Absolute quantitation of seed allergens using tandem mass spectrometry

Severin E. Stevenson

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Hotel Indigo
Research Triangle Park, NC

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Overview

- Quantitative proteomics strategies
  - "Bottom-up" proteomics techniques
  - Quantitative outputs
  - Absolute quantitation strategy (AQUA®)
- Seed allergens
  - Statistics and concerns
- Allergen quantitation using LC-MS/MS
  - Environmental vs. genetical variables in determining soybean allergen levels

“Bottom-up” quantitative proteomic techniques

- **Analyzes protein fragments (peptides) from proteolysis**
- **Peptides are fragmented during tandem mass spectrometry analysis (MS/MS)**
- **LC-retention time, peptide mass and fragment masses provide specificity for peptide of interest**

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Quantitative proteomic outputs

- **Chromatography-dependent quantitation (peak integration)**
  - **Whole peptide signal measured as it elutes (dMS)**
  - **Peptide fragment signal measured as it elutes (MRM)**
- **Chromatography-independent quantitation (spectral counting)**
  - **Count the number of times a protein is identified by database searching of MS/MS spectra**

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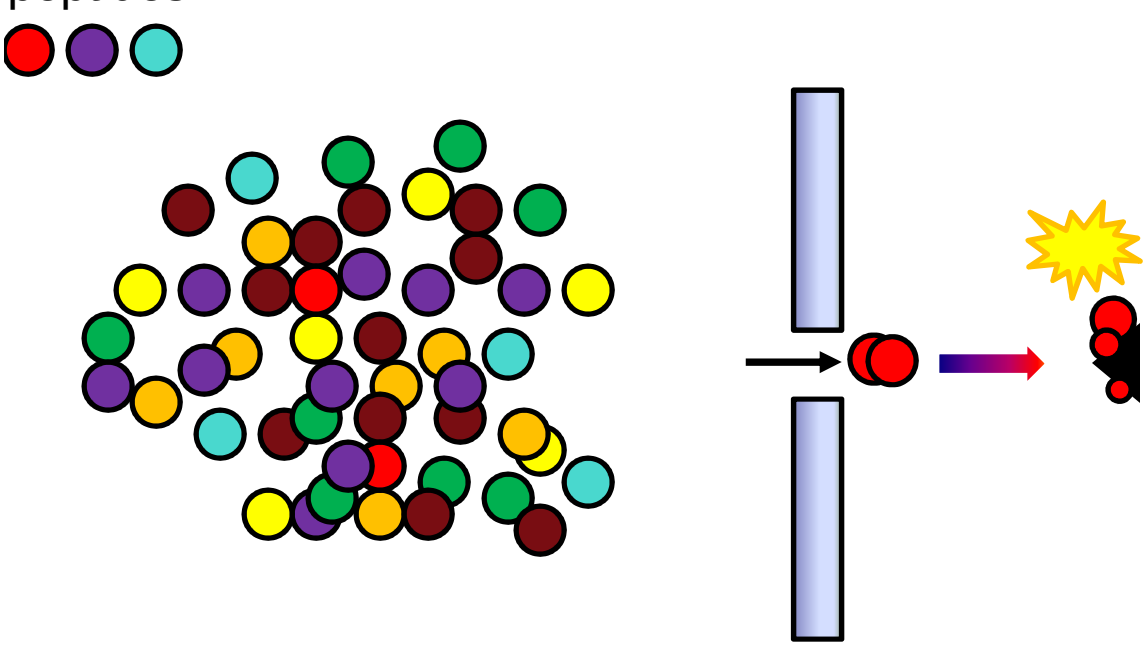
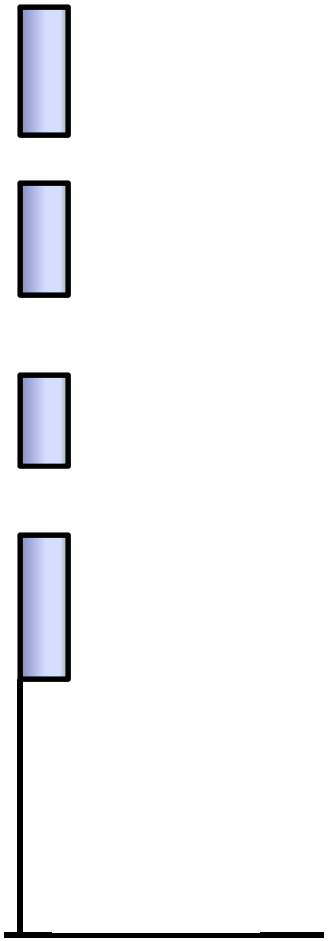
Quantitation by Multiple Reaction Monitoring (MRM)

peptides





Occurs within milli seconds-
Capable of measuring >10 different peptides every second
<u>Hundreds of peptides can be quantified in a single experiment</u>

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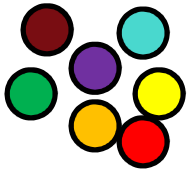
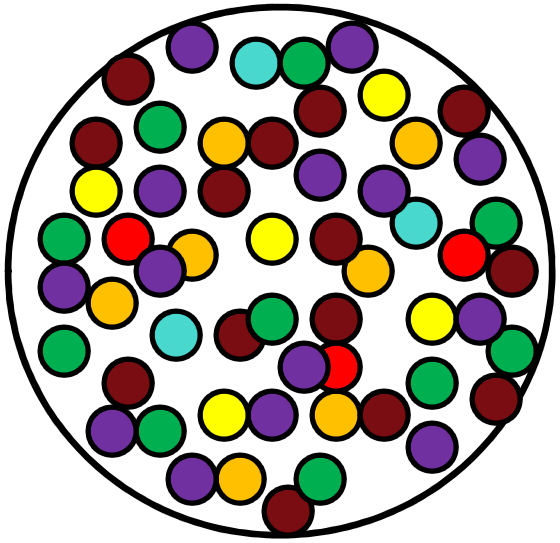
Quantitation by Multiple Reaction Monitoring (MRM)

peptides



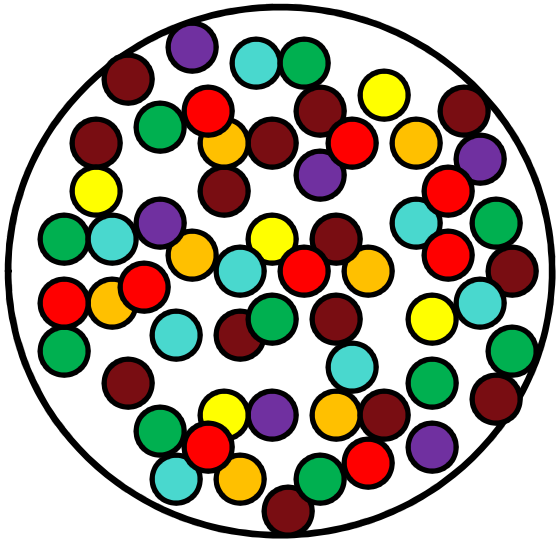
Control

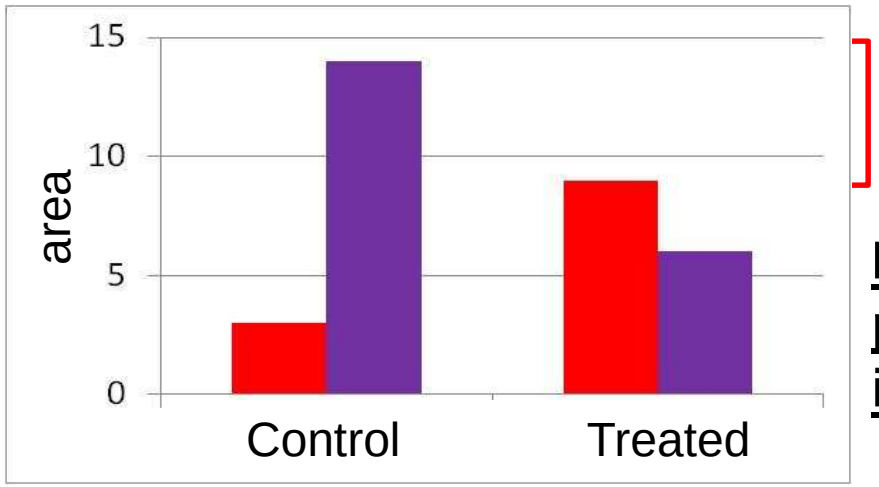
Proteome



Treated

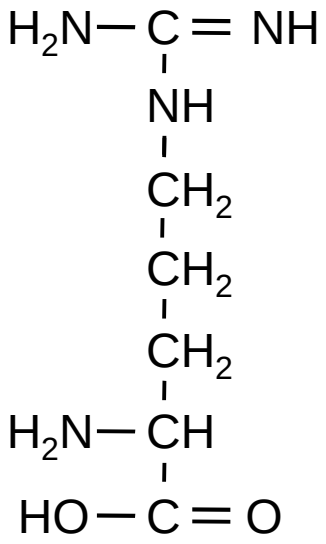
Proteome

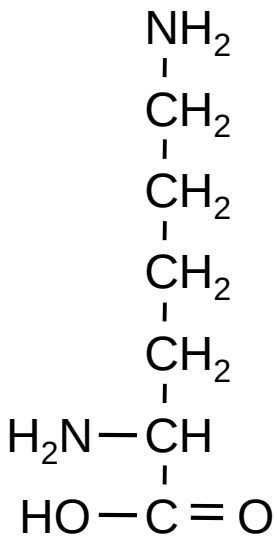




<u>Doping with peptide standards can provide absolute quantitative information</u>

AQUA® peptides are synthetic peptides with a single stable isotope-labeled amino acid





	Arginine (R) – 174.2	Lysine (K) – 146.2
Isotope labeled	**$^{13}C_6H_{14}{}^{15}N_4O_2$ – 184.2**	**$^{13}C_6H_{14}{}^{15}N_2O_2$ – 154.2**
Native peptide	LSAEFGSLR – 978.5	VSDDEFNNYK – 1229.5
AQUA peptide	LSAEFGSL**R*** – 988.5	VSDDEFNNY**K*** – 1237.5

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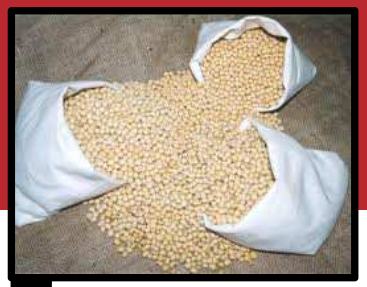
AQUA-MRM
Absolute quantitation

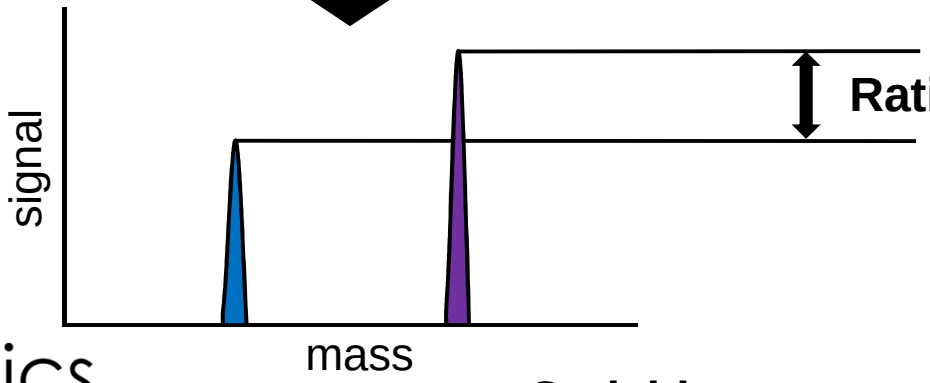


Seed allergen (peptides)

Labeled internal standard

Mix

mass spectrometry can distinguish them



Ratio gives quantity

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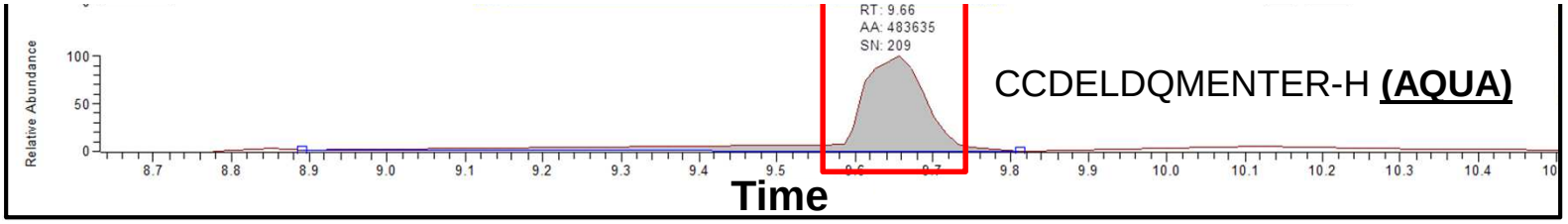
Stoichiometry correlates peptide and protein abundances

Near isobaric peptides may not be distinguished during low resolution analysis
-Retention times finalize peptide ID-




```
                                CCDELDQMENTER
A5Z1R0 QYDSYDIRSTRSSDQQQRCCDELNEMENTQRCMCEALQQIMENQCDRLQDRQMVQQFKRE 120
A5Z1Q6 QYDSYDIRSTRSSDQQQRCCDELNEMENTQRCMCEALQQIMENQCDRLQDRQMVQQFKRE 120
Q647G9 QYDSYDIRSTRSSDQQQRCCDELNEMENTQRCMCEALQQIMENQCDRLQDRQMVQQFKRE 120
A5Z1Q5 QYDSYDIRSTRSSDQQQRCCDELDQMENTERCMCEALQQIMENQCDRLQDRQMVQQFKRE 120
       ************************:::****:******************************
```

1 - CCDELNEMENTQR	1584.61 Da	792.81 m/z
2 - CCDELDQMENTER	1585.60 Da	793.30 m/z

0.5 Da difference

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Summary

- “Bottom-up” quantitative proteomic strategies measure peptide abundances
- Tandem mass spectrometry analysis has multiple quantitative outputs
- MRM identifies peptides with three levels of specificity
  - Precursor mass, fragment mass and retention time
- MRM with AQUA® peptides provides absolute levels of peptides and proteins

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Some legumes, tree nuts, and grains elicit an allergic response in humans

- 4-6% of the US population has a food allergy
- 90% of all food allergy reactions are caused by 8 foods: milk, **soy**, eggs, **wheat, peanuts, tree nuts,** fish and shellfish
- Food safety regulatory agencies would like to monitor allergen levels in new crops, especially GM crops

Rationale

- **Natural variation in allergen expression in conventional soy germplasm is unknown**
- **Understanding this range of variation would be useful for determining the “tolerable” levels of allergen expression**
- **These data could also be useful for developing guidelines for acceptable variation in allergen expression in GM crops**

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Environmental and genetical effect on soybean allergen levels

- **9 locations, 3 genotypes in and around Iowa (ILSI-HESI Protein Allergen Technical Committee)**
- **6 locations, 4 genotypes across North America (DuPont-Pioneer)**

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Soy allergens of interest

- **Glycinin G1 (Bx)**
- **Glycinin G2 (A2)**
- **Glycinin G3 (A)**
- **Glycinin G4 (A5)**
- **Beta-conglycinin, α subunit**
- **Kunitz trypsin inhibitor 1**
- **Kunitz trypsin inhibitor 3**
- **Gly m Bd 28K**
- **Gly m Bd 30K (34 kDa maturing seed protein)**

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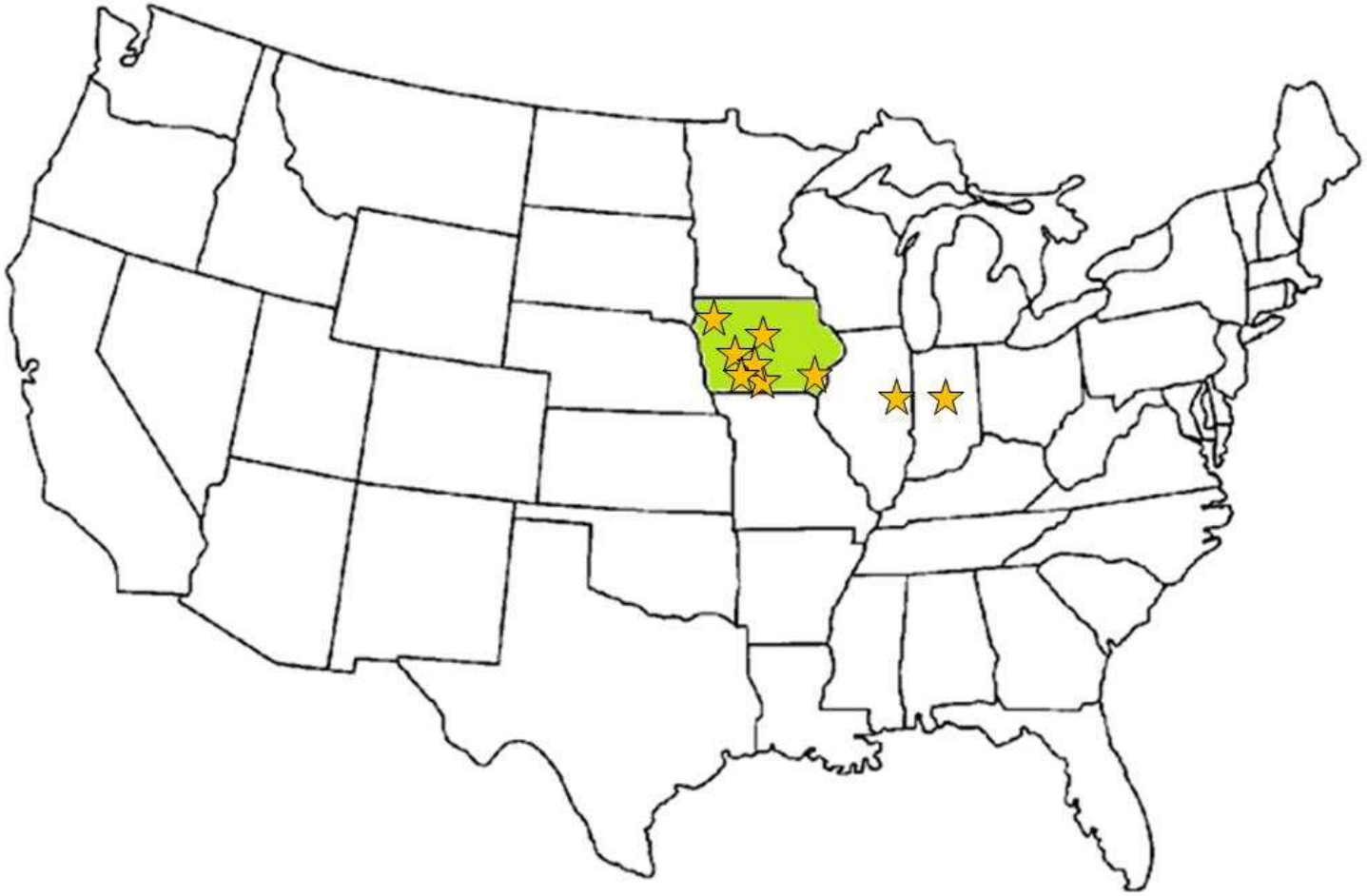
HESI-PATC project

3 cultivars (Stine varieties) x 9 locations



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HESI-PATC project
Experimental design

- **Seeds provided by Bayer Crop Sciences**
- **3 biological replicates (each replicate, 5 seeds)**
- **Protein isolated, quantified, processed for AQUA- MRM**
- **LC-MS/MS on a TSQ Vantage EMR triple quadrupole mass spectrometer**

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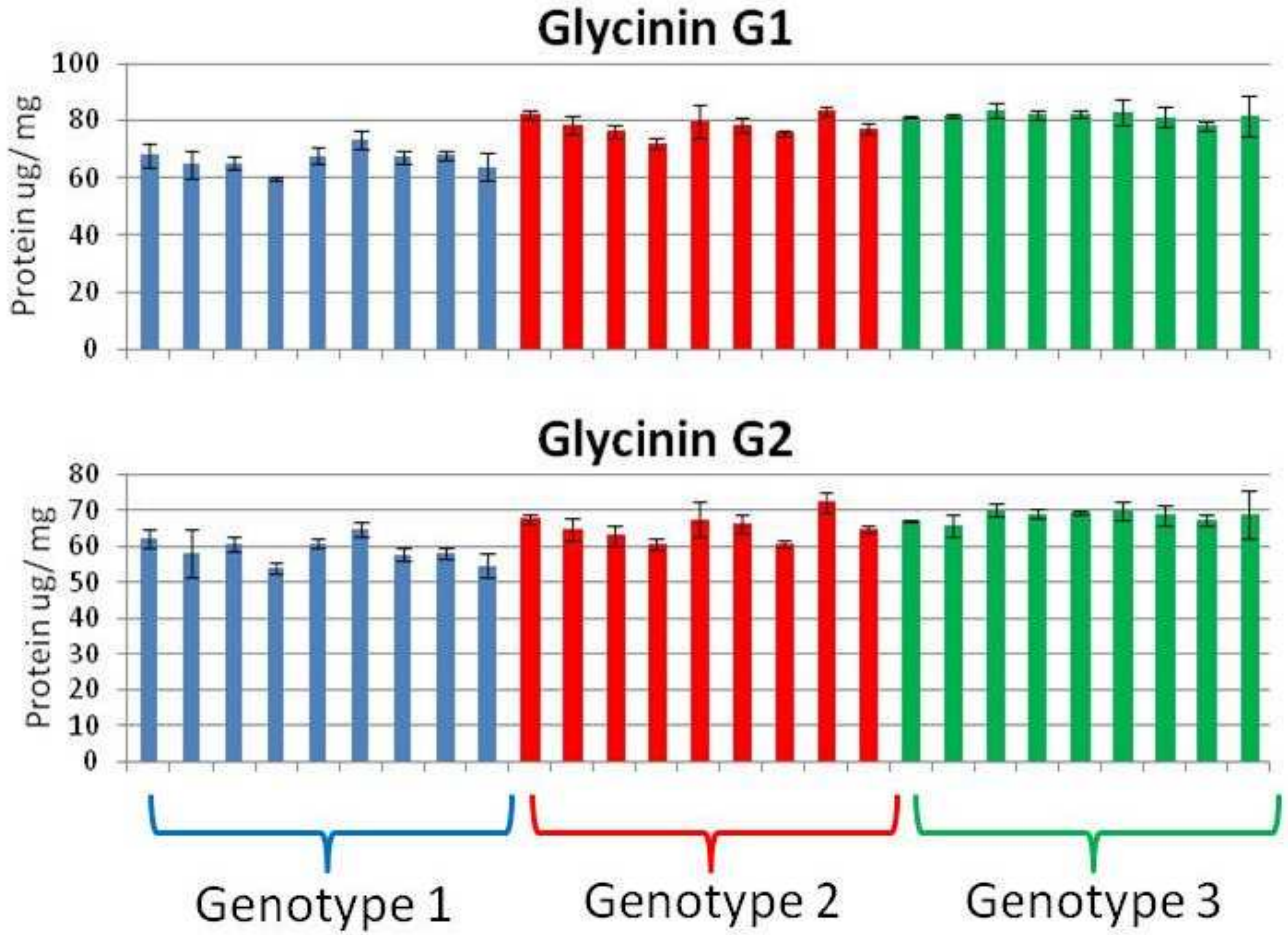
Glycinins G1 and G2 are visibly different among genotypes



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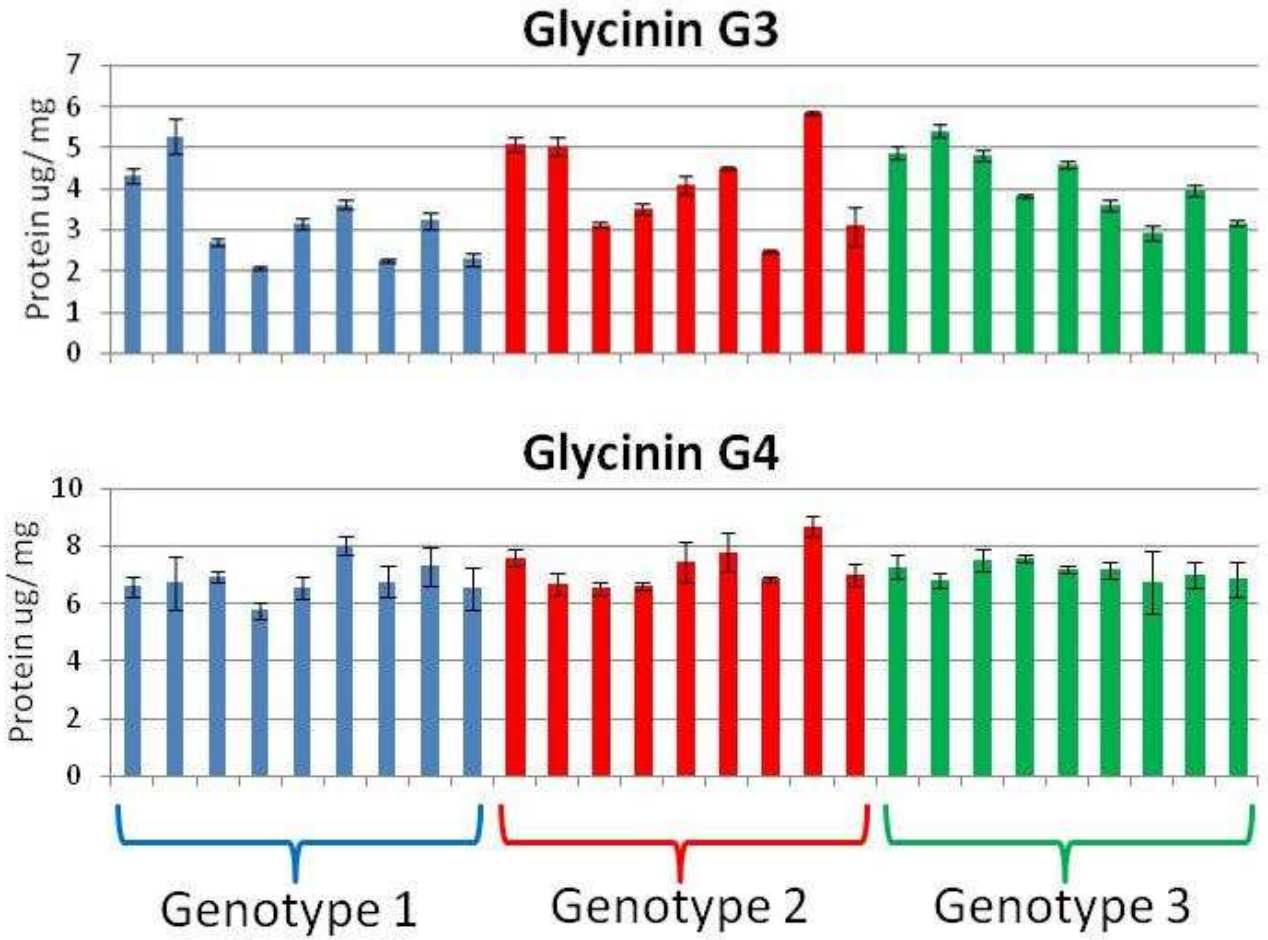
Glycinins G3 and G4 are visibly different among environments



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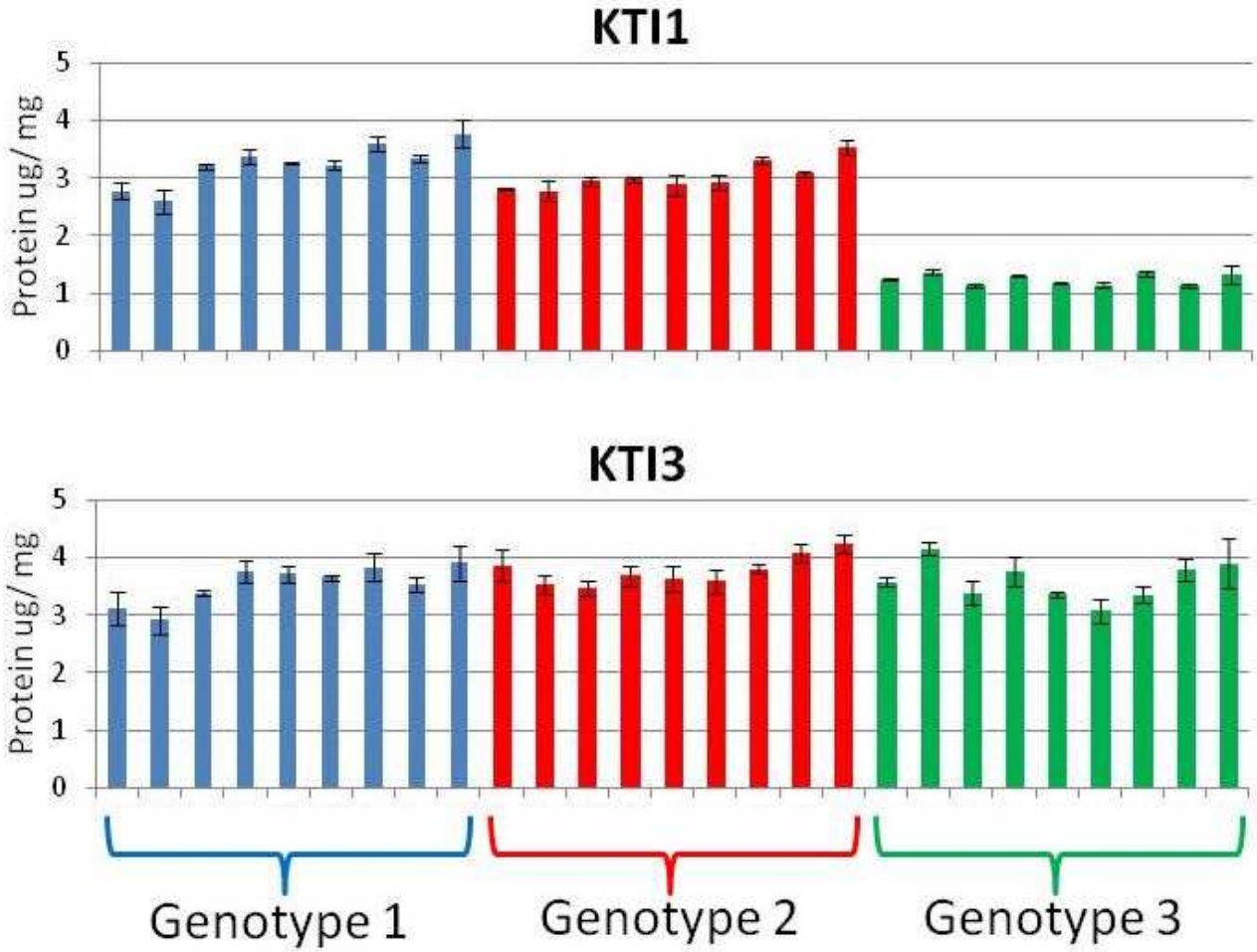
Kunitz trypsin inhibitor family members are affected by different variables



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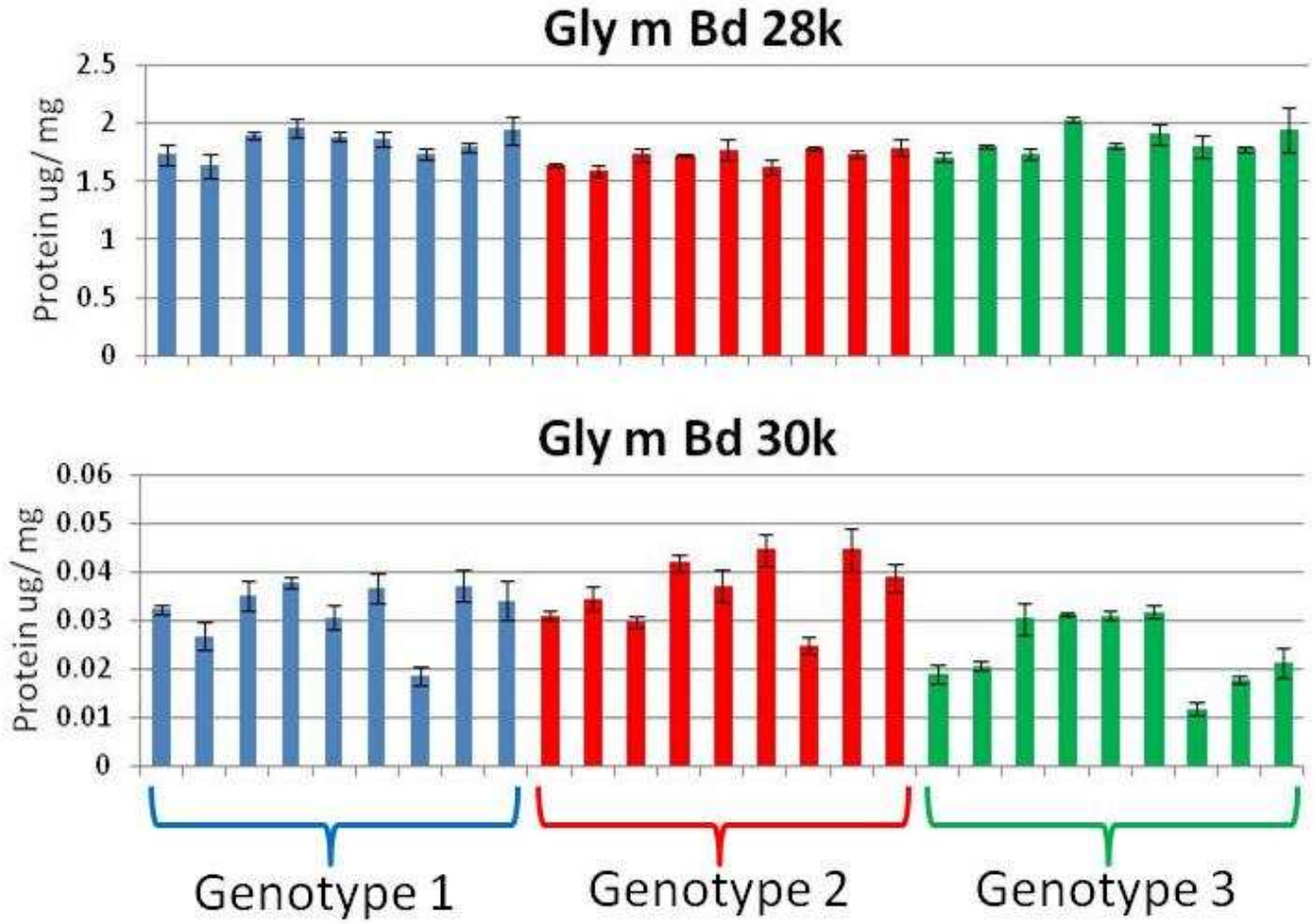
Gly m Bd 28k and 30k are affected by both genetics and environment



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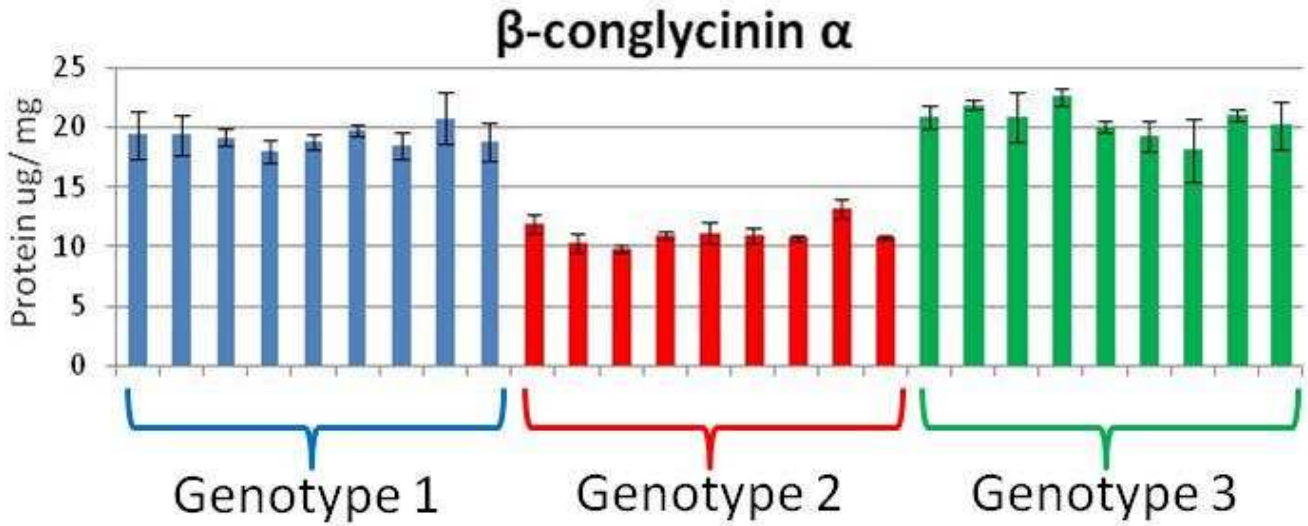
Beta-conglycinin alpha subunit shows marked genotypic variation



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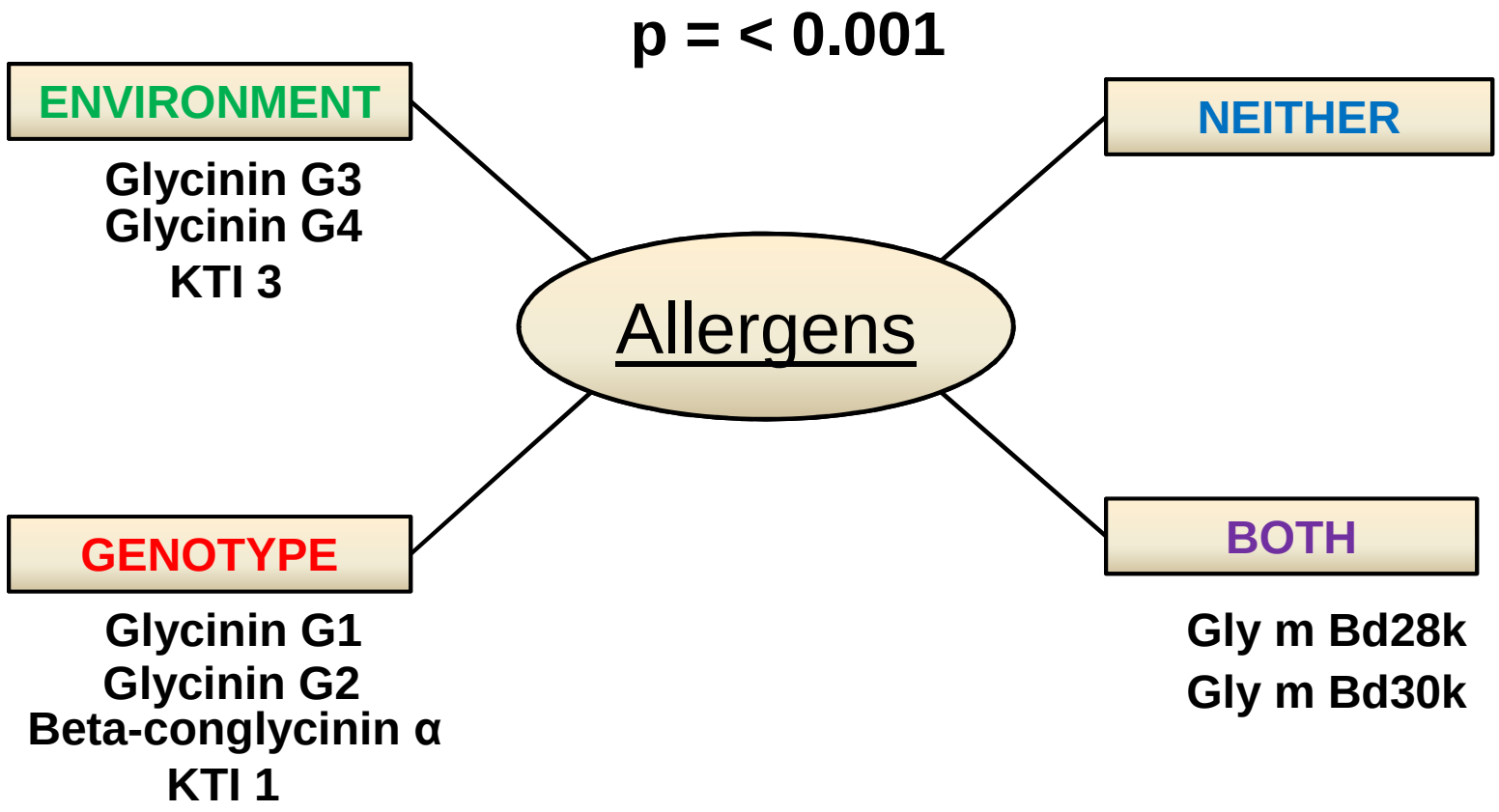
Sources of variation based on 1-way ANOVA
-Mostly Iowa environments (Bayer)-



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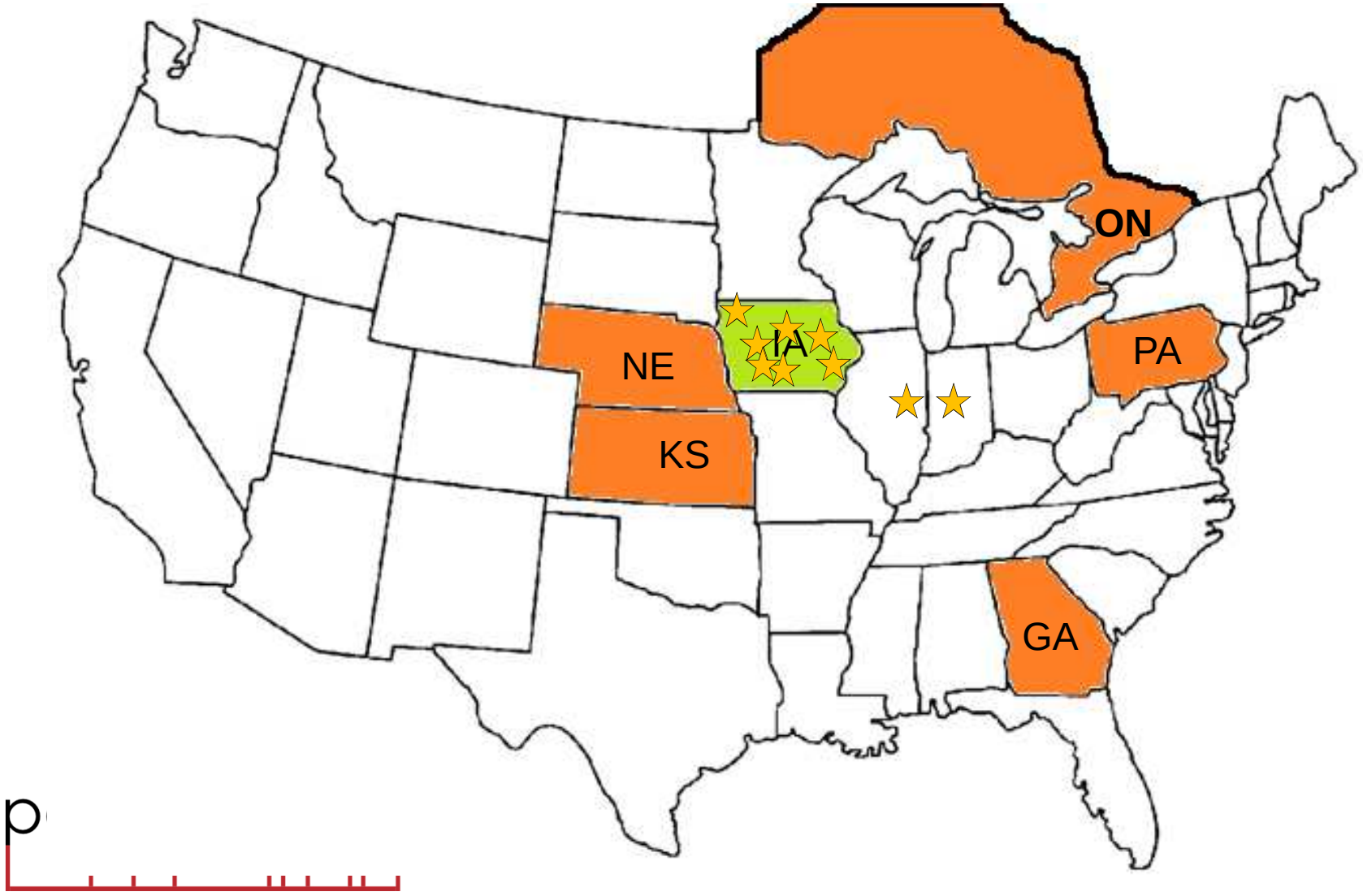
DuPont-Pioneer project

4 cultivars x 6 locations



DuPont-Pioneer project
Experimental design

- **Ground seed provided by DuPont-Pioneer**
- **4 extraction replicates were made**
- **Protein isolated, quantified, processed for AQUA- MRM**
- **LC-MS/MS on a TSQ Vantage EMR triple quadrupole mass spectrometer**

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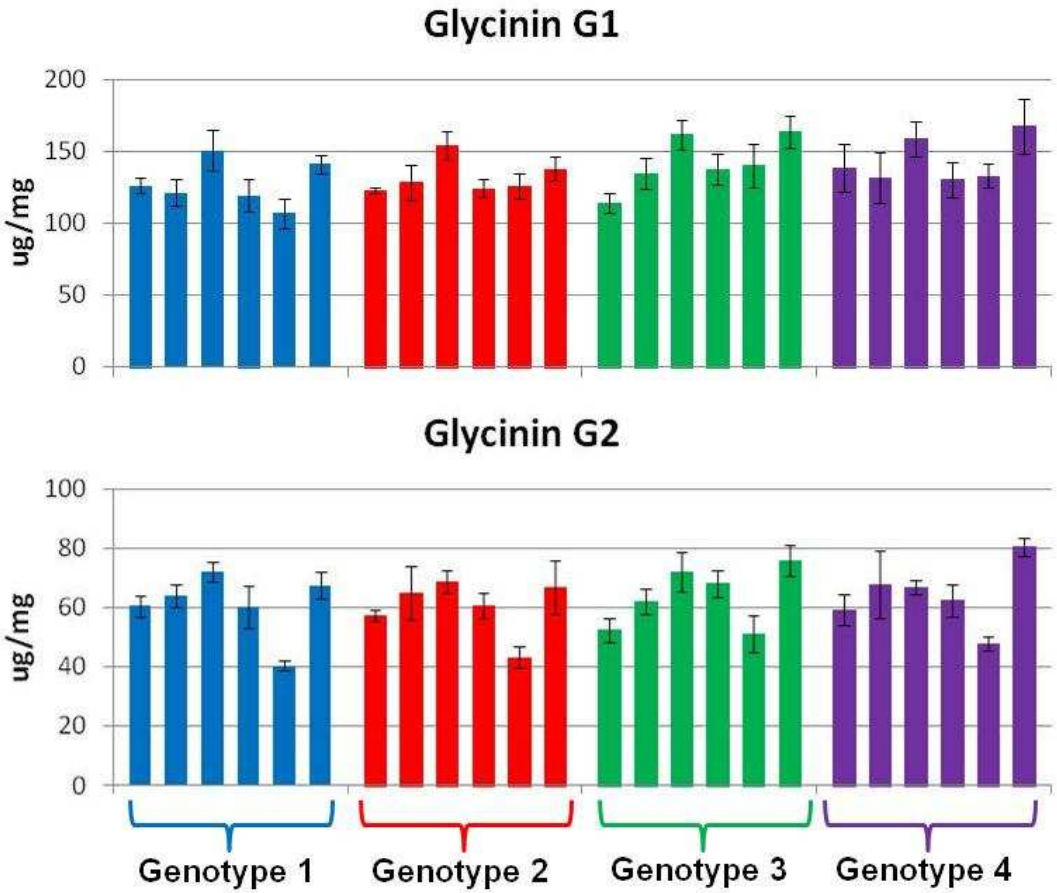
Glycinin G1 and G2 are visibly different among environments by not among genotypes



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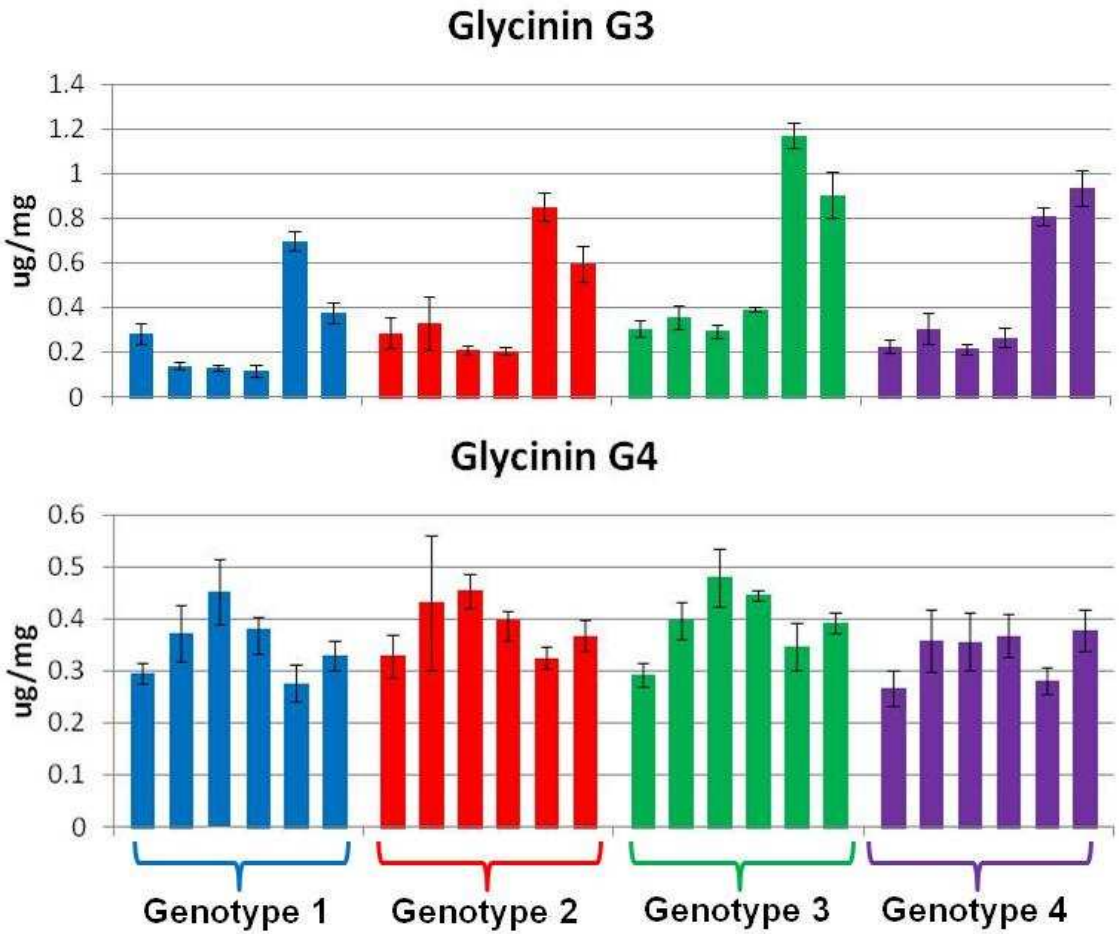
S. Stevenson et. al., Frontiers in Plant Proteomics, 2012., *submitted.*

Glycinin G3 shows large variation among environments



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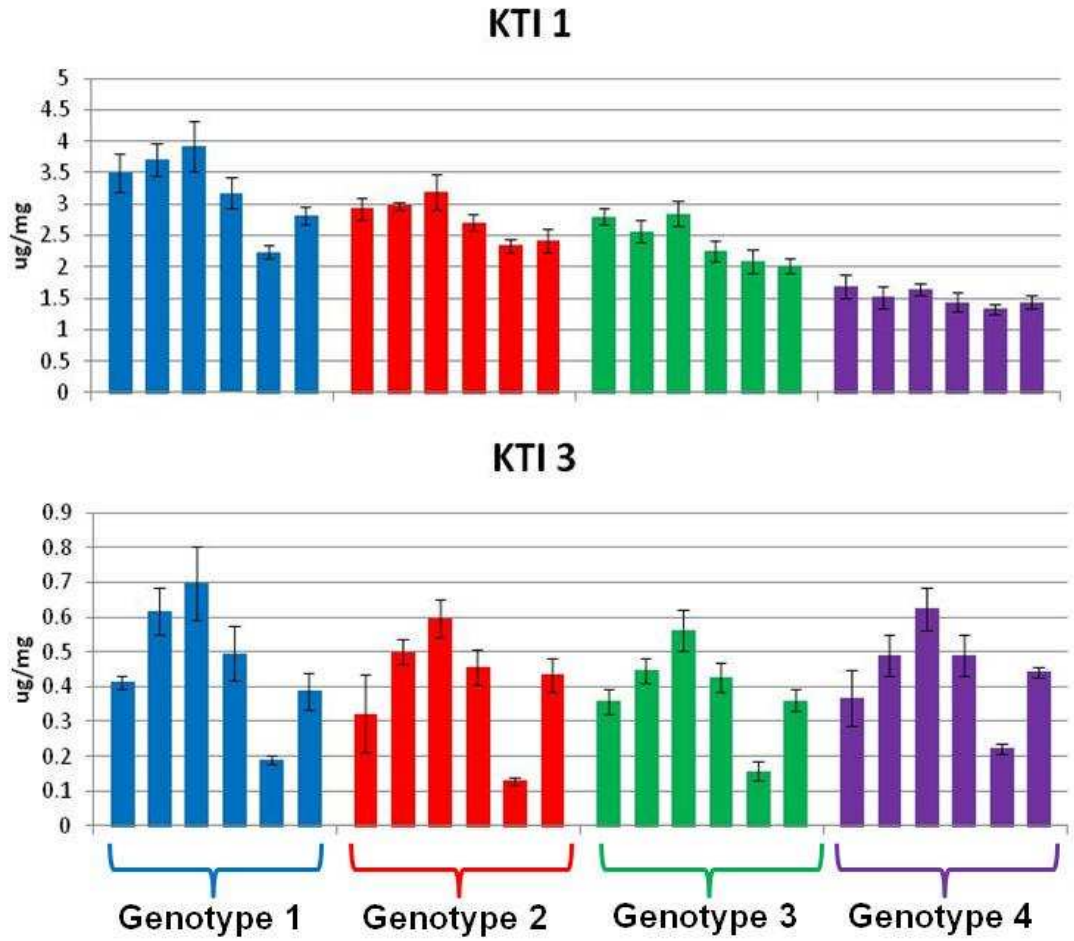
S. Stevenson et. al., Frontiers in Plant Proteomics, 2012., *submitted.*

KTI family members are again affected differently by the two variables



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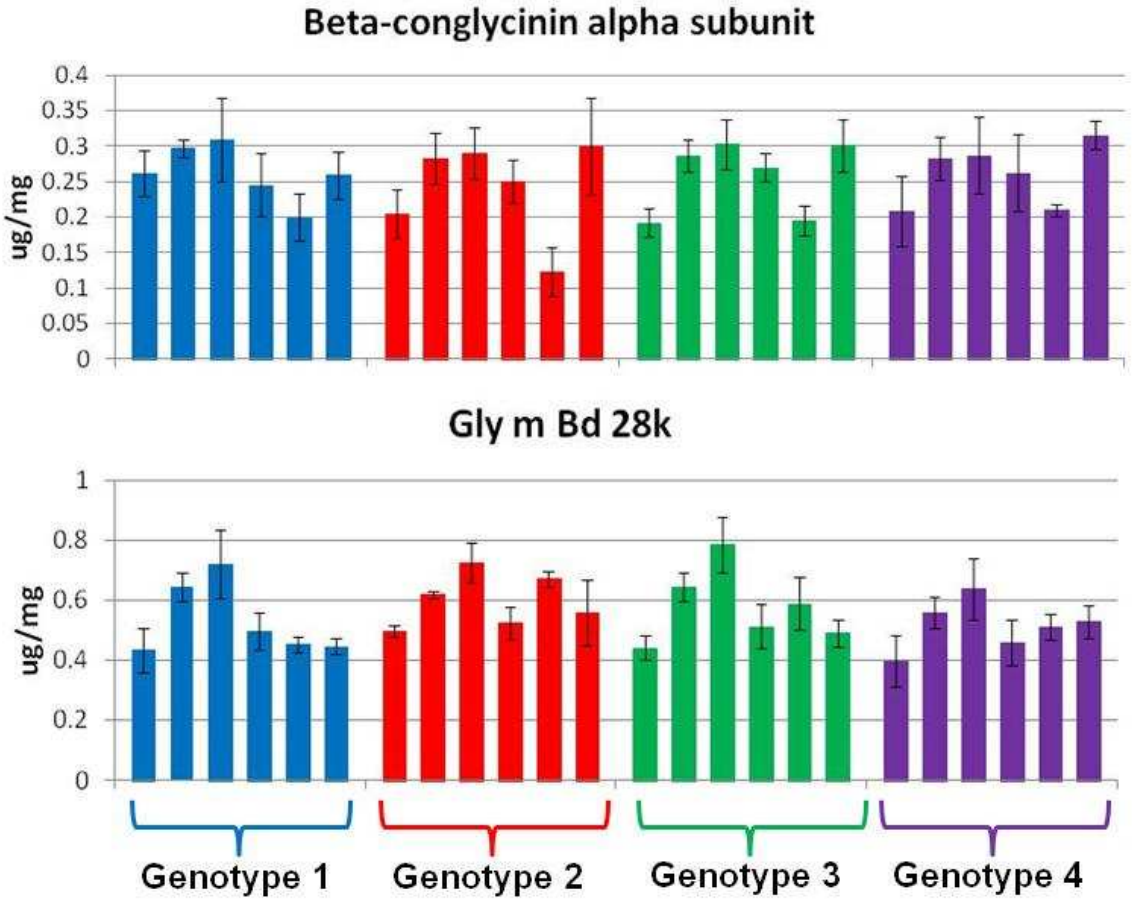
S. Stevenson et. al., Frontiers in Plant Proteomics, 2012., *submitted.*

Beta-conglycinin and Gly m Bd 28k are visibly affected by environment



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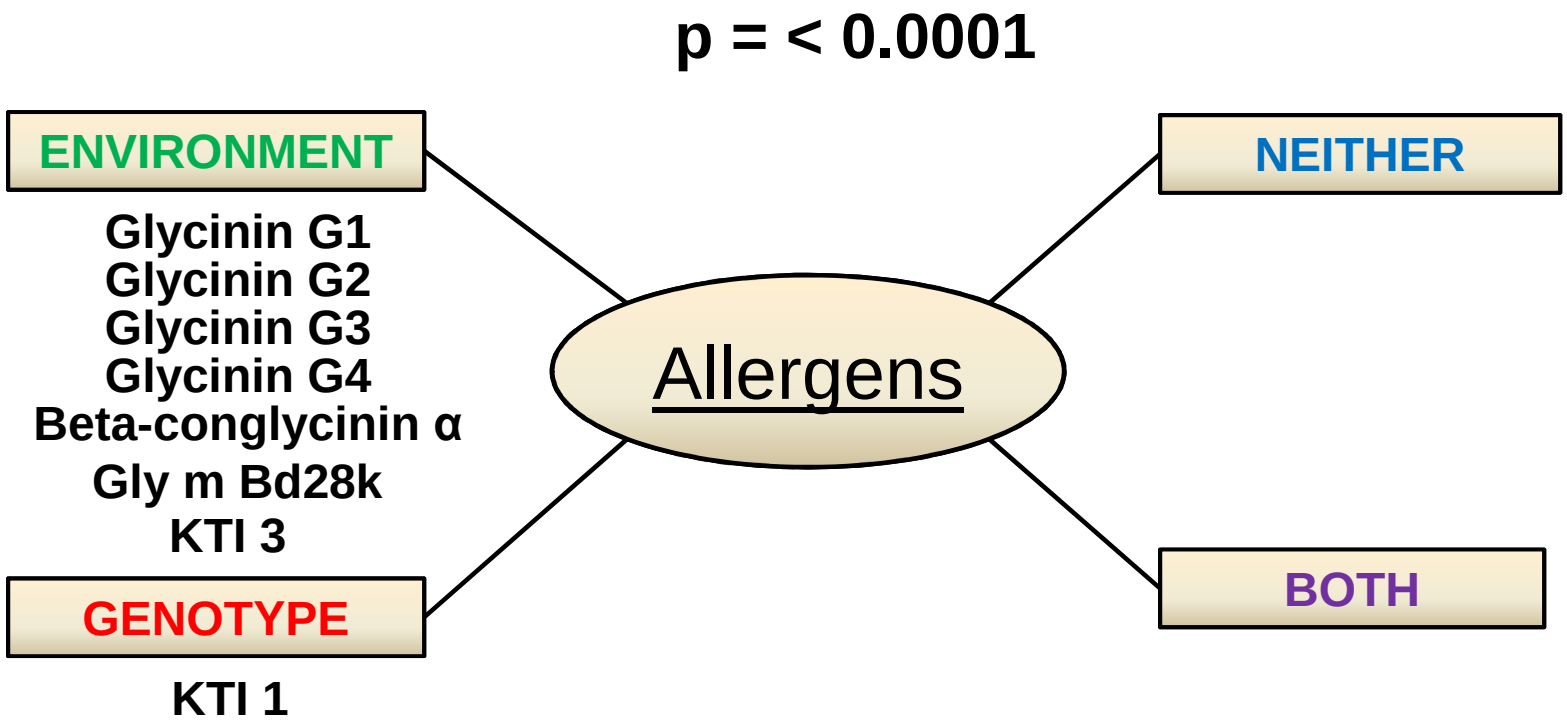
S. Stevenson et. al., Frontiers in Plant Proteomics, 2012., *submitted.*

Sources of variation based on 1-way ANOVA
-Multi-state environments (Pioneer)-



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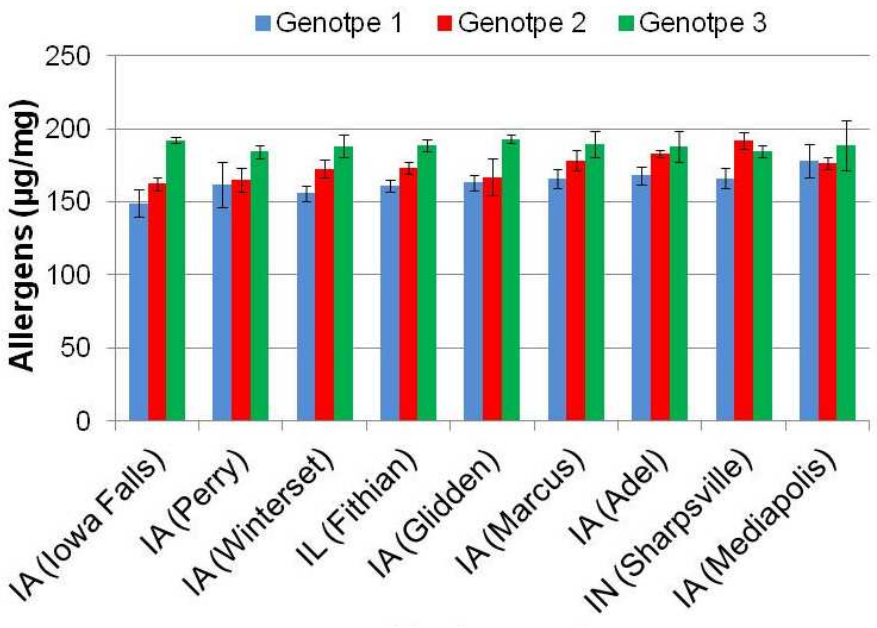
Variation in summed allergen content

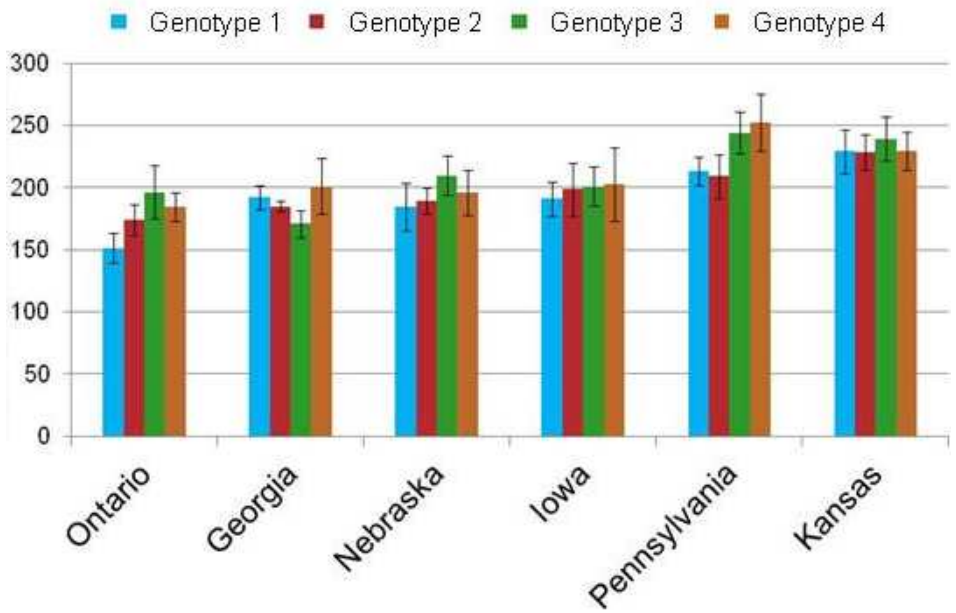
HESI-PATC
Mostly Iowa Study



Environment p = 0.94

Genotype p = 7.65E-7

DuPont-Pioneer
Multi-state Study



Environment p = 7.57E-5

Genotype p = 0.52

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Summary

HESI-PATC Study (mostly Iowa) - Genetic background contributed more to allergen variation than the environment

DuPont-Pioneer Study (N. America) - Environment contributed more to allergen variation than genetic background

Environmental effect on allergen expression is greater than genetics when geographical distribution is vast

Allergens (even gene paralogs) were affected individually by genetics and environment – perhaps due to diverse functions for allergens

AQUA-MRM is accurate, precise and quantitative

- **High-throughput and multiplexable**
- **Capable of quantifying hundreds of proteins in a single 30 minute acquisition**
- **Selective and specific**

Thanks

Funding

ILSI-HESI-PATC ("Iowa" phase 2 study)

Gregory S. Ladics[1], Gary A. Bannon[2], Scott McClain[3], Laura Privalle[4], Nicola Stagg[5], Corinne Herouet-Guicheney[6]

Pioneer Hi-Bred International[1], Monsanto[2], Syngenta Company[3], BASF Plant Science[4], Dow AgroSciences[5], Bayer SAS Bayer CropScience[6]

Collaboration

DuPont-Pioneer ("North America" study)

Gregory S. Ladics[1], Bonnie Hong[2], Xiaoxiao Kong[2]

[1]DuPont Agricultural Biotechnology; [2]Pioneer HiBred International

Thelen Lab-University of Missouri

Jay Thelen

Carlotta Peters

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