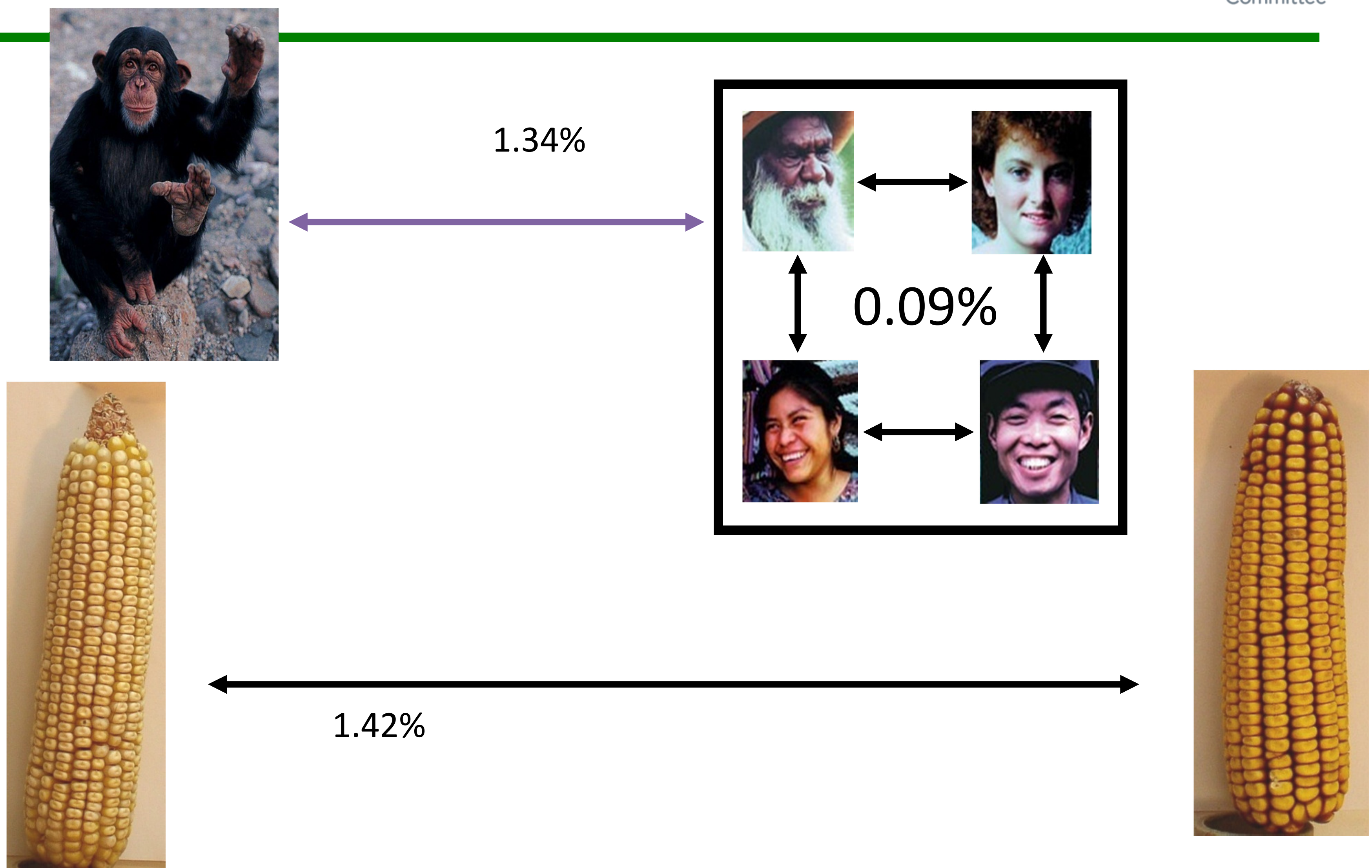
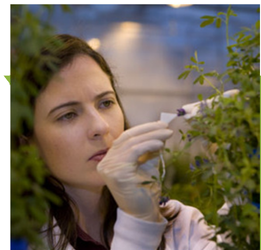


Slide by Ed Buckler (Zhao PNAS 2000; Tenallion et al., PNAS 2001)

Conventional breeding accumulates desired variation



1,000 F_1

2,000,000 F_2 - Disease & habit

50,000 F_3

4,000 F_4 - Quality

1,000 F_5

500 F_6 - Yield

50 F_7

8 F_8 – Regional trials

1 Variety

Elimination of undesired types

Soybean variety trial

http://www.plantpath.wisc.edu/soyhealth/bsr/bsrvar.htm

Modified from: http://www.generationcp.org/plantbreeding/index.php?id=052

Gene transfer, 1950's style

Ln-9 *gene for leaf rust resistance from* Aegilops *to wheat*

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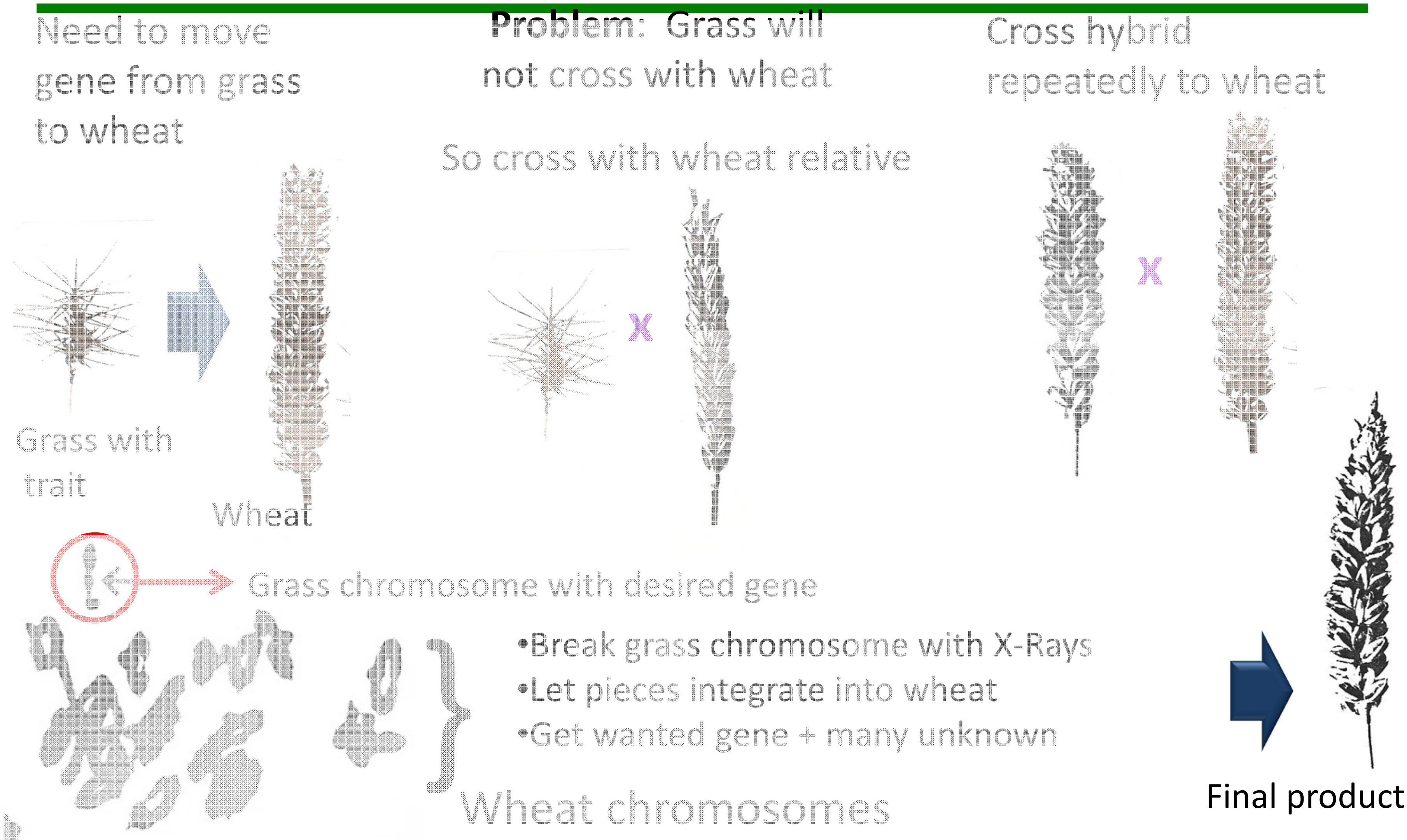

Sears, E.R. 1956. The transfer of leaf-rust resistance form *Aegilops umbellulata to wheat. Genetics in Plant Breeding Brookhaven Symposium #9*.

Summary of traits transferred from other species

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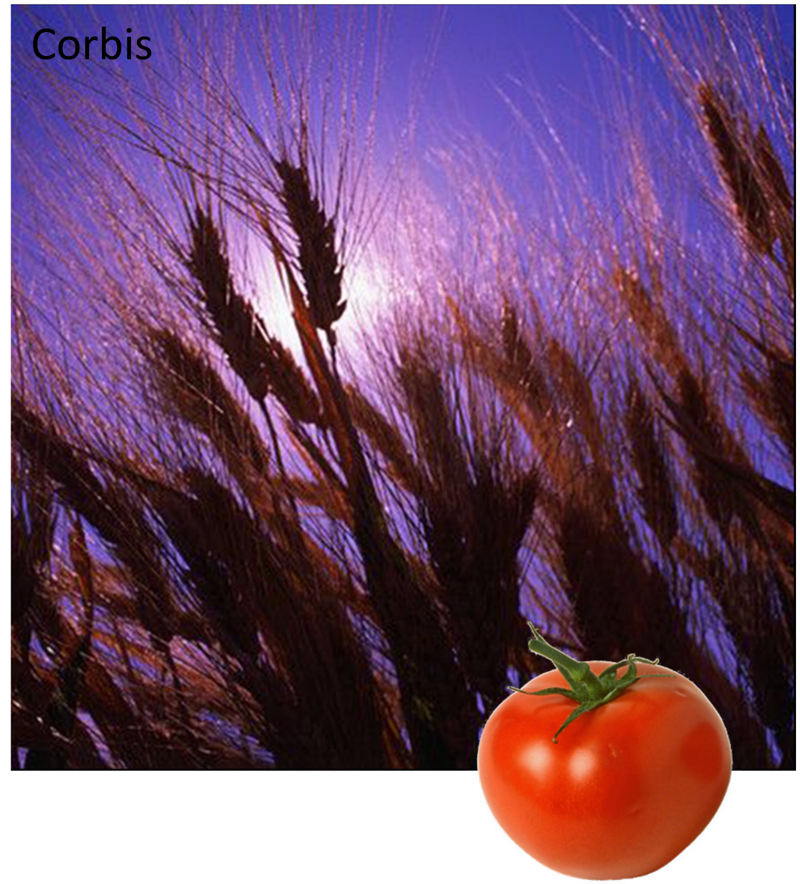
- 111 genes introduced into 19 crops over 20 years
  - Tomato = 55
  - Rice & potato = 12 each
    - Hajjar & Hodkin, 2007
- Wheat
  - Dozens of genes from 6 genera
    - Jones et al., 1995



- Hajjar & Hodgkin (2007) The use of wild relatives in crop improvement: A survey of developments over the last 20 years. EUPHYTICA 156: 1-13
- Jones et al. (1995) Use of alien genes for the development of disease resistance in wheat. ANN. REV PHYTOPATHOL 33:429-443.

Gene transfer via rDNA

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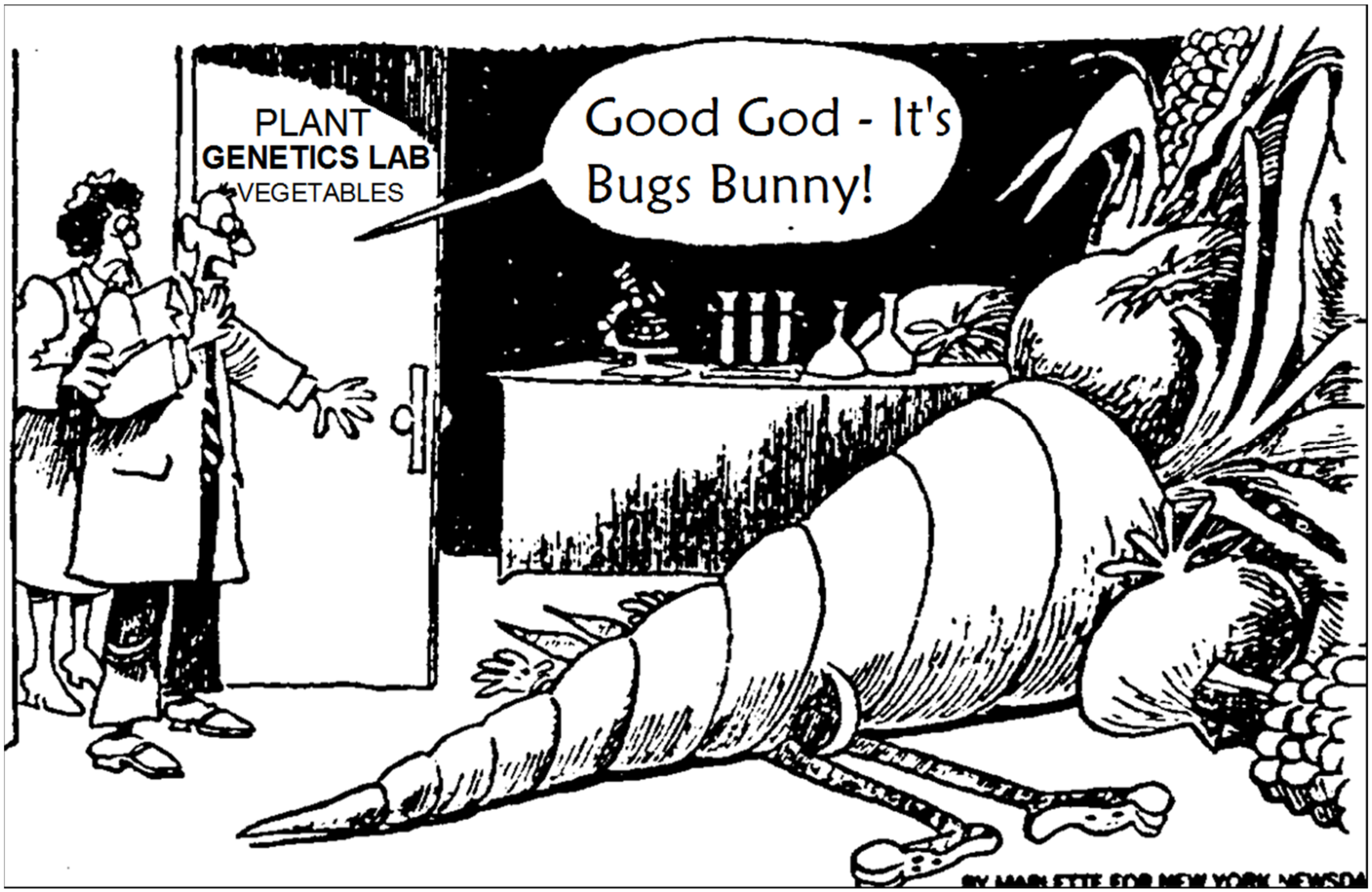

Comparative safety assessment of GM crops

2 Types of changes

- Intentional
  - The product of the transgene and its related metabolites
- Assess for
  - Toxicity
  - Allergenicity
  - Nutritional equivalence

Comparative safety assessment of GM crops

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2 Types of changes

- Unintentional
  - The product of the transgene can serve as a substrate for novel (& toxic) metabolites
  - Mutations during the transformation process???

What are we guarding against?

- “Additionally, plants, …, have metabolic pathways that no longer function because of mutations that occurred during evolution.”

of some of these
cants.

Ensure no nearby flanking genes

Avoid filler DNA, vector sequences, etc.

- “…, such silent pathways may be activated by the introduction or rearrangement of regulatory elements, or by the inactivation of repressor genes by point mutations, insertional mutations, or chromosomal rearrangements.”

Avoid all SNPs

Ensure fertility is not affected

The Safety of Foods Developed by Biotechnology

David A. Kessler, Michael R. Taylor, James H. Maryanski, Eric L. Flamm, Linda S. Kahl

Science, 256, (Jun. 26, 1992), pp. 1747-1749

What are we guarding against?

- “Additionally, plants, …, have metabolic

Problem:

- Cannot exclude other mutations that happen during the transformation process and which could cause unintended effects

insertional mutations, or chromosomal rearrangements.”

The Safety of Foods Developed by Biotechnology

David A. Kessler, Michael R. Taylor, James H. Maryanski, Eric L. Flamm, Linda S. Kahl

Science, 256, (Jun. 26, 1992), pp. 1747-1749

Testing for unintended differences

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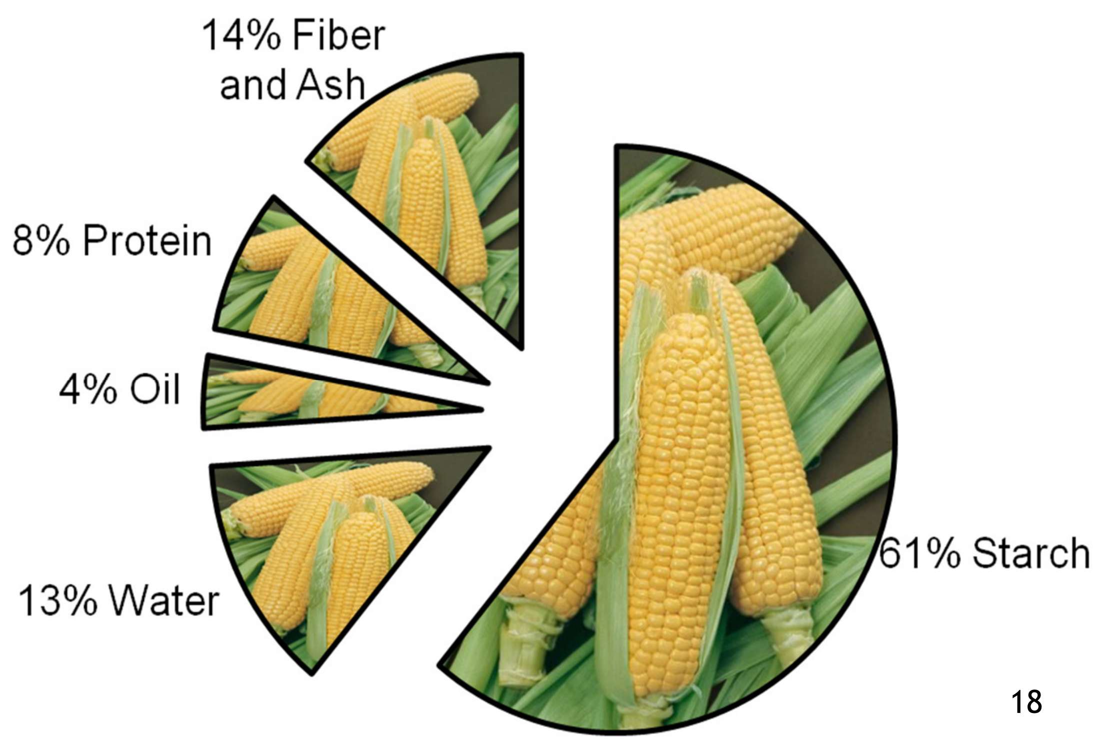
- No test for unknown differences
- Must infer the presence of unknown differences
  - Differences in growth
  - Differences in chemical composition based on hypotheses



Compositional anaysis

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- Compositional analysis of key components is a part of all safety assessments
  - CODEX Alimentarius, 2004
  - OECD, 1999

We cannot buy this. It is full of ingredients.

Two types of regulatory procedures

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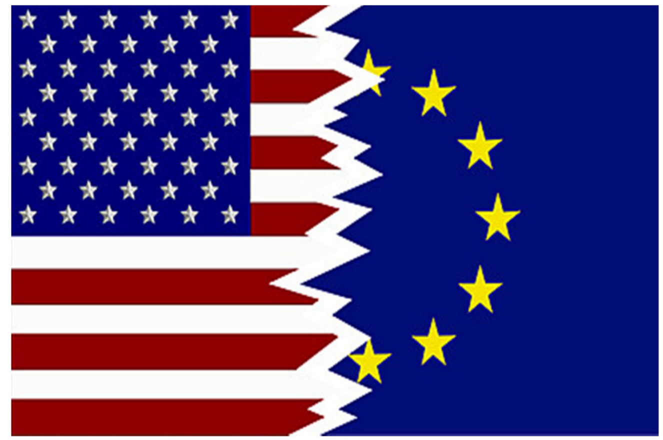

- Product-based
  - Eg, USA, Canada
- Process-based
  - European Union
  - Australia

- Both procedures require composition data
  - Food and Chemical Toxicology 42 (2004) 1047–1088
  - Increased or decreased levels of nutrients, anti-nutrients and/or natural toxicants

Finding changes that are unintended and hazardous

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- If 2 things are identical in composition
  - It is impossible for 1 to be safe and the other unsafe

ID of differences

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- If 2 things are not identical in composition, and 1 is safe and the other unsafe
  - The hazard MUST be related to the difference in composition
- 1st step is to ID all compositional differences

Proximates

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- Moisture
  - Weight loss after drying
- Crude Protein
  - N X 6.25
- Crude Fat
  - Usually Ether Extractable
- Ash
  - Remainder after combustion in a furnace (5000 C)
- Fibre – Crude
  - Remainder after acid treatment and drying)

Fibre

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- Crude Fibre
  - Not meaningful except for literature comparison
- Total Dietary Fibre
  - That not digested by humans
  - Important in human nutrition
- Acid Detergent Fibre
  - Cellulose + lignin
  - Indigestible to animals
- Neutral Detergent Fibre - ADF fraction

Amino acids

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- Essential for non-ruminant animals
  - Arginine, histidine, isoleucine, lysine, leucine, methionine (+ cystine), phenalanine, threonine, valine and tryptophan
- Glysine, tyrosine and serine are also important
- Most limiting amino acids for animals are lysine, methionine and threonine

Fatty acids

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- Some fatty acids are essential in the diet for animals
  - Linoleic acid (18:2)
  - Linolenic acid (18:3,alpha [omega 3])
  - Others of interest: Linolenic (gamma),
  - palmitic (16:0) and palmitoleic (16:1)

Vitamins

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- Fat Soluble
  - A, D, E, K,
- Water Soluble
  - B1, B2, B6, C, folacin, niacin and pantothenic acid
- The key vitamins depend on what the feed from the unmodified plant is recognized for

Minerals

- Major minerals
  - calcium, phosphorus, magnesium, potassium and sodium
- Trace minerals
  - iron, selenium, manganese, copper and zinc
- Other than Ca and P, the key minerals depend on what the feed from the unmodified plant is recognized for

Key Nutrients, Anti-nutrients and Natural Toxicants

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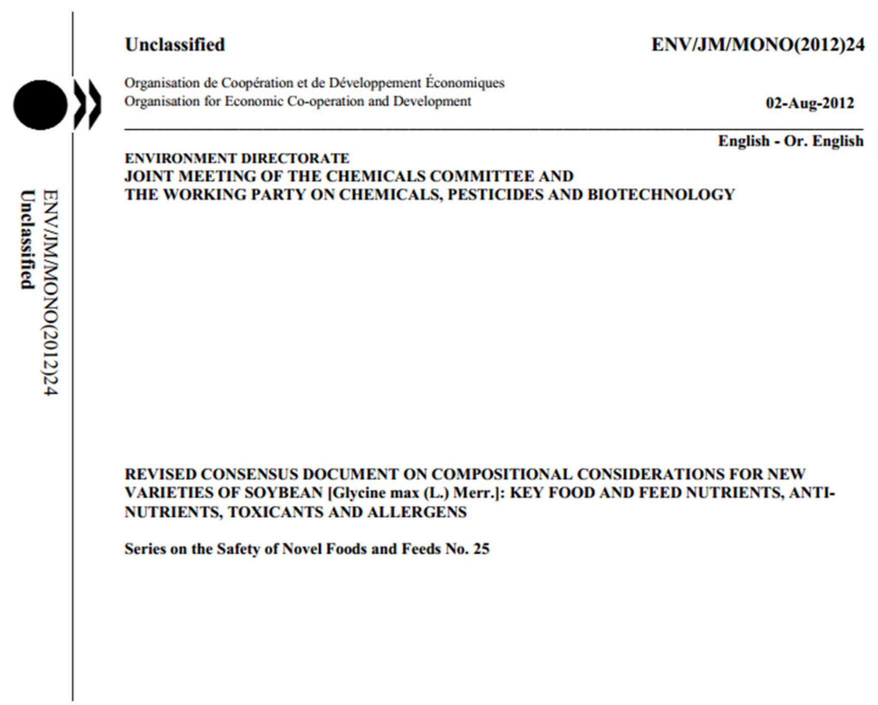
- OECD has published crop specific consensus documents that list:
  - Key Nutrients
  - Anti-nutrients
  - Secondary metabolites
  - Natural toxicants
- For other crops, data on these components
  - extracted from the literature



Unclassified ENV/JM/MONO(2012)24

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development 02-Aug-2012

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

ENV/JM/MONO(2012)24
Unclassified

REVISED CONSENSUS DOCUMENT ON COMPOSITIONAL CONSIDERATIONS FOR NEW VARIETIES OF SOYBEAN [Glycine max (L.) Merr.]: KEY FOOD AND FEED NUTRIENTS, ANTI-NUTRIENTS, TOXICANTS AND ALLERGENS

Series on the Safety of Novel Foods and Feeds No. 25

What analytes are measured?

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- Depends upon the crop
  - OECD consensus documents are a starting point
  - Levels of nutrients, anti-nutrients, toxicants, appropriate secondary metabolites (based on contributions the crop makes to the diet)
- Depends upon the nature of the trait(s) of interest
  - Some traits may influence metabolism (modified fatty acids, stress-tolerance)
- Hypothesis-driven decisions should be used in analyte selection

Putting it all together

Always compared to the non-GM version

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- **Proximates:** crude protein, crude fat, ash, carbohydrates by calculation, and moisture
- **Fiber:** ADF, NDF, and total dietary fiber (TDF);
- **Minerals:** calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc
- **Amino acids:** alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine
- **Fatty acids:** palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, arachidic acid, eicosenoic acid, and behenic acid
- **Vitamins:** B1, B2, B6, E, niacin, and folic acid
- **Anti-nutrients:** phytic acid and raffinose
- **Secondary metabolites:** ferulic acid and p-coumaric acid

http://www.cfsan.fda.gov/~rdb/bnfm087.html

Replicated trials for compositional analyses

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- Replicated trials in time and space
  - The GM crop and its comparator
    - Usually the isoline
- Geographical area where crop will be grown
- GM and comparator must be grown at same time
  - Same agronomic practices to the extent possible

BT maize in Colombia

Harvest, storage, processing and analysis

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- Harvest, storage and processing conducted in such a manner to maintain the integrity of the experimental plant material
- Compositional analysis is conducted using validated analytical methods
- The resulting data are subjected to an appropriate statistical analysis resulting in probability estimates

Analyzed metabolites

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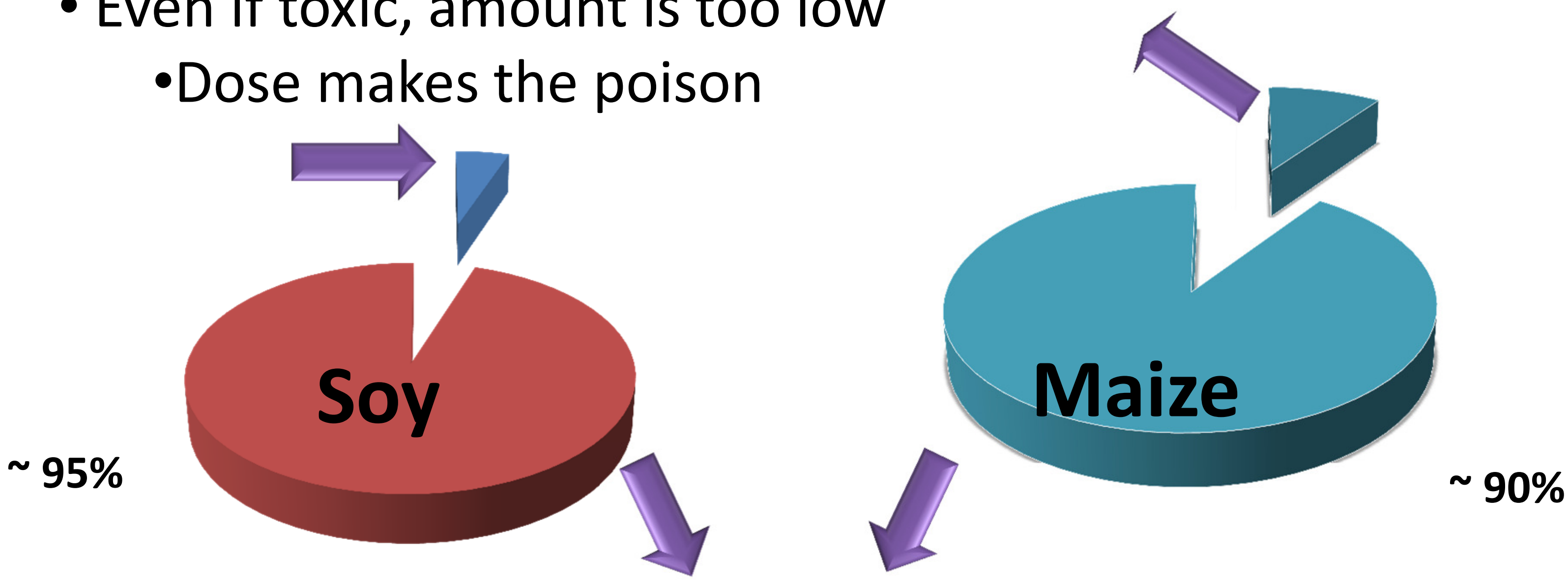
Not all metabolites are in the OECD lists

- 1000's of compounds
- Each in very low quantities
- Even if toxic, amount is too low
  - Dose makes the poison



The OECD lists dozens of key compounds

- Change in amount or relative ratios = sign of an unintended change

Sources of compositional variability

- Genetics
- Agronomic practices
- Environment

Evaluation of compositional data

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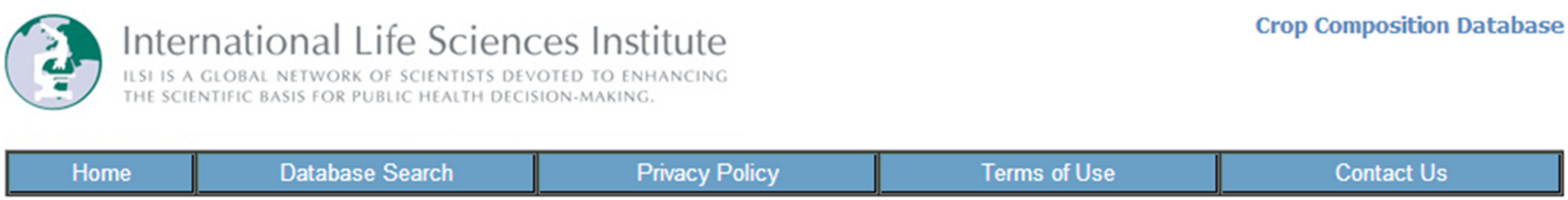
- First, evaluate against the comparator
  - A non-GM isoline or closely related line
- For significant differences (5% of parameters should be)
  - Compare to other data bases
  - OECD, USDA, NARO WHO, NRC, refereed literature
  - Plant composition should be within expected/reported ranges



- If there are still differences
  - a logical scientific justification must be presented involving safety data, or
  - new safety data must be generated

Isoflavones in soybean

Eldridge and Kwolek. 1983. JAFC 31:394-396

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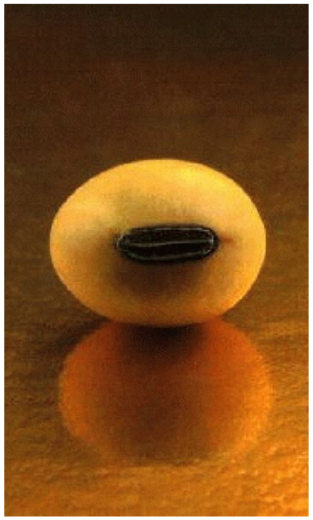
- Isoflavones are physiologically active

Variety	Place	mg isoflavones/10 g
Hardin	Girard, IL	47 a
Hardin	Urbana, IL	82 a
Hardin	Pontiac, IL	156 b
Hardin	Dekalb, IL	171 b
Hardin	Urbana, IL	116 a
Amcor	Urbana, IL	150 b
Century	Urbana, IL	250 c
Sprite	Urbana, IL	309 d



Genetics contributes to wide compositional (phenotypic) variability

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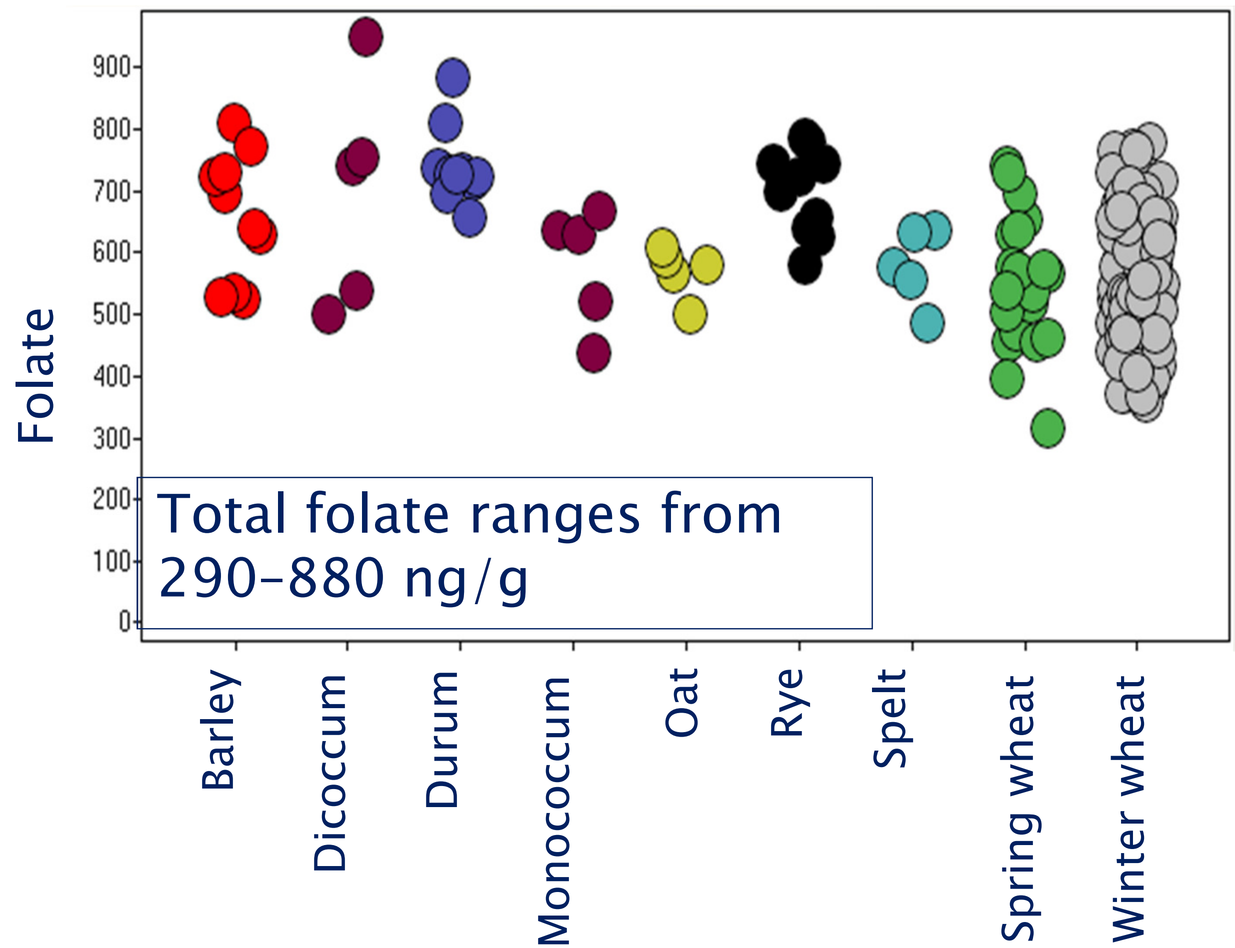

From: Peter Shewry, ILSI CCW, 2012

Conventional breeding leads to compositional variability

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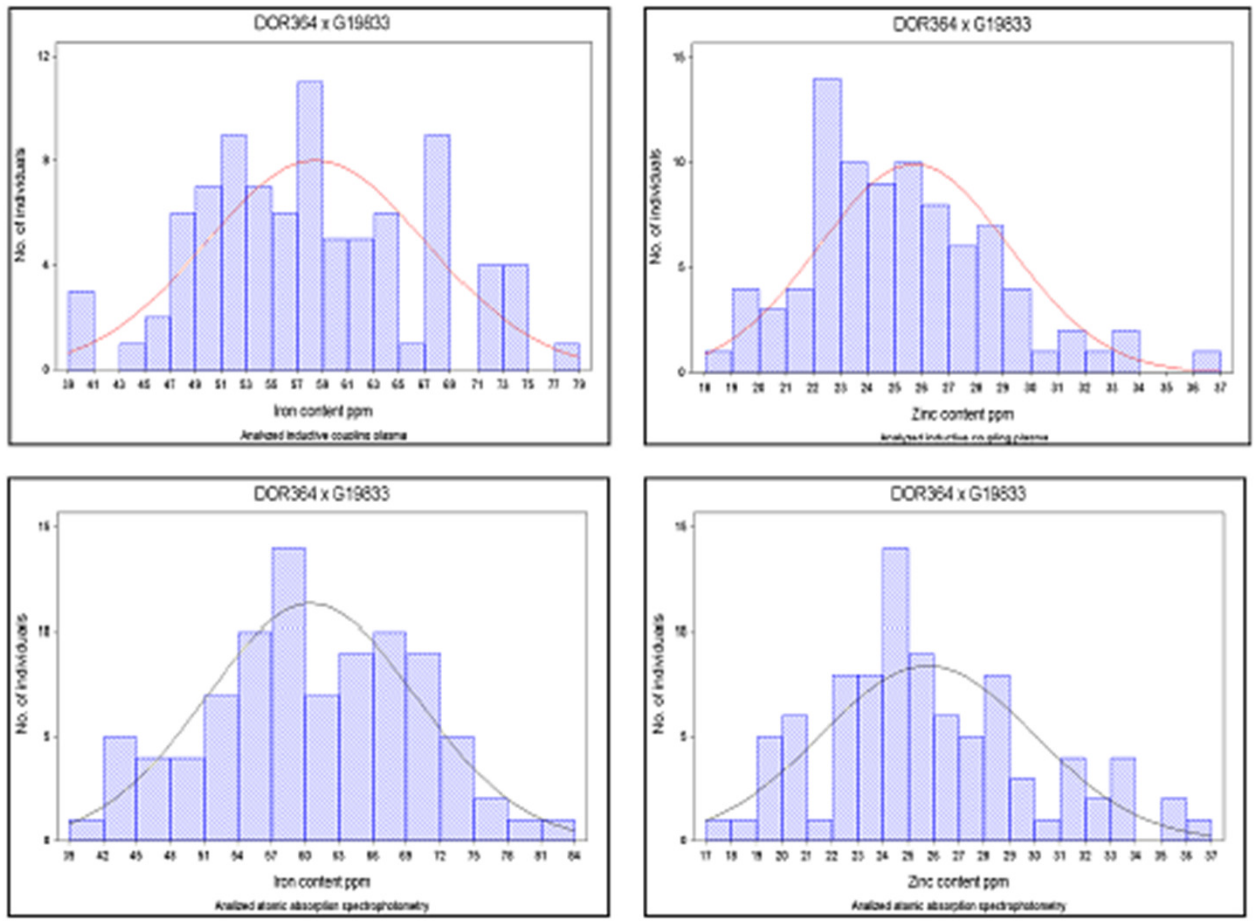
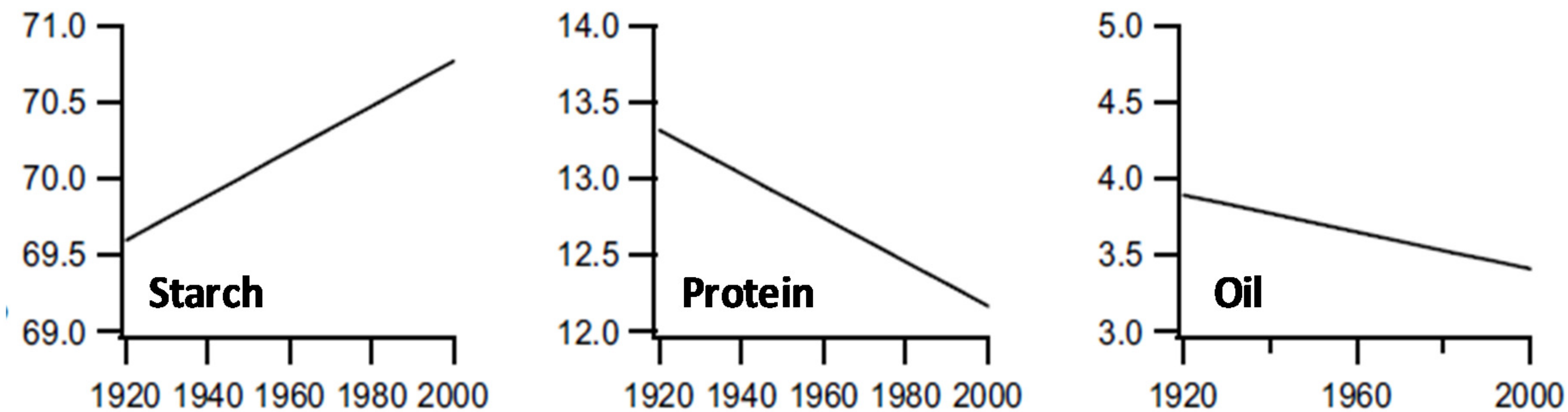
Mineral variability in DOR364   G19833 Recombinant Inbred Lines

Fe range 39 – 84 ppm   Zn range 17-37 ppm



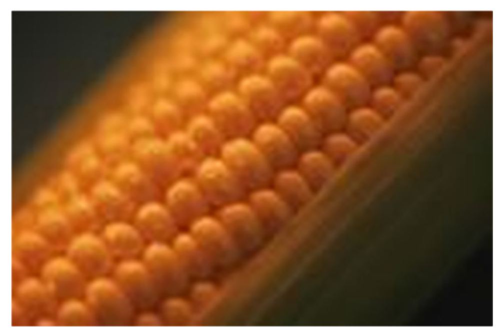
Blair et al., 2009. Presented at ILSI CCW September 2012

Genetics contributes to changes over time



Analysis of corn cultivars representing 80 years of breeding shows changes in composition over time in conventional maize

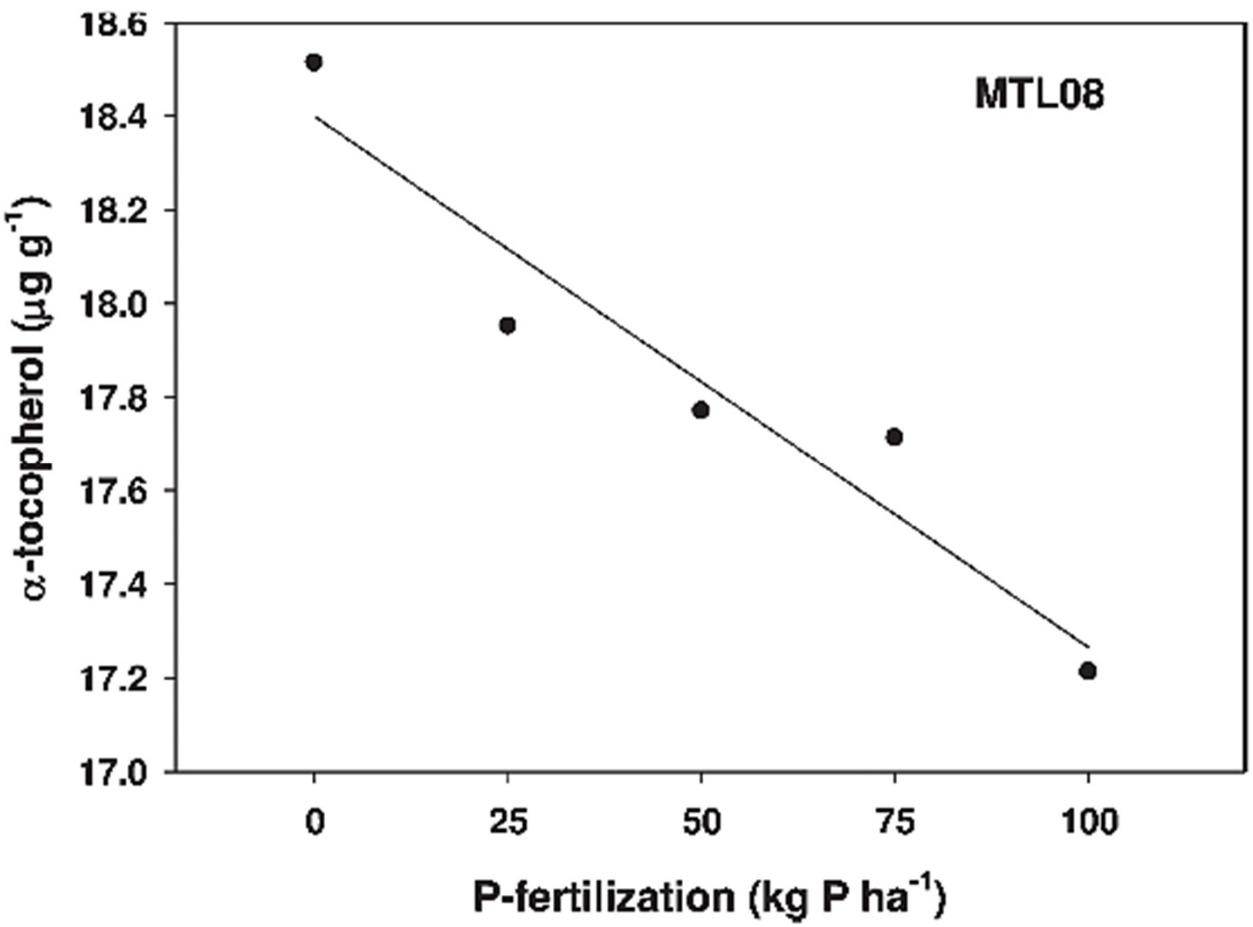
Scott et al. (2006) Maydica 51, 417



Agronomic practices contribute to compositional variability

Soybean tocopherol levels decrease as P-fertilization increases

Seeding rates and row spacing also affect soybean tocopherol levels



From Seguin et al., 2010

The environment contributes to wide compositional variability

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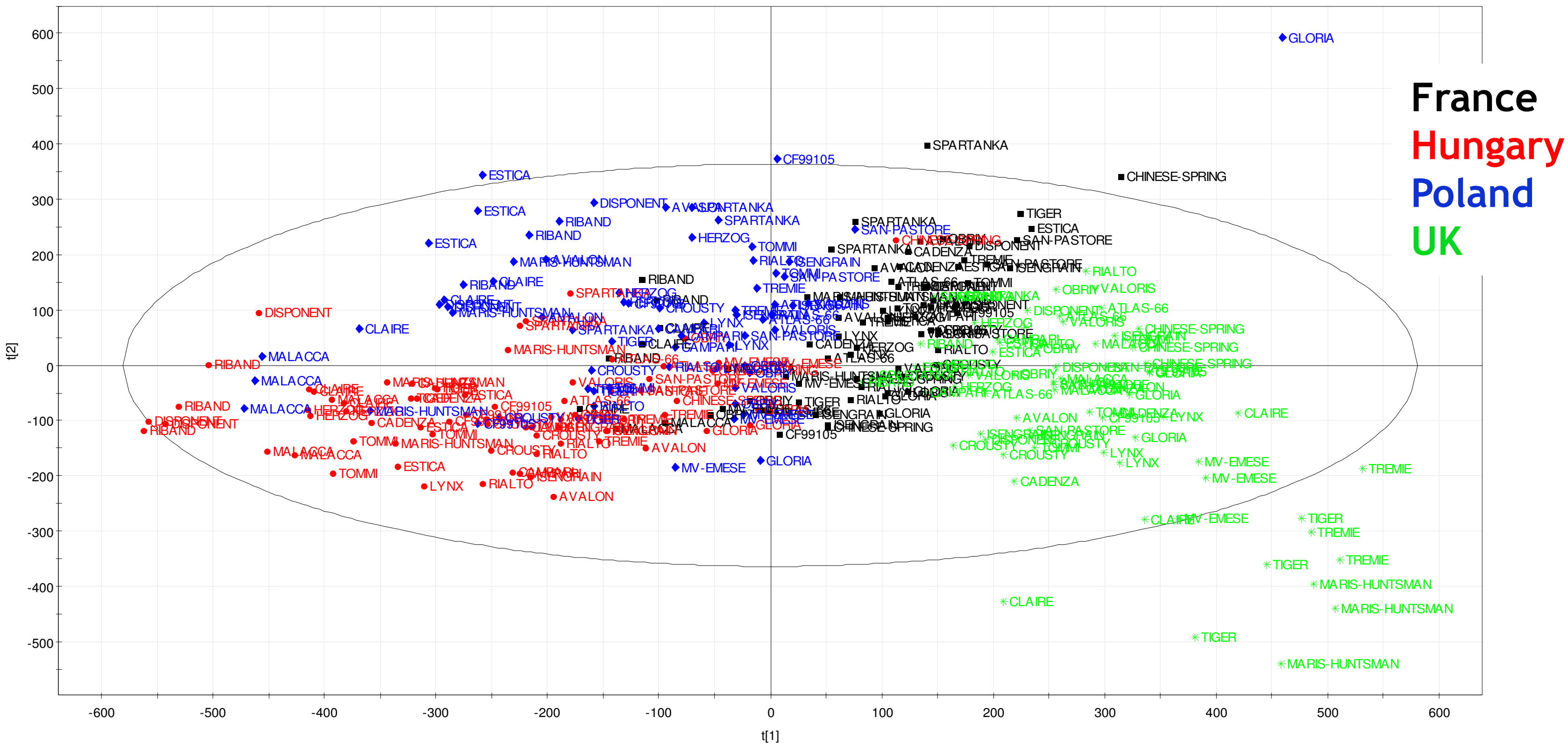

PCA analysis- 26 conventional wheat lines, 4 locations in 2007

- **Data clusters according to location, not variety**

From: Peter Shewry, ILSI CCW, 2012

The environment contributes to wide compositional variability

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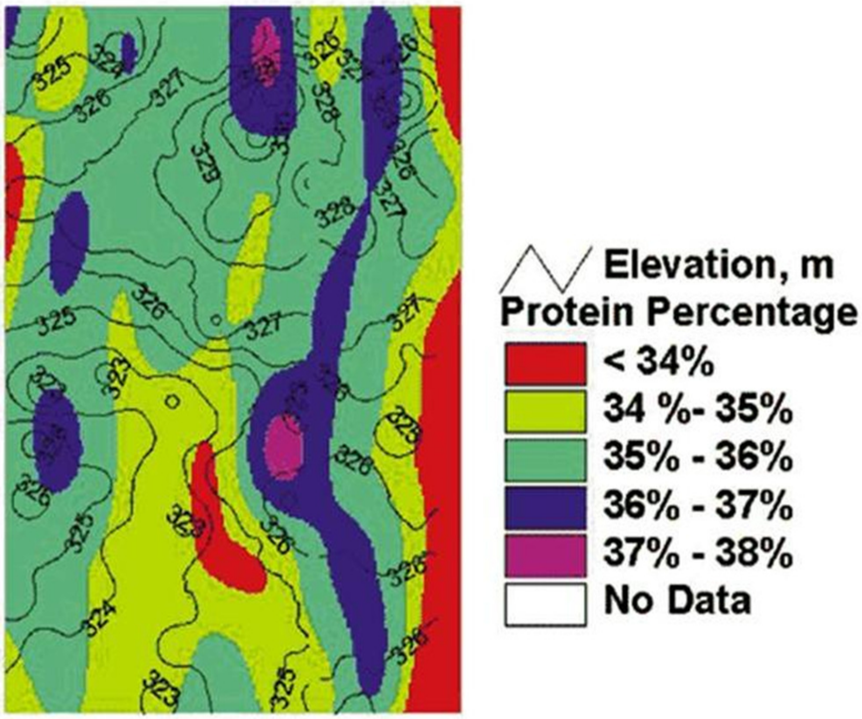
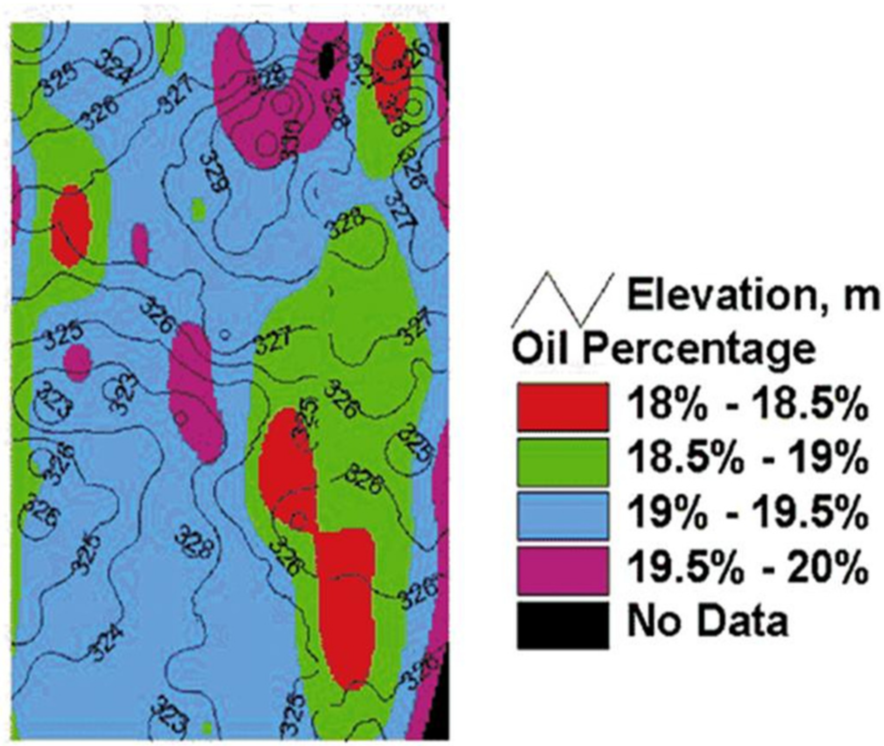
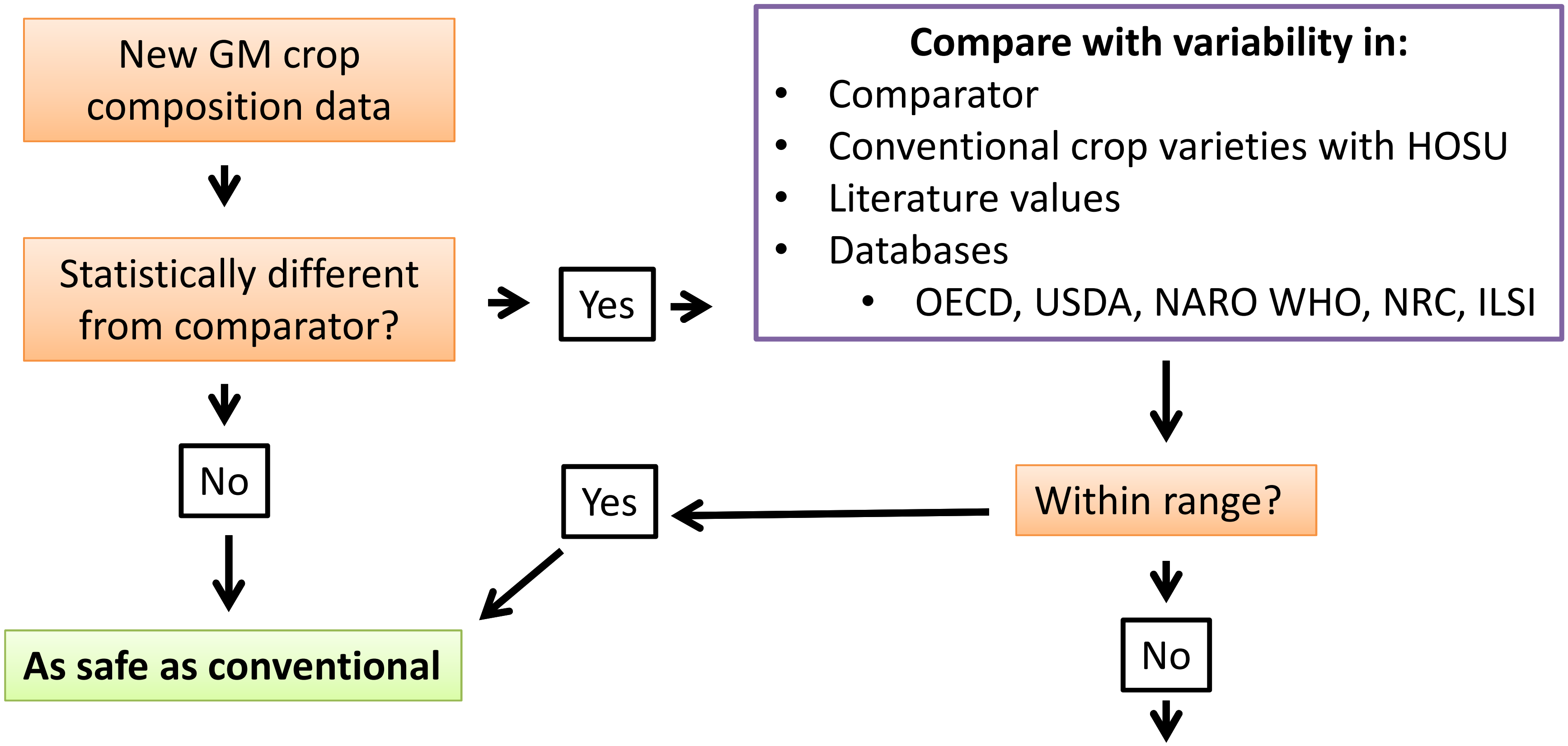




Distribution of protein and oil content in a 23-ha soybean field in Iowa, USA

http://www.ipm.iastate.edu/ipm/icm/1999/5-5-1999/soycompvar.html

Interpretation of results
Comparative safety assessment



If difference is biologically relevant, then

- a logical scientific justification must be presented involving safety data, or
- new safety data must be generated

B. Price, ILSI CCW 2012; P. Brune, IFBiC/OECD workshop 2013

Using variability to provide context in compositional assessments

- Statistical vs biological relevance
- Variability and context

Statistical vs biological significance

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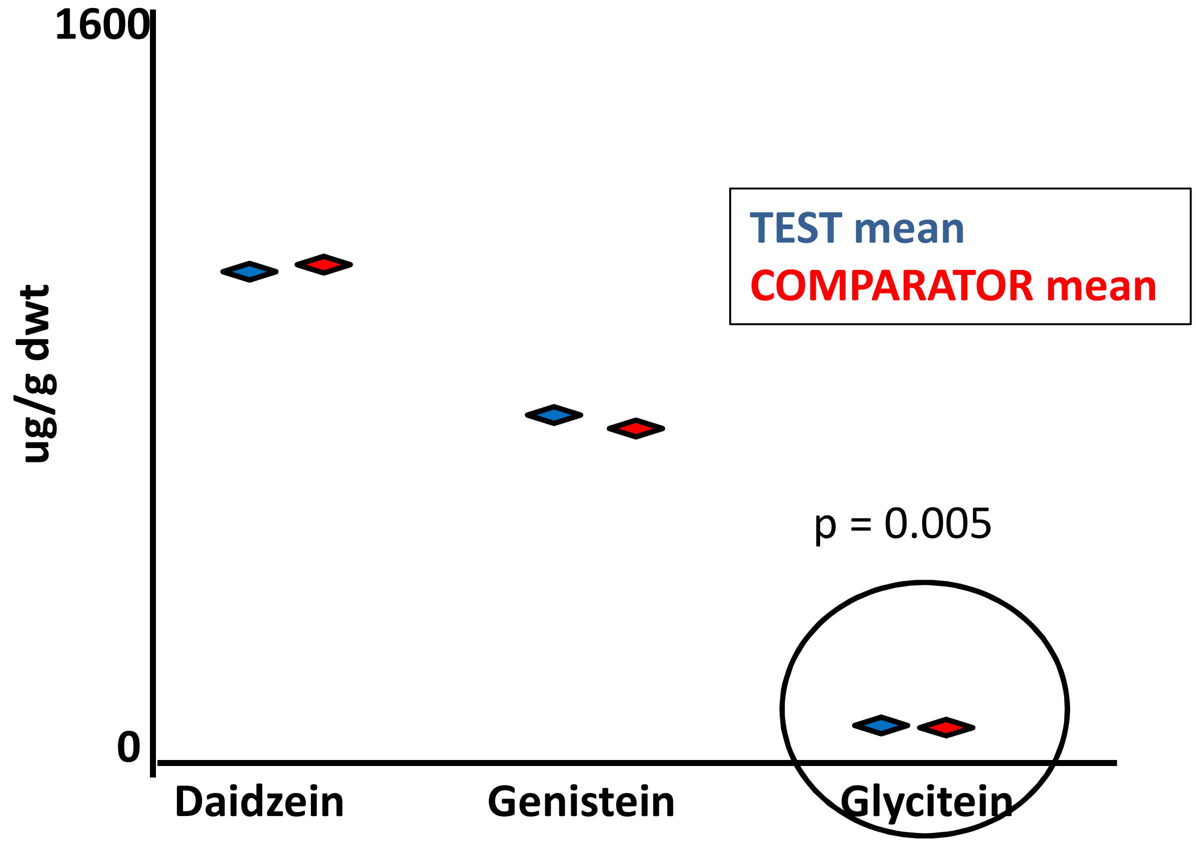
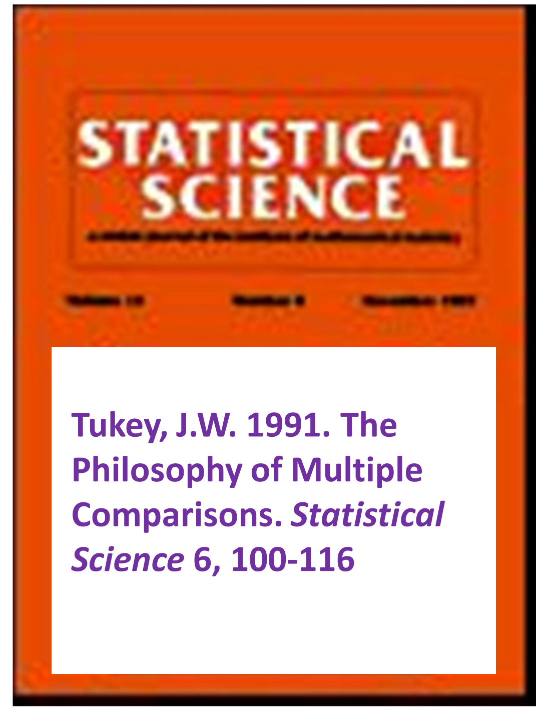
- Which analyte is different between the test and control?

- Is this difference relevant?



Statistical significance ≠ biological relevance

- ***"All we know about the world teaches us that the effects of A and B are always different – in some decimal place – for any A and B."***
- Statistical significance depends on many factors including:
  - The test and control being compared
  - The design of the experiment
    - e.g., # of replicates
  - The variation in the data



Tukey, J.W. 1991. The Philosophy of Multiple Comparisons. *Statistical Science* 6, 100-116

Statistical significance ≠ biological relevance

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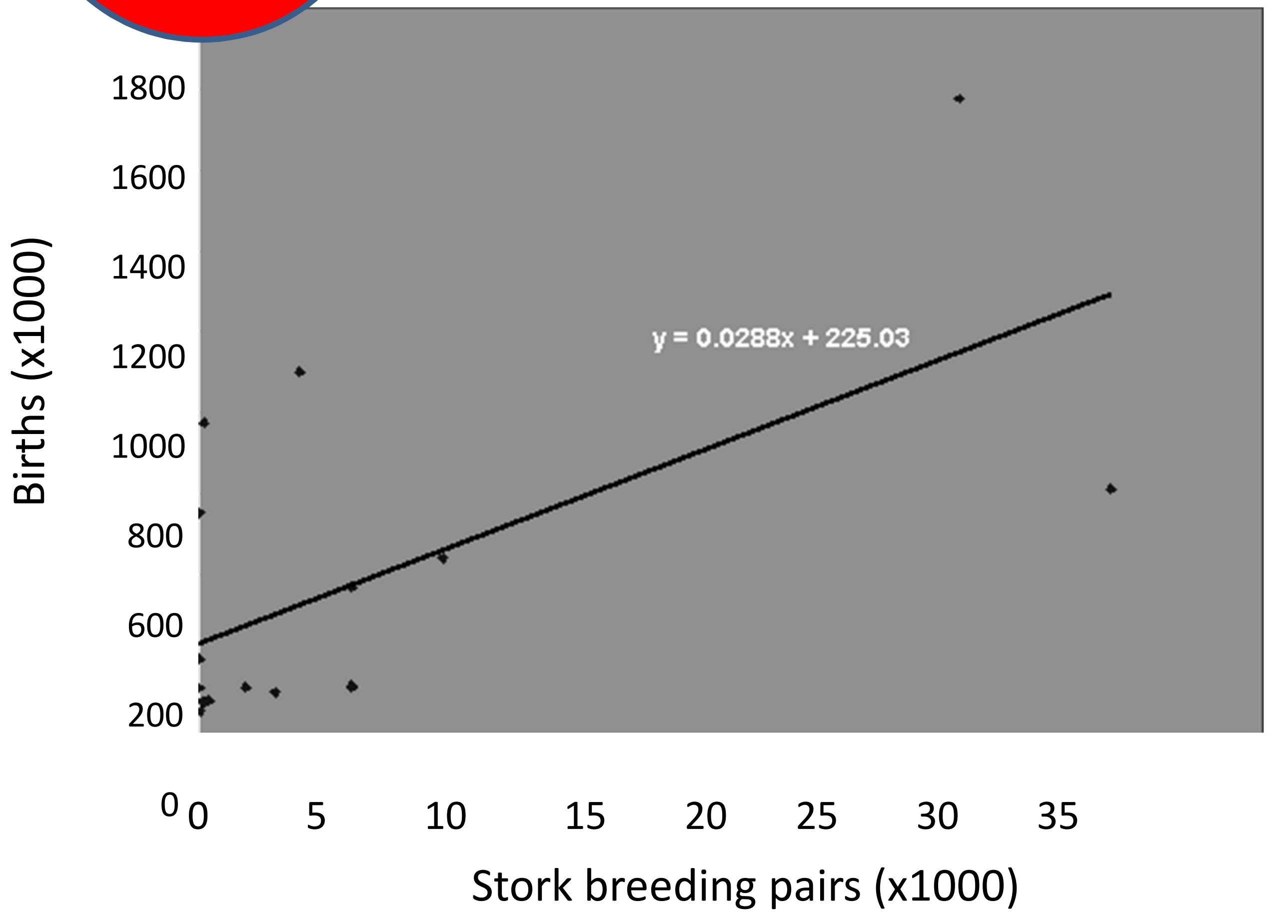
Country	Storks (pairs)	Birth rate (10^3 yr)		Country	Storks (pairs)	Birth rate (10^3 yr)
Albania	100	83		Hungary	5000	124
Austria	300	87		Italy	5	551
Belgium	1	118		Poland	30,000	610
Bulgaria	5000	117		Portugal	1500	120
Denmark	9	59		Romania	5000	367
France	140	774		Spain	8000	439
Germany	3300	901		Switzerland	150	82
Greece	2500	106		Turkey	25,000	1576
Holland	4	188				

Matthews, R. 2000. Storks deliver babies ($p = 0.008$). Teaching Statistics 22:36-38.

Statistical significance ≠ biological relevance



- R = 0.62, p = 0.008
- 0.008 = 1/125
- I.e., there is 1 chance in 125 that results are due to random chance
- Misinterpretation that there is 124/125 = 99.2% certainty that storks really deliver babies

Statistical analysis identifies differences

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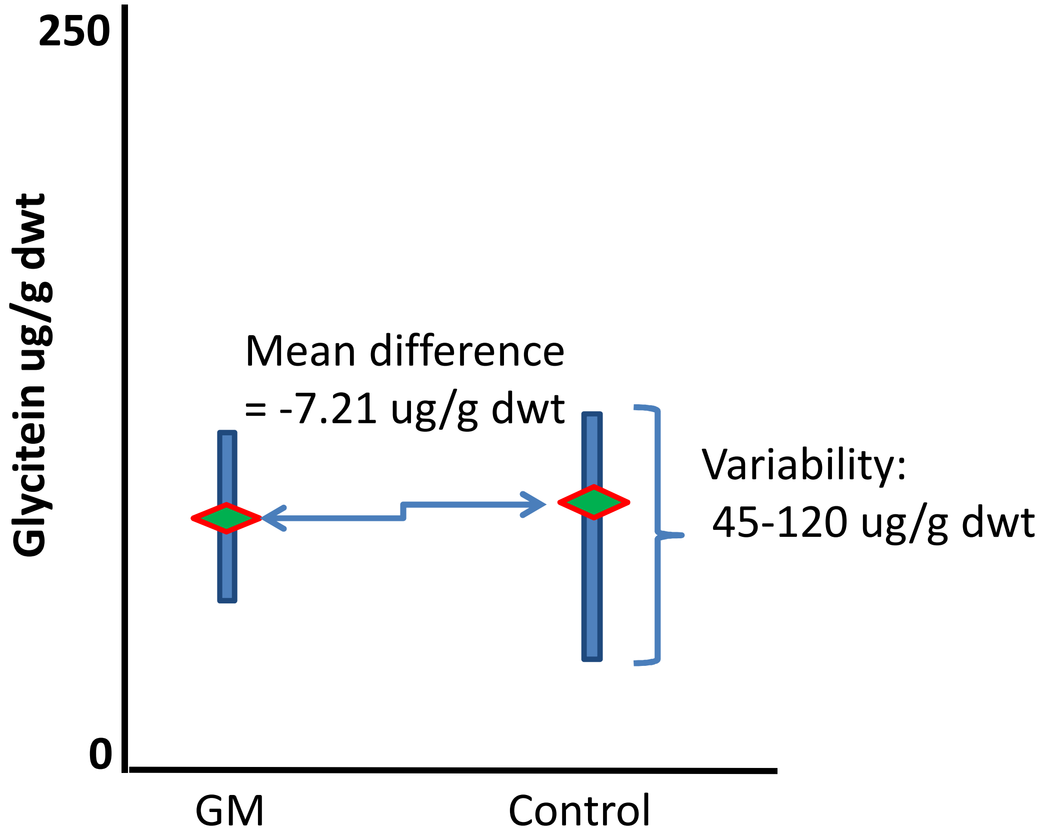
Comparing the differences to **natural variability** provides a context of biological relevance



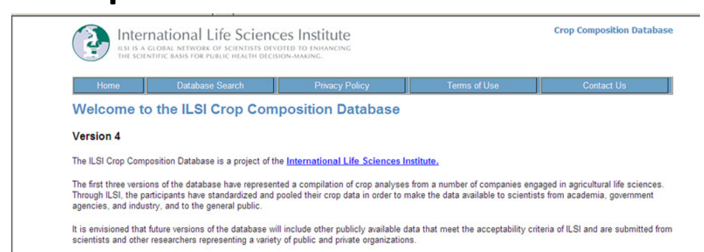
***Science is inevitably about magnitudes*.**

Cohen, J. (1990). *American Psychologist*, **45**, 1304-1312)

Defining natural variability is key to determining if a difference has a safety implication!

ILSI Crop Composition Database- Helps capture variability of component levels across geographies, environments, and years

- Contains over 100,000 data points on corn, cotton and soybeans
- Compositional data has been added from Australia, Philippines, and Canada
- Updating include canola, new data on crops
- Web-based User Interface
  - www.cropcomposition.org
  - Free public access



Analyte Type	Analyte	Minimum Value	Maximum Value	Mean Value	Units
Bio Actives	Total Daidzein	60.0	2,453.5	834.8	mg/kg DW
Bio Actives	Total Genistein	144.3	2,837.2	976.8	mg/kg DW
Bio Actives	Total Glycitein	15.3	310.0	156.6	mg/kg DW

Describing natural variability places differences into biological context

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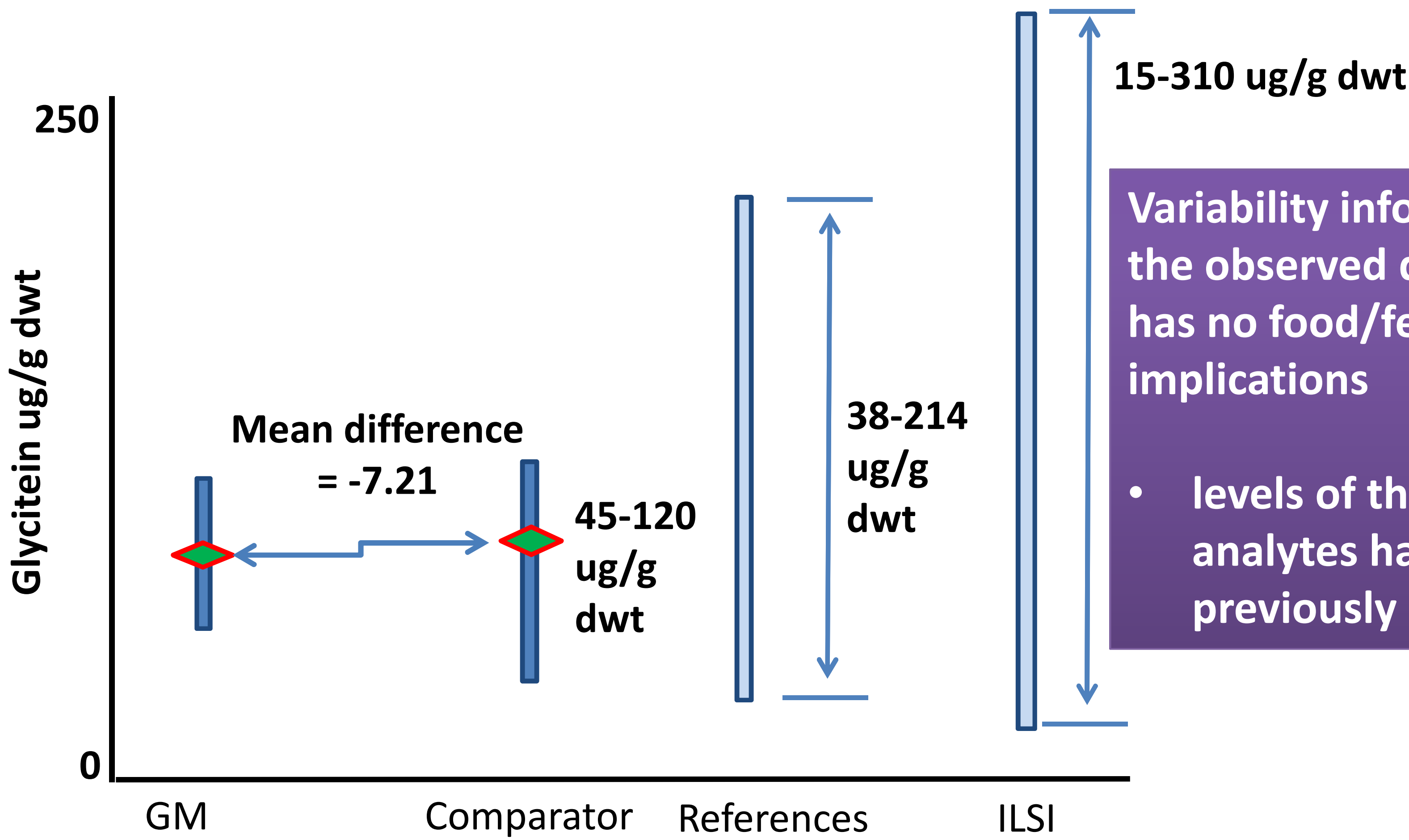


Variability informs that the observed difference has no food/feed safety implications

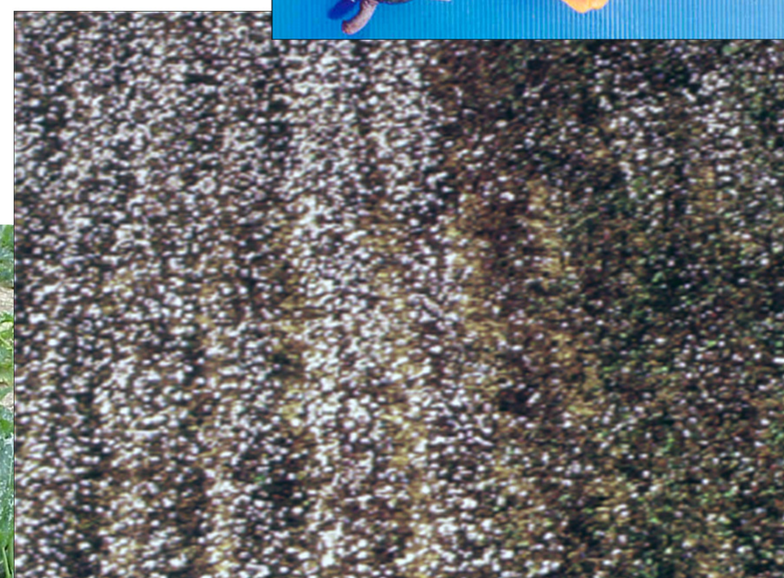
- levels of these analytes have been previously consumed

FDA 1995 – 2012

- Evaluation of 129 events
  - 11 different crops
- *All* have been
  - "'Not materially different in composition, safety or any other relevant factor of varieties now grown, marketed or consumed in the US"
- I.e., are 'substantially equivalent'
- No other country has found differences either

urtesy Bill Price, FDA, retired
:p://www.ilsi.org/FoodBioTech/Documents/2012%20TF12%20Plant%20Compositional%20Analysis%20Workshop%20Presentation%20PDFs
4_Price%20(IFBiC%202012%20TF12%20Workshop).pdf

Why no difference?



Many steps where off-types and unintended effects get eliminated

- During transgenic selection

- When transgenic crossed into varieties and inbreds

- Selection for phenotype
  - Eliminate off-types
- Selection for phenotype
  - Eliminate off-types
- Selection for phenotype
  - Eliminate off-types

- During seed production

Making modern transgenics via rDNA

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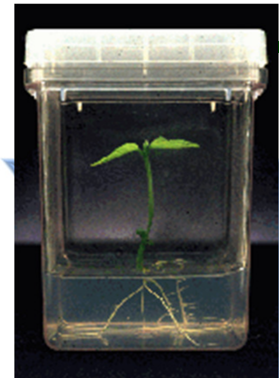


1,000's — T_0 – Molecular characterization

10,000's — T_1 - Gene expression

1000's — T_2 - Phenotype

100's — T_3 -

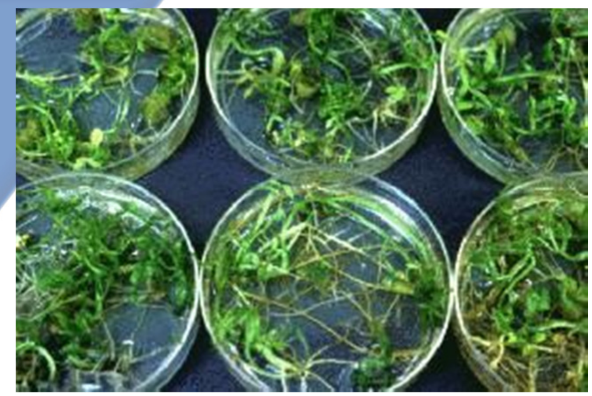
T_4 - Gene Stability

10's — T_5 - Potential lead events

T_6 – Composition & agronomic

T_8 – Composition & agronomic

1 Lead event



Elimination of

- Undesired types
- Unintended types

The trait introgression process reduces potential for unintended effects

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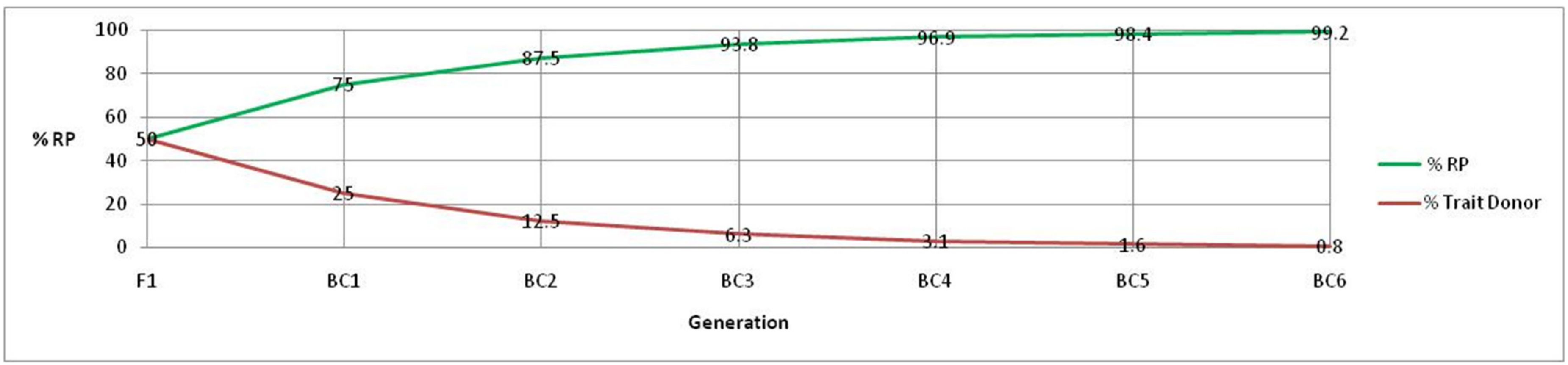
GM varieties contain >99% genetics of recurrent parent

Transgenic (TG) Trait Donor

Elite Germplasm with TG



Seed production

Additional selection as seed is increased

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Breeder seed

- Selection for phenotype
  - Eliminate off-types

Foundation seed

- Selection for phenotype
  - Eliminate off-types

Certified seed

- Selection for phenotype
  - Eliminate off-types

Conventional breeding has never activated novel toxic pathways

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- Of millions of conventional varieties, only 9 have been reported to have unintended effects
  - Dermatitis and stomach aches
  - One of the safest technologies
- All involved elevated levels of **known** toxins
  - Part of OECD list
  - When crops have known toxins, testing of new varieties has become customary

- What about unknown toxins?
- In all the history of breeding
  - A toxin that did not exist at the genus level has NEVER appeared unintentionally

- Previous report that a novel toxin was found in a potato somatic hybrid
    - Laurila et al., 1996. Plant Sci 118:145-155
  - Missed the fact that same toxin was previously described in some genotypes of potato
    - Jadav et al. 1981. CRC Critical Reviews in Toxicology pp 21-104.

Variability vs technique

Breeding ▪ Mutagenesis ▪ Transgenic

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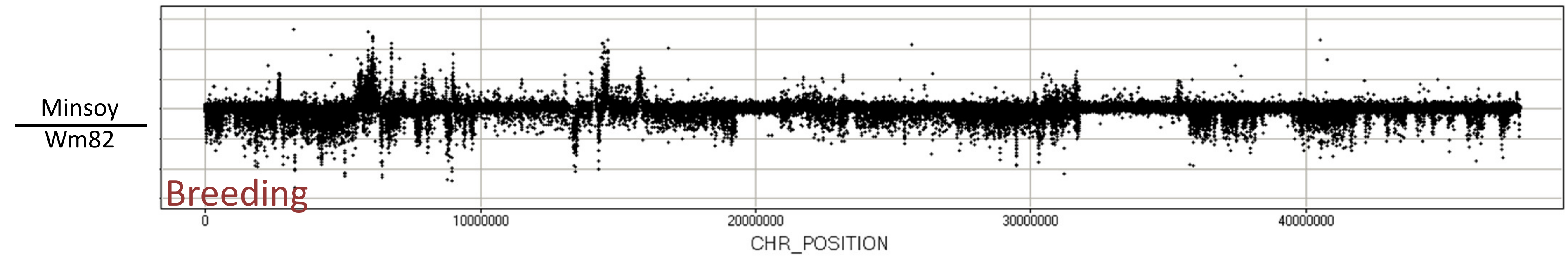
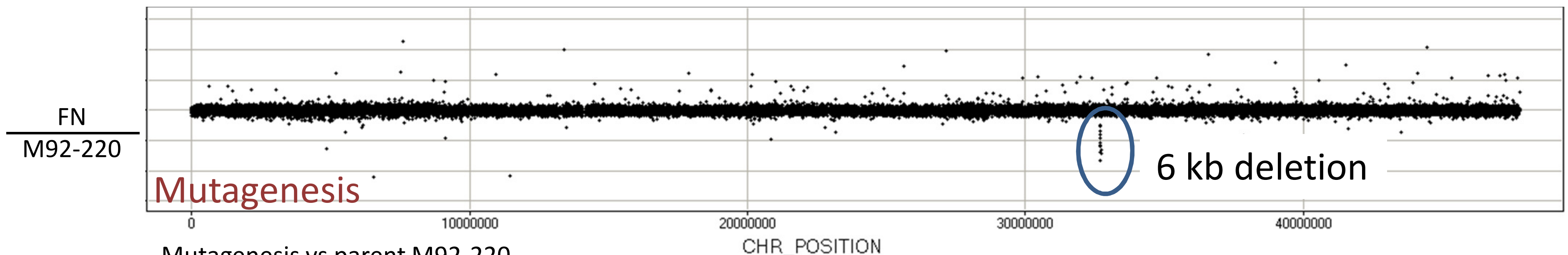
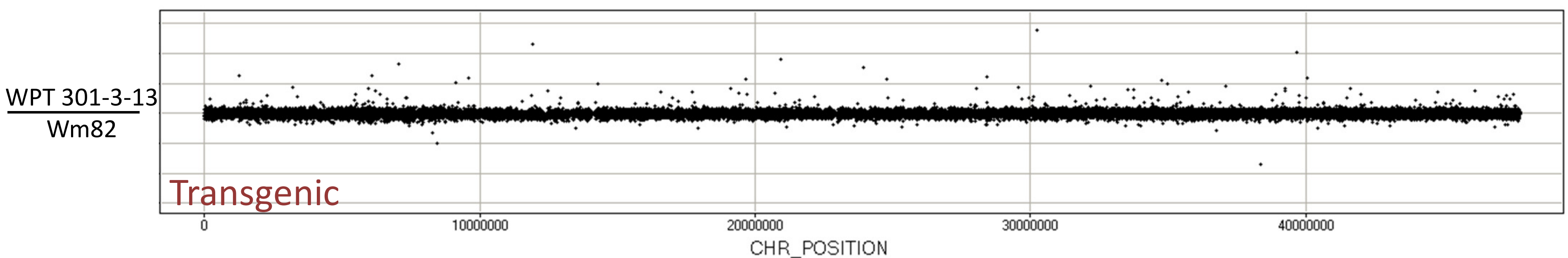




Mutagenesis vs parent M92-220



Transgenic vs parent, Wm82

Slide by Robert Stupar, University of Minnesota

How does variability impact our safety assessments of GM crops?

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- We consume variability every day
  - it is important to health!
- Defining variability
  - shows what has safely been used/consumed
- Evaluating the relevance of variation due to GM involves considering natural variation
  - i.e., what has been safely consumed

Take-home messages

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- Compositional analysis is an additional safeguard against unintended changes
  - The probability of an unwanted change is low
- Crops have been extensively modified during domestication and subsequent breeding
  - Almost all changes in composition *per se* are not dangerous
    - Novel metabolic pathways do not arise suddenly
    - Breeding is not known for toxin generation
- The system eliminates unwanted types at many steps of the process
- Engineering makes fewer changes than breeding does
- Genotype and environment contribute more compositional variability than the insertion of a transgene

IFBiC Composition Issues
Task Force 12

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International Food Biotechnology Committee

Academia • Government • Industry

* TF12 Co-Chair

Gerard Barry, IRRI, Philippines
Andrew Bartholomaeus, University of Canberra & University of Queensland, Australia
Philip Brune*, Syngenta Crop Protection
Janet Collins, DuPont
Russ Essner, Bayer Crop Science
Nancy Gillikin, BASF Plant Science
Angela Hendrickson Culler*, Monsanto Company
Rod Herman, Dow AgroSciences

Owen Hoekenga, USDA, ARS
Joanne Holden, USDA, ARS (Ret.)
Greg Ladics, DuPont
Carl Maxwell, DuPont
Wayne Parrott, University of Georgia
Bill Price, US FDA (Ret.)
William Ridley, Saint Louis University
Jannavi Srinivasan, US FDA
Kate Walker, ILSI

http://www.ilsi.org/FoodBioTech/Pages/2012PlantCompositionWorkshop.aspx

Journal of Agricultural and Food Chemistry Plant Physiology
September 4 issue (Vol. 61, No. 35), pp. 8243-8355

Importance of Science-Based Policies

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- Science-based regulation is needed to
  - Ensure safety is not compromised
  - Ensure that over-regulation does not prevent useful products from reaching the consumer
- Science-based policies require
  - Understanding what is done and its limitations
  - Understanding why the tests are done
- Strong science can
  - Minimize regional differences in interpretation, implementation and requirements
  - Help harmonize trade