Quantification of eCry3.1Ab and Phosphomannose Isomerase in Maize Tissues Derived from Transformation Event 5307

Data requirement:	Not applicable
Author:	Mark Bednarcik
Date:	Study completed on March 2, 2011
Syngenta Study No.:	5307-08-01
Report No.:	SSB-016-09 A2
Performing Laboratory:	Syngenta Crop Protection, LLC Product Safety PO Box 12257 3054 East Cornwallis Rd. Research Triangle Park, NC 27709-2257, USA

STATEMENTS OF DATA CONFIDENTIALITY CLAIMS

The following statement applies to submissions to the United States Environmental Protection Agency (US EPA).

Statement of No Data Confidentiality Claim

No claim of confidentiality is made for any information contained in this report on the basis of its falling within the scope of Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Section 10 (d) (1) (A), (B), or (C).

Company: *Syngenta Seeds, Inc.*

Company Representative:

Demetra Vlachos — March 1, 2011

Demetra Vlachos
Regulatory Affairs Manager

Date

These data are the property of Syngenta Seeds Inc. and, as such, are considered to be confidential for all purposes other than compliance with the regulations implementing FIFRA Section 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other provision of common law or statute or in any other country.

The following statement applies to submissions to regulatory agencies and other competent authorities other than the US EPA and all other viewers.

This Document Contains Confidential Business Information

This document contains information that is proprietary to Syngenta and, as such, is considered to be confidential for all purposes other than compliance with the relevant registration procedures.

Without the prior written consent of Syngenta, this information may (i) not be used by any third party including, but not limited to, any other regulatory authority for the support of regulatory approval of this product or any other product, and (ii) not be published or disclosed to any third party including, but not limited to, any authority for the support of regulatory approval of any products.

Its submission does not constitute a waiver of any right to confidentiality that may exist in any other country.

STATEMENT CONCERNING GOOD LABORATORY PRACTICES

This study was conducted in compliance with the relevant provisions of Good Laboratory Practice Standards (40 CFR Part 160, U.S. EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act with the following exceptions:

- The production, collection, and shipping of the plant material were conducted outside the GLP portion of this study.
- An expired solution was used during a portion of the study.

Study Director

Mark J. Bednarcik — March 2, 2011

Mark Bednarcik
Product Safety
Syngenta Crop Protection, LLC

Date

Submitted by

Demetra Vlachos — March 1, 2011

Demetra Vlachos
Regulatory Affairs Manager
3054 East Cornwallis Rd.
PO Box 12257
Research Triangle Park, NC 27709-2257, USA

Date

Sponsor

Catherine Kramer — March 2, 2011

Catherine Kramer
Product Safety
Syngenta Crop Protection, LLC

Date

QUALITY ASSURANCE STATEMENT

Study Title: Quantification of eCry3.1Ab and Phosphomannose Isomerase in Maize Tissues Derived from Transformation Event 5307

Study Director: Mark Bednarcik

Study Number: 5307-08-01

Report Number: SSB-016-09 A2

Pursuant to Good Laboratory Practice Regulations (40 CFR Part 160), this statement verifies that the aforementioned study was inspected and/or audited and the findings reported to Management and to the Study Director by the Quality Assurance Unit on the dates listed below.

Inspection/Audit Type	Inspection/Audit Dates	Reporting Dates
Audit Protocol	19-JUN-2008 - 19-JUN-2008	19-JUN-2008
Inspect Analytical	10-FEB-2009 - 10-FEB-2009	12-FEB-2009
Audit Study Data	13-APR-2009 - 16-APR-2009	21-APR-2009
Audit Final Report, 1st audit	12-AUG-2009 - 14-AUG-2009	14-AUG-2009
Audit Final Report, 2nd audit	20-AUG-2009 - 20-AUG-2009	20-AUG-2009
Inspect Sample Verification	18-NOV-2009 - 18-NOV-2009	07-DEC-2009
Inspect Analytical	07-OCT-2010 - 07-OCT-2010	12-OCT-2010
Audit Amended Final Report, 1st audit	21-OCT-2010 - 22-OCT-2010	22-OCT-2010
Audit Amended Final Report, 2nd audit	26-OCT-2010 - 26-OCT-2010	26-OCT-2010

Prepared By: Connie L. Connor Date: March 2, 2011

Connie L. Connor
Senior Quality Assurance Auditor
Syngenta Crop Protection, LLC

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

BSA	bovine serum albumin
DW	dry weight
ELISA	enzyme-linked immunosorbent assay
FW	fresh weight
GLP	Good Laboratory Practice
LOD	limit of detection
LOQ	limit of quantitation
OD	optical density
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PMI	phosphomannose isomerase
PVP-360	polyvinylpyrrolidone 360
SBI	Syngenta Biotechnology, Inc.
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
TMB	3,3′,5,5′-tetramethylbenzidine
Tween 20	polyoxyethylene sorbitan monolaurate

Report Amendment No. 1: October 27, 2010

The Summary on page 9, Table 6 on page 17, and Table D4 on page 37 have updated data on the concentrations of eCry3.1Ab in kernel tissue. The summary on page 9 has been revised for clarity.

On pages 12 and 13, the LOD and LOQ information for eCry3.1Ab has been revised for clarity.

On pages 12, 13, 16, 23, 26 (Appendix B), 43 (Appendix F) a reference was added describing the validation of eCry3.1Ab quantitation in kernels.

On page 22, two additional associates were added to the list of Contributing Scientists.

Appendix F, page 43, has been updated for clarity and to reflect the extraction efficiency of eCry3.1Ab in kernels.

In addition, information regarding the method validation parameters for eCry3.1Ab and PMI has been inserted within Appendices B and C.

Report Amendment No. 2: March 2, 2011

The footnote on page 44 has been revised for accuracy by adding “and anthesis”.

SUMMARY

The purpose of this study was to measure the concentrations of the proteins eCry3.1Ab and phosphomannose isomerase (PMI) in several tissues of maize plants derived from transformation Event 5307, grown in four different locations in 2008. The concentrations of eCry3.1Ab and PMI were measured by enzyme-linked immunosorbent assay (ELISA) in various plant tissues from a 5307 maize hybrid sampled at four developmental stages: whorl, anthesis, maturity and senescence. As controls, plants of a nontransgenic, near-isogenic hybrid were also grown, and samples were collected and analyzed concurrently with the samples of the 5307 maize hybrid.

The maize plants used for this study were grown according to local agronomic practices at four different locations in the United States corn-belt. At each location, ten plants of a 5307 hybrid were collected at each of four growth stages. Five of the ten plants were retained as whole-plant samples; leaves and roots were sampled from the remaining five plants. At maturity and senescence stages, kernel samples were also collected along with the leaf and root samples. Pollen samples were collected and pooled from at least ten plants of each hybrid. All samples were analyzed by ELISA to measure the concentrations of eCry3.1Ab and PMI in the 5307 maize tissues. The tissues of the nontransgenic maize hybrid were also analyzed concurrently with those of the 5307 maize hybrid to identify matrix effects on the ELISAs.

The concentrations of eCry3.1Ab across all locations and plant stages on a fresh-weight basis ranged from < LOQ to 71.21 µg/g in leaves, and from 0.40 µg/g to 9.29 µg/g in roots. Across all locations and plant stages, the concentrations of eCry3.1Ab ranged from 1.70 µg/g to 28.64 µg/g in whole plants and 1.60 µg/g to 7.29 µg/g in kernels on a fresh-weight basis. Concentrations of eCry3.1Ab in pollen samples across locations ranged from < LOQ to 0.09 µg/g on a fresh-weight basis.

The concentrations of PMI across all locations and plant stages on a fresh-weight basis ranged from < LOD to 1.66 µg/g in leaves, and from < LOQ to 1.07 µg/g in roots. Across locations, the concentrations of PMI ranged from 0.15 µg/g to 2.13 µg/g in whole plants, and from 0.50 µg/g to 2.38 µg/g in kernels on a fresh-weight basis. Concentrations of PMI across locations in pollen samples ranged from 5.16 µg/g to 6.06 µg/g on a fresh-weight basis.

The concentrations of eCry3.1Ab and PMI measured in this study represent the levels of these proteins in 5307 maize in various tissue types at four developmental stages across four different locations.

Concentrations of eCry3.1Ab were either detectable or quantifiable in all tissues analyzed. Concentrations of PMI were either detectable or quantifiable in all tissues analyzed except for some leaf tissue samples at senescence stage.

INTRODUCTION

The purpose of this study was to measure the concentrations of eCry3.1Ab and PMI proteins present in maize (*Zea mays*) tissues and whole plants derived from transformation Event 5307 collected at four developmental stages grown in four separate locations of the United States corn-belt, including a geographic region similar to Canada, in 2008.

Using the techniques of modern molecular biology, Syngenta has transformed maize to produce Event 5307 maize, a new cultivar that has insecticidal activity against certain corn rootworm (*Diabrotica spp.*) species. Maize plants derived from transformation Event 5307 ("5307 maize") contain the gene *ecry3.1Ab* encoding an eCry3.1Ab protein and the gene *manA* encoding the enzyme phosphomannose isomerase (PMI). The eCry3.1Ab protein is an engineered chimera of modified Cry3A (mCry3A) and Cry1Ab proteins. The gene *manA* was obtained from *Escherichia coli* strain K-12 and the protein it encodes was utilized as a plant selectable marker during development of 5307 maize.

The data from this study provide a profile of the concentrations of eCry3.1Ab and PMI present in leaves, roots, kernels, pollen, and whole plants of 5307 maize throughout the life of the plant. The concentrations of eCry3.1Ab and PMI in various tissues were quantified by enzyme-linked immunosorbent assay (ELISA). A corresponding nontransgenic, near-isogenic control hybrid, grown concurrently, was also analyzed to evaluate any impact of the plant matrices on the assay.

MATERIALS AND METHODS

Test, Control, and Reference Substances

The test substance for this study was the seed of a 5307 maize hybrid. The control substance was the seed of a nontransgenic, near-isogenic hybrid (Table 1).

Table 1. Pedigree codes of the test and control substances used in this study

Description	Pedigree Code
5307 maize	NP2171 × NP2460 (5307)
Nontransgenic, near-isogenic maize	NP2171 × NP2460

Seed lots of the test and control substances were subject to Stewardship Quality Control testing according to the current Syngenta standards.

A pedigree chart illustrating the production of the hybrids shown in Table 1 can be found in Appendix A.

The microbially produced reference protein used to produce the standard curves for each eCry3.1Ab ELISA was ECRY3.1AB-0208 (Nelson 2008a). The microbially produced

reference protein used to produce the standard curves for each PMI ELISA was PMI-0105 (Nelson 2008b).

Verification of Plant Genotypes

TaqMan PCR (Ingham *et al*. 2001) was used to verify the presence or absence of the intended transgenes in individual plants prior to sampling. In the event that a plant failed the testing process, the plant was excluded from the study.

Plant Tissue Production and Collection

Maize plants used in this study were grown in 2008 at four separate field trial locations; Bloomington, IL; Sadorus, IL; Shirley, IL; and Stanton, MN, according to standard local agronomic practices. At each location, the field trials contained two plots; one for each of the test and control hybrids.

Plants were collected for analysis at four growth stages: whorl, anthesis, maturity, and senescence. At each stage and from each location, ten plants of the 5307 hybrid and two plants of the nontransgenic hybrid were collected and harvested. The harvested plants were shipped overnight on ice packs to Syngenta Biotechnology, Inc. (SBI) Research Triangle Park, NC, USA. Upon receipt, five of the transgenic plants and one of the nontransgenic plants were kept as whole-plant samples. Leaf and root samples were collected from the remaining five transgenic plants and from the one remaining nontransgenic plant. At the maturity and senescence stages, kernels were collected in addition to the leaf and root samples. Samples were stored at -80°C ± 10°C until processed for analysis. At the anthesis stage, pollen was collected and pooled from a minimum of 10 plants per plot, resulting in one composite pollen sample per hybrid. Each pollen sample was air-dried and sieved to remove non-pollen debris greater than 100 μm and shipped overnight on dry ice to SBI where the samples were stored at -80°C ± 10°C. Table 2 shows the plant tissues collected for analysis by growth stage.

Table 2. Tissues collected for analysis

Growth stage	Tissues collected
Whorl	leaves, roots, whole plants
Anthesis	leaves, roots, whole plants, pollen
Maturity	leaves, roots, whole plants, kernels
Senescence	leaves, roots, whole plants, kernels

Plant Tissue Processing

In the presence of dry ice, each tissue sample (leaves, roots, whole plants, and kernels) except pollen was individually processed to a fine powder.

A subsample from each homogenous, powdered sample was lyophilized, and stored at -80°C ± 10°C. The percent dry weight of each sample was determined from the weight of

the sample before lyophilization (fresh weight) and after lyophilization (dry weight). A percent dry weight value for each sample was calculated as follows:

$$\frac{\text{Dry weight (g)}}{\text{Fresh weight (g)}} \times 100$$

The percent dry weight values were used to convert protein concentrations between gram dry weight and gram fresh weight.

Each pollen sample was air-dried and sieved to remove non-pollen debris greater than 100 µm and shipped overnight on dry ice to SBI where the samples were stored at -80°C ± 10°C.

Protein Extraction and ELISA Analysis

Protein extractions were performed on representative aliquots of the lyophilized leaf, root, whole plant and kernel samples, and fresh frozen pollen samples. The extracts were analyzed by ELISA to quantify the amount of eCry3.1Ab and PMI in each sample (Tijssen 1985). Sample extracts were analyzed in triplicate, and standard curves were generated with known amounts of the corresponding reference protein. Standard curves were generated for each ELISA plate. Nontransgenic plant tissue extracts were analyzed in parallel to evaluate any impact of the plant matrix on the ELISA.

The extraction and ELISA procedures are described in Appendices B and C.

The mean absorbance for each sample was plotted against the standard curve to obtain the concentration of eCry3.1Ab and PMI per milliliter of extract (ng/ml).

The concentrations of the analyzed proteins in each sample (µg/g) were calculated as follows:

$$\frac{\text{(ng/ml)} \times \text{(dilution factor)} \times \text{(volume of buffer [ml])}}{\text{(amount of tissue [g])} \times 1000}$$

The percent dry weight values were used to convert protein concentrations between gram dry weight and gram fresh weight as follows:

$$\text{µg/g fresh weight} = \text{µg/g dry weight} \times (\%\ \text{dry weight} \div 100)$$
$$\text{µg/g dry weight} = \text{µg/g fresh weight} \div (\%\ \text{dry weight} \div 100)$$

LOQ and LOD Determination

The LOQ and LOD for eCry3.1Ab in leaves, roots, and pollen were determined in a separate study (Murray 2008). The LOQ and LOD for eCry3.1Ab in kernels were

determined in a separate study (Schwartz 2010). The LOQ and LOD for PMI in each sample type were determined during the course of this study.

The limit of quantitation (LOQ) for PMI in each sample type was based on the average protein concentration corresponding to the lowest quantifiable point on the standard curve (ng/ml) and the acceptable dilution factor. The acceptable dilution factor for each sample type is the most concentrated dilution at which the protein of interest in the sample extracts can accurately be quantified by ELISA.

The limit of detection (LOD) for PMI in each sample type was based on the lowest concentration of reference protein used in the standard curve (ng/ml) and the acceptable dilution factor. The acceptable dilution factor for each sample type is the most concentrated dilution at which the OD of a nontransgenic extract is lower than the OD of the lowest concentration of reference protein used in the standard curve.

Extraction Efficiency

The extraction efficiencies of eCry3.1Ab (Murray 2008 and Schwartz 2010) and PMI (deFontes 2009) were used to estimate the amount of each transgenic protein recovered from a routine extraction of a tissue sample.

The extraction efficiency for each tissue type, except pollen, was used to calculate the estimated absolute concentration of the transgenic proteins for each individual sample analyzed as shown for eCry3.1Ab:

$$\frac{\text{amount of eCry3.1Ab (µg/g) from 1}^{st}\text{ extraction}}{\text{amount of eCry3.1Ab (µg/g) from all extractions}} \times 100$$

Statistical Methods

All calculations, including means and standard deviations, were performed using Microsoft Office Excel 2003.

RESULTS AND DISCUSSION

Concentrations of eCry3.1Ab in Tissues at Several Developmental Stages

The means and ranges of eCry3.1Ab concentrations in leaf, root, whole-plant, kernel, and pollen samples from applicable stages are presented on a dry- and a fresh-weight basis in Tables 3–7. All values presented in this section, except pollen, have been adjusted to the estimated absolute concentration of eCry3.1Ab using the extraction efficiencies determined for various maize tissues shown in Table F1 of Appendix F. Due to non-quantifiable levels in sequential extractions of eCry3.1Ab in pollen, an extraction efficiency value could not

be determined for this matrix. The concentrations of eCry3.1Ab in each individual sample of the various tissues analyzed are reported in Appendix D (Tables D1–D5).

Table 3. Concentrations of eCry3.1Ab in leaves of 5307 maize at four stages on a dry-weight (DW) and a fresh-weight (FW) basis

N = 5 at all stages.

		µg/g DW		µg/g FW	
Stage	**Location**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Whorl	Shirley, IL	110.47 ± 19.31	88.65–133.74	20.81 ± 3.95	16.81–25.50
	Sadorus, IL	134.04 ± 13.06	112.64–143.65	22.91 ± 2.12	19.36–24.55
	Bloomington, IL	106.34 ± 3.70	100.59–109.58	23.04 ± 2.15	21.37–26.77
	Stanton, MN	221.00 ± 35.64	195.41–279.79	28.23 ± 3.99	23.76–33.80
	Across all Locations	142.96 ± 53.44	88.65–279.79	23.75 ± 3.16	16.81–33.80
Anthesis	Shirley, IL	74.70 ± 7.81	64.09–83.22	18.61 ± 2.46	15.16–21.50
	Sadorus, IL	98.05 ± 3.62	92.79–101.60	24.09 ± 2.09	22.06–27.59
	Bloomington, IL	83.24 ± 16.75	71.21–112.62	19.13 ± 3.77	15.90–25.64
	Stanton, MN	81.35 ± 18.20	61.37–103.32	19.08 ± 4.90	13.83–25.17
	Across all Locations	84.34 ± 9.85	61.37–112.62	20.23 ± 2.59	13.83–27.59
Maturity	Shirley, IL	95.16 ± 12.58	73.60–105.60	51.74 ± 11.27	42.80–71.21
	Sadorus, IL	42.65 ± 19.36	18.56–64.08	20.92 ± 9.51	7.25–31.60
	Bloomington, IL	35.30 ± 27.34	1.46–62.98	16.74 ± 12.06	0.89–28.40
	Stanton, MN	23.04 ± 8.30	11.45–31.35	11.92 ± 3.72	7.08–14.98
	Across all Locations	49.04 ± 31.79	1.46–105.60	25.33 ± 17.99	0.89–71.21
Senescence	Shirley, IL	11.86 ± 9.39	3.15–26.50	8.79 ± 7.13	2.42–20.29
	Sadorus, IL	–	<LOQ–8.22[a]	–	<LOQ–5.95[a]
	Bloomington, IL	–	<LOQ–9.16[a]	–	<LOQ–7.29[a]
	Stanton, MN	3.50 ± 2.36	2.05–7.61	2.12 ± 1.40	1.31–4.60
	Across all Locations	–	<LOQ–26.50	–	<LOQ–20.29

– = Not Applicable. It was not possible to calculate the mean as some values were below the LOQ.

[a] LOQ = 0.10 µg/g DW and 0.02 µg/g FW

Values have been adjusted for extraction efficiency (Table F1).

Table 4. Concentrations of eCry3.1Ab in roots of 5307 maize at four stages on a dry-weight (DW) and a fresh-weight (FW) basis

N = 5 at all stages.

		µg/g DW		µg/g FW	
Stage	**Location**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Whorl	Shirley, IL	41.87 ± 4.95	34.29–47.27	4.69 ± 0.77	3.67–5.81
	Sadorus, IL	37.34 ± 5.97	30.47–45.53	4.99 ± 1.14	3.77–6.58
	Bloomington, IL	42.97 ± 6.32	33.56–50.89	6.01 ± 0.69	5.14–6.76
	Stanton, MN	48.70 ± 11.82	41.18–69.66	6.48 ± 1.71	4.99–9.29
	Across all Locations	42.72 ± 4.67	30.47–69.66	5.54 ± 0.84	3.67–9.29
Anthesis	Shirley, IL	14.43 ± 1.43	13.16–16.75	2.55 ± 0.21	2.35–2.79
	Sadorus, IL	19.18 ± 2.72	15.41–22.56	3.20 ± 0.52	2.46–3.73
	Bloomington, IL	14.12 ± 1.50	12.39–15.89	2.39 ± 0.39	1.88–2.81
	Stanton, MN	25.05 ± 3.27	21.33–29.59	4.95 ± 0.76	3.95–5.99
	Across all Locations	18.20 ± 5.12	12.39–29.59	3.27 ± 1.17	1.88–5.99
Maturity	Shirley, IL	16.67 ± 3.48	12.80–22.25	4.48 ± 1.02	3.40–5.97
	Sadorus, IL	7.24 ± 1.05	5.60–8.40	1.49 ± 0.34	1.02–1.82
	Bloomington, IL	13.10 ± 3.12	9.37–17.63	3.60 ± 1.26	2.07–5.41
	Stanton, MN	10.83 ± 5.00	3.18–15.07	2.35 ± 1.02	1.20–3.76
	Across all Locations	11.96 ± 3.96	3.18–22.25	2.98 ± 1.32	1.02–5.97
Senescence	Shirley, IL	16.65 ± 2.87	14.84–21.74	5.56 ± 1.49	3.62–7.81
	Sadorus, IL	5.28 ± 2.51	2.30–8.25	0.91 ± 0.45	0.40–1.52
	Bloomington, IL	11.97 ± 1.27	10.41–13.83	3.60 ± 0.60	2.88–4.32
	Stanton, MN	2.61 ± 1.46	1.40–4.67	0.85 ± 0.50	0.44–1.62
	Across all Locations	9.13 ± 6.38	1.40–21.74	2.73 ± 2.28	0.40–7.81

Values have been adjusted for extraction efficiency (Table F1).

Table 5. Concentrations of eCry3.1Ab in whole plants of 5307 maize at four stages on a dry-weight (DW) and a fresh-weight (FW) basis

N = 5 at all stages.

		µg/g DW		µg/g FW	
Stage	**Location**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Whorl	Shirley, IL	101.44 ± 10.78	92.73–114.57	13.06 ± 1.93	11.41–16.19
	Sadorus, IL	97.85 ± 12.17	85.98–112.19	18.62 ± 6.42	12.10–28.64
	Bloomington, IL	78.43 ± 2.85	75.16–81.16	14.52 ± 1.66	13.09–16.48
	Stanton, MN	166.58 ± 8.14	155.38–178.22	16.90 ± 2.77	15.28–21.75
	Across all Locations	111.08 ± 38.36	75.16–178.22	15.78 ± 2.47	11.41–28.64
Anthesis	Shirley, IL	50.33 ± 5.77	42.00–55.67	9.72 ± 0.80	8.32–10.31
	Sadorus, IL	37.58 ± 7.15	29.51–46.14	10.14 ± 2.01	8.08–13.12
	Bloomington, IL	34.74 ± 3.44	31.79–40.70	6.32 ± 0.54	5.92–7.22
	Stanton, MN	29.90 ± 9.41	14.18–38.33	6.26 ± 1.88	3.10–8.02
	Across all Locations	38.14 ± 8.72	14.18–55.67	8.11 ± 2.11	3.10–13.12
Maturity	Shirley, IL	18.49 ± 12.77	6.37–38.94	9.95 ± 7.46	3.37–21.96
	Sadorus, IL	21.80 ± 7.63	13.57–33.10	13.22 ± 5.28	6.55–21.03
	Bloomington, IL	14.70 ± 3.21	11.94–19.97	8.50 ± 2.86	5.83–13.31
	Stanton, MN	9.11 ± 1.35	7.97–11.36	3.76 ± 0.58	3.36–4.79
	Across all Locations	16.03 ± 5.45	6.37–38.94	8.86 ± 3.93	3.36–21.96
Senescence	Shirley, IL	12.32 ± 2.21	8.86–14.92	4.89 ± 0.65	3.78–5.41
	Sadorus, IL	6.28 ± 2.13	4.27–9.75	2.98 ± 1.09	2.22–4.86
	Bloomington, IL	6.07 ± 2.06	3.92–9.41	2.82 ± 0.65	1.97–3.72
	Stanton, MN	8.39 ± 9.57	3.41–25.46	3.72 ± 3.88	1.70–10.65
	Across all Locations	8.27 ± 2.90	3.41–25.46	3.60 ± 0.94	1.70–10.65

Values have been adjusted for extraction efficiency (Table F1).

Table 6. Concentrations of eCry3.1Ab in kernels of 5307 maize at two stages on a dry-weight (DW) and a fresh-weight (FW) basis

N = 5 at all stages.

		µg/g DW		µg/g FW	
Stage	**Location**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Maturity	Shirley, IL	6.99 ± 1.70	5.00–9.64	5.26 ± 1.35	3.51–7.29
	Sadorus, IL	8.00 ± 0.59	7.00–8.45	5.53 ± 0.40	4.98–6.08
	Bloomington, IL	6.14 ± 1.62	4.21–8.14	4.96 ± 1.25	3.47–6.41
	Stanton, MN	3.63 ± 1.90	2.37–6.95	2.49 ± 1.12	1.60–4.39
	Across all Locations	6.19 ±1.87	2.37–9.64	4.56 ± 1.40	1.60–7.29
Senescence	Shirley, IL	3.68 ± 0.50	3.02–4.42	2.74 ± 0.26	2.38–3.10
	Sadorus, IL	5.28 ± 1.07	4.08–6.76	3.65 ± 0.74	2.90–4.66
	Bloomington, IL	3.80 ± 0.68	2.92–4.74	3.07 ± 0.51	2.45–3.84
	Stanton, MN	5.02 ± 1.07	3.83–6.41	3.49 ± 0.58	2.74–4.31
	Across all Locations	4.45 ± 0.82	2.92–6.76	3.24 ± 0.41	2.38–4.66

Values have been adjusted for extraction efficiency (Table F1).

Table 7. Concentrations of eCry3.1Ab in pollen of 5307 maize at anthesis on a dry-weight (DW) and a fresh-weight (FW) basis

N = 1; pooled pollen sample

Location	µg/g DW	µg/g FW
Shirley, IL	0.10	0.09
Sadorus, IL	0.10	0.09
Bloomington, IL	<LOQ[a, b]	<LOQ[a, b]
Stanton, MN	ND[c]	ND[c]

[a] eCry3.1Ab concentrations in pollen were detectable but not quantifiable
[b] LOQ = 0.10 µg/g DW and 0.08 µg/g FW
[c] ND = Not Determined, sample unavailable
Values NOT adjusted for extraction efficiency.

Concentrations of PMI in Tissues at Several Developmental Stages

The means and ranges of PMI concentrations in leaf, root, whole-plant, kernel, and pollen samples from applicable stages are presented on a dry- and a fresh-weight basis in Tables 8–12. All values presented in this section have been adjusted to the estimated absolute concentration of PMI using the extraction efficiencies estimated for various maize tissues shown in Table F2 of Appendix F. The concentrations of PMI in each sample of the various tissues analyzed are reported in Appendix E (Tables E1–E5).

Table 8. Concentrations of PMI in leaves of 5307 maize at four stages on a dry-weight (DW) and a fresh-weight (FW) basis

N = 5 at all stages.

		µg/g DW		µg/g FW	
Stage	**Location**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Whorl	Shirley, IL	4.00 ± 0.45	3.50–4.60	0.75 ± 0.10	0.64–0.88
	Sadorus, IL	5.51 ± 0.59	5.08–6.52	0.94 ± 0.11	0.84–1.13
	Bloomington, IL	3.28 ± 0.36	2.97–3.88	0.71 ± 0.10	0.59–0.82
	Stanton, MN	6.54 ± 1.01	5.96–8.33	0.84 ± 0.16	0.69–1.10
	Across all Locations	4.83 ± 1.47	2.97–8.33	0.81 ± 0.10	0.59–1.13
Anthesis	Shirley, IL	2.60 ± 1.46	1.74–5.20	0.65 ± 0.36	0.43–1.28
	Sadorus, IL	3.31 ± 0.19	3.09–3.60	0.81 ± 0.08	0.72–0.92
	Bloomington, IL	2.68 ± 0.13	2.60–2.90	0.62 ± 0.03	0.59–0.66
	Stanton, MN	3.04 ± 0.40	2.54–3.49	0.71 ± 0.12	0.58–0.86
	Across all Locations	2.91 ± 0.33	1.74–5.20	0.70 ± 0.08	0.43–1.28
Maturity	Shirley, IL	2.29 ± 0.90	1.04–3.50	1.19 ± 0.37	0.69–1.66
	Sadorus, IL	0.45 ± 0.26	0.16–0.79	0.22 ± 0.12	0.06–0.39
	Bloomington, IL	–	<LOQ–1.00[a]	–	<LOQ–0.44[a]
	Stanton, MN	0.23 ± 0.17	0.09–0.42	0.12 ± 0.09	0.05–0.22
	Across all Locations	–	<LOQ–3.50	–	<LOQ–1.66
Senescence	Shirley, IL	–	<LOD–0.54[b]	–	<LOQ–0.42[b]
	Sadorus, IL	–	<LOD–<LOQ[b]	–	<LOD–<LOQ[b]
	Bloomington, IL	–	<LOD–0.12[b]	–	<LOD–0.09[b]
	Stanton, MN	–	<LOD–0.09[b]	–	<LOD–0.06[b]
	Across all Locations	–	<LOD–0.54	–	<LOD–0.42

– = Not Applicable. It was not possible to calculate the mean as some values were below the LOQ or LOD.

[a] LOQ = 0.06 µg/g DW and 0.03 µg/g FW at maturity

[b] LOQ = 0.05 µg/g DW and 0.03 µg/g FW; LOD = 0.01 µg/g DW and FW at senescence

Values have been adjusted for extraction efficiency (Table F2).

Table 9. Concentrations of PMI in roots of 5307 maize at four stages on a dry-weight (DW) and a fresh-weight (FW) basis

N = 5 at all stages.

		µg/g DW		µg/g FW	
Stage	**Location**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Whorl	Shirley, IL	1.96 ± 0.23	1.71–2.27	0.22 ± 0.05	0.16–0.30
	Sadorus, IL	2.09 ± 0.54	1.30–2.80	0.28 ± 0.10	0.16–0.40
	Bloomington, IL	1.47 ± 0.10	1.38–1.60	0.21 ± 0.03	0.15–0.25
	Stanton, MN	2.91 ± 0.72	1.97–3.75	0.39 ± 0.10	0.24–0.49
	Across all Locations	2.11 ± 0.60	1.30–3.75	0.28 ± 0.08	0.15–0.49
Anthesis	Shirley, IL	1.20 ± 0.26	0.97–1.63	0.21 ± 0.05	0.16–0.29
	Sadorus, IL	1.55 ± 0.11	1.43–1.72	0.26 ± 0.02	0.24–0.30
	Bloomington, IL	1.40 ± 0.10	1.24–1.50	0.24 ± 0.03	0.21–0.26
	Stanton, MN	2.62 ± 0.48	1.99–3.13	0.51 ± 0.06	0.42–0.57
	Across all Locations	1.69 ± 0.63	0.97–3.13	0.31 ± 0.14	0.16–0.57
Maturity	Shirley, IL	2.07 ± 0.33	1.56–2.49	0.55 ± 0.06	0.46–0.61
	Sadorus, IL	1.69 ± 0.57	1.15–2.64	0.35 ± 0.16	0.21–0.63
	Bloomington, IL	1.70 ± 0.34	1.19–2.08	0.46 ± 0.11	0.26–0.53
	Stanton, MN	1.95 ± 0.93	0.92–2.88	0.44 ± 0.18	0.17–0.64
	Across all Locations	1.85 ± 0.19	0.92–2.88	0.45 ± 0.08	0.17–0.64
Senescence	Shirley, IL	2.26 ± 0.80	0.96–3.02	0.78 ± 0.33	0.23–1.07
	Sadorus, IL	–	<LOQ–1.85[a]	–	<LOQ–0.34[a]
	Bloomington, IL	1.16 ± 0.46	0.67–1.88	0.35 ± 0.15	0.16–0.55
	Stanton, MN	0.72 ± 0.74	0.14–1.99	0.24 ± 0.26	0.05–0.69
	Across all Locations	–	<LOQ–3.02	–	<LOQ–1.07

– = Not Applicable. It was not possible to calculate the mean as some values were below the LOQ.
[a] LOQ = 0.10 µg/g DW and 0.03 µg/g FW at senescence
Values have been adjusted for extraction efficiency (Table F2).

Table 10. Concentrations of PMI in whole plants of 5307 maize at four stages on a dry-weight (DW) and a fresh-weight (FW) basis

N = 5 at all stages.

		µg/g DW		µg/g FW	
Stage	**Location**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Whorl	Shirley, IL	3.03 ± 0.25	2.66–3.30	0.39 ± 0.05	0.34–0.45
	Sadorus, IL	4.89 ± 0.28	4.43–5.11	0.91 ± 0.20	0.68–1.13
	Bloomington, IL	3.13 ± 0.52	2.59–3.94	0.58 ± 0.09	0.43–0.68
	Stanton, MN	5.85 ± 1.04	5.18–7.69	0.59 ± 0.10	0.50–0.72
	Across all Locations	4.23 ± 1.38	2.59–7.69	0.62 ± 0.22	0.34–1.13
Anthesis	Shirley, IL	7.99 ± 0.51	7.56–8.83	1.57 ± 0.32	1.36–2.13
	Sadorus, IL	3.63 ± 0.47	3.18–4.35	0.98 ± 0.12	0.87–1.14
	Bloomington, IL	3.04 ± 0.39	2.63–3.54	0.55 ± 0.07	0.49–0.64
	Stanton, MN	2.87 ± 0.87	2.00–4.10	0.60 ± 0.18	0.44–0.86
	Across all Locations	4.38 ± 2.43	2.00–8.83	0.93 ± 0.47	0.44–2.13
Maturity	Shirley, IL	1.10 ± 0.25	0.68–1.29	0.57 ± 0.11	0.41–0.73
	Sadorus, IL	2.15 ± 0.32	1.62–2.43	1.30 ± 0.30	0.78–1.57
	Bloomington, IL	1.85 ± 0.44	1.40–2.56	1.07 ± 0.36	0.66–1.56
	Stanton, MN	2.20 ± 0.24	1.91–2.47	0.91 ± 0.15	0.74–1.05
	Across all Locations	1.83 ± 0.51	0.68–2.56	0.96 ± 0.31	0.41–1.57
Senescence	Shirley, IL	1.38 ± 0.42	0.89–2.02	0.55 ± 0.13	0.38–0.71
	Sadorus, IL	0.57 ± 0.18	0.39–0.86	0.28 ± 0.10	0.15–0.41
	Bloomington, IL	1.17 ± 0.20	0.85–1.38	0.56 ± 0.14	0.34–0.71
	Stanton, MN	0.74 ± 0.47	0.43–1.55	0.34 ± 0.19	0.20–0.65
	Across all Locations	0.97 ± 0.37	0.39–2.02	0.43 ± 0.14	0.15–0.71

Values have been adjusted for extraction efficiency (Table F2).

Table 11. Concentrations of PMI in kernels of 5307 maize at two stages on a dry-weight (DW) and a fresh-weight (FW) basis

N = 5 at all stages.

		µg/g DW		µg/g FW	
Stage	**Location**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Maturity	Shirley, IL	2.19 ± 0.56	1.62–2.94	1.57 ± 0.35	1.16–2.05
	Sadorus, IL	1.96 ± 0.36	1.37–2.34	1.18 ± 0.21	0.83–1.41
	Bloomington, IL	1.49 ± 0.29	1.04–1.83	1.06 ± 0.20	0.74–1.28
	Stanton, MN	2.66 ± 0.89	1.92–3.82	1.64 ± 0.57	1.16–2.38
	Across all Locations	2.08 ± 0.49	1.04–3.82	1.36 ± 0.29	0.74–2.38
Senescence	Shirley, IL	1.04 ± 0.21	0.67–1.18	0.78 ± 0.16	0.50–0.93
	Sadorus, IL	1.13 ± 0.10	0.97–1.20	0.78 ± 0.05	0.70–0.82
	Bloomington, IL	1.13 ± 0.34	0.70–1.62	0.91 ± 0.26	0.59–1.28
	Stanton, MN	1.15 ± 0.15	0.95–1.37	0.80 ± 0.05	0.74–0.87
	Across all Locations	1.11 ± 0.05	0.70–1.62	0.82 ± 0.06	0.50–1.28

Values have been adjusted for extraction efficiency (Table F2).

Table 12. Concentrations of PMI in pollen of 5307 maize at anthesis on a dry-weight (DW) and a fresh-weight (FW) basis

N = 1; pooled pollen sample

Location	**µg/g DW**	**µg/g FW**
Shirley, IL	7.23	6.06
Sadorus, IL	5.79	5.20
Bloomington, IL	6.52	5.16
Stanton, MN	ND[a]	ND[a]
Across all Locations	6.51 ± 0.72[b]	5.47 ± 0.51[b]

[a] ND = Not Determined, sample unavailable
[b] Mean ± SD

Values have been adjusted for extraction efficiency (Table F2).

No circumstances occurred during the conduct of this study that would have adversely affected the quality or integrity of the data generated.

CONCLUSIONS

The concentrations of eCry3.1Ab and PMI measured in this study represent the levels of these proteins in 5307 maize in various tissue types at four developmental stages across four different locations.

Concentrations of eCry3.1Ab were either detectable or quantifiable in all tissues analyzed. Concentrations of PMI were either detectable or quantifiable in all tissues analyzed except for some leaf tissue samples at senescence stage.

RECORDS RETENTION

Raw data, the original copy of this report, and other relevant records are archived at Syngenta Crop Protection, LLC, 3054 East Cornwallis Rd., Research Triangle Park, NC 27709-2257, USA.

CONTRIBUTING SCIENTISTS

All analytical work was conducted by Mark Bednarcik B.S., Jim Branson, Robert Champon B.S., Emmanuel Ferew, Philip Garibaldi B.S., Hong Pham B.A., and Steven Testerman at the Product Safety Laboratory, Research Triangle Park, NC, USA.

CRITICAL DATES

Study initiation date:	June 20, 2008
Experimental start date:	October 1, 2008
Experimental end date:	October 11, 2010

REFERENCES

Ingham DJ, Beer S, Money S, Hansen G. 2001. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *BioTechniques* 31:136–140.

Tijssen P. 1985. Processing of data and reporting of results of enzyme immunoassays. In *Practice and Theory of Enzyme Immunoassays*. *Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 15.* Amsterdam, Netherlands: Elsevier Science Publishers. pp. 385–421.

U.S. EPA. 1989. Good Laboratory Practice Standards. 40 CFR Part 160.

UNPUBLISHED REFERENCES

deFontes, J. 2009. *Determination of Extract Dilution Factors and Extraction Efficiencies for ELISA Analysis of Transgenic Proteins in Matrices Derived from, or Including, Maize Events 3272, Bt11, MIR162, MIR604 and GA21*. Report Number: SSB-009-09 (unpublished). Research Triangle Park, NC. Syngenta Biotechnology. 35 pp.

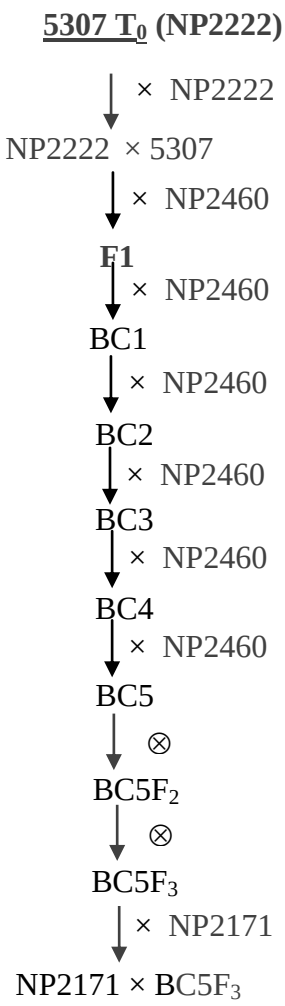
Murray, J. 2008. *Validation of a Quantitative eCry3.1Ab ELISA for Analysis of Maize Leaf, Root, Kernel, and Pollen Extracts.* Report Number: SSB-021-08 (unpublished). Research Triangle Park, NC. Syngenta Biotechnology: 47 pp. US EPA MRID No. 47753902.

Nelson, A. 2008a. *Characterization of Test Substance ECRY3.1AB-0208 and Certificate of Analysis.* Report Number: SSB-010-08 (unpublished). Research Triangle Park, NC. Syngenta Biotechnology: 18 pp. US EPA MRID No. 47734704.

Nelson, A. 2008b. *Characterization of Phosphomannose Isomerase Test Substance PMI-0105 and Certificate of Analysis.* Report No. SSB-025-07 (unpublished). Research Triangle Park, NC: Syngenta Biotechnology. 18 pp.

Schwartz, B. 2010. *Validation of the ELISA Method for Quantification of eCry3.1Ab Protein in Maize Kernel.* Report No. TK0051524 (unpublished). Research Triangle Park, NC: Syngenta Biotechnology.

APPENDIX A: PEDIGREE CHART FOR 5307 SEED



Control Hybrid
NP2171 × NP2460

APPENDIX B: eCry3.1Ab QUANTIFICATION – EXTRACTION AND ELISA PROCEDURES

Buffers

The buffers used for extraction and enzyme-linked immunosorbent assay (ELISA) analysis of eCry3.1Ab are listed in the following table:

Name of buffer	Constituents
Phosphate-buffered saline (PBS)	140 mM sodium chloride, 8.24 mM sodium phosphate dibasic, 1.81mM sodium phosphate monobasic, pH 6.75
Borate buffer (pollen)	0.1 M Sodium tetraborate decahydrate, 0.2% PVP-360, 7.69 mM sodium azide, 1.2% Concentrated hydrochloric acid, 0.5% Tween 20; pH approximately 7.5. Complete Protease Inhibitor Cocktail (Roche Applied Science) added on day of extraction
Borate buffer (leaves, roots, whole plants, kernels)	0.1 M Sodium tetraborate decahydrate, 0.2% PVP-360, 7.69 mM sodium azide, 0.5% Tween 20; titrated to pH 10.0. Complete Protease Inhibitor Cocktail (Roche Applied Science) added on day of extraction
Dilution buffer	PBS, 0.05% Tween 20, 1% BSA, 0.02% sodium azide
Wash buffer	10 mM Tris, 0.05% Tween 20, 0.02% sodium azide

eCry3.1Ab Extraction

Leaves, Roots, Whole Plants, and Kernels. A ratio of 3 ml of borate buffer pH 10.0 was added to 100 mg of lyophilized tissue. The samples were vortexed, placed on wet ice for at least 30 minutes, homogenized using an Omni-Prep Homogenizer, and centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at 2°C to 8°C until analysis.

Pollen. For each sample, 25 mg of fresh frozen pollen was weighed into a 2-ml Eppendorf tube containing three 4-mm glass beads and stored at -80°C ± 10°C for at least two hours. Each tube was then placed into a Four Station Titer Plate/Micro Tube Grinding Mill and set at ~3000 strokes per minute for approximately 45 seconds. The tubes were then placed on wet ice and 1.5 ml of borate buffer, pH 7.5 was added to each sample. The samples were mixed and set on wet ice for at least 30 minutes then centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at 2°C to 8°C until analysis.

eCry3.1Ab Quantification

The eCry3.1Ab ELISA kit was manufactured at Beacon Analytical Systems (BAS), Portland, ME. The assay is a double-antibody sandwich assay in which the eCry3.1Ab protein is affixed to the wells of a microtiter plate using a monoclonal, anti-mCry3A antibody that binds to the mCry3A domains of the eCry3.1Ab protein. The primary antibody was diluted and added to each well of a 96-well microtiter plate. The plate was then blocked using a proprietary method. Dilutions of each tissue extract and appropriate serial dilutions of eCry3.1Ab reference protein (ECRY3.1AB-0208 [Nelson 2008a]),

prepared in dilution buffer, were applied to the pre-coated plates at a total volume of 100 µl/well. The plates were incubated at room temperature on a titre plate shaker at 400 rpm for 1 hour. The plates were washed five times with wash buffer in a BioTek ELx405 Microplate Washer. After washing the plates, a secondary, rabbit polyclonal anti-Cry1Ab antibody (provided in the kit) was then used to bind the Cry1Ab domain of the eCry3.1Ab protein at 100 µl/well. The plates were incubated at room temperature on a titre plate shaker at 400 rpm for one hour and washed five times as described above.

After the plates were washed, a tertiary donkey anti-rabbit conjugated with alkaline phosphatase diluted in dilution buffer was added to each of the wells (100 µl/well) and incubated at room temperature on a titre plate shaker at 400 rpm for one hour. The plates were then washed five times as described above, and alkaline phosphatase substrate solution provided in the kit was added at a volume of 100 µl/well. The plates were incubated for 30 minutes at room temperature on a titre plate shaker at 400 rpm. The reaction was stopped by the addition of 3N sodium hydroxide (100 µl/well), and absorbance of the reaction was measured at a dual wavelengths (405 and 492 nm) with a Tecan Sunrise Microplate Reader. The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2, using a four-parameter algorithm. The results for kernel samples were analyzed with SoftMax Pro, v. 5.2, using a four-parameter algorithm.

Validation of eCry3.1Ab ELISA Extraction Efficiency and Sensitivity

Protein extraction efficiency and method sensitivity (dilution factor, limit of detection, and limit of quantitation) were validated for each matrix outside the scope of this study (Murray 2008 and Schwartz 2010). Method sensitivity data are summarized below.

Minimum dilution factor. The minimum dilution factor for each matrix was determined by analyzing a dilution series of nontransgenic extracts spiked with a known quantity of eCry3.1Ab reference protein. The most concentrated dilution of spiked sample extract that yielded a percent recovery between 70% and 120%, and was followed by two subsequent dilutions with recoveries in the same range was selected as the minimum acceptable dilution factor.

Extraction efficiency. The efficiency of the eCry3.1Ab protein extraction method was evaluated in each matrix through exhaustive extractions of eCry3.1Ab protein from transgenic samples. Each extraction was analyzed by ELISA to determine the concentration of eCry3.1Ab protein present. The extraction efficiencies (%) were calculated using the following formula:

$$\frac{\text{Amount of eCry3.1Ab (ng/ml) from 1st extraction}}{\text{Total eCry3.1Ab (ng/ml) from all extractions}} \times 100$$

Limit of detection for Leaf, Root, and Pollen. The OD_{LOD} for each matrix was evaluated by comparing the average optical density (OD) plus three standard deviations of an unspiked, nontransgenic sample extract with the average OD of nontransgenic sample extracts spiked with varying concentrations of eCry3.1Ab reference protein.

$$OD_{LOD} = \text{mean OD} + (3 \times \text{standard deviation})$$

The standard curve concentration corresponding to the OD_{LOD} value was multiplied by the minimum dilution factor to yield the LOD (ng/ml). If concentration corresponding to the OD_{LOD} value was less than 1.25 ng/ml which is the lower quantitative range of the assay, the LOD was set at 1.25 ng/ml.

The LOD (µg/g tissue) was calculated using the following formula:

$$\frac{\text{LOD}(\frac{ng}{ml}) \times (\text{Volume of buffer [ml]})}{(\text{amount of tissue [g]}) \times 1000}$$

Limit of quantitation for Leaf, Root, and Pollen. The limit of quantitation (LOQ) for each matrix was evaluated by spiking nontransgenic sample extracts with known concentrations of eCry3.1Ab reference protein, and measuring the percent recovery of eCry3.1Ab protein to determine the minimum dilution factor.

The percent recovery for each spiked sample was calculated using the following formula:

$$\frac{\text{mean protein concentration of spiked extract } (\frac{ng}{ml})}{\text{spiked protein concentration } (\frac{ng}{ml})} \text{ x } 100$$

For each matrix, the LOQ (ng/ml) was calculated by multiplying the LOD (ng/ml) by the minimum acceptable dilution factor. The LOQ (µg/g tissue) was calculated using the following formula:

$$\frac{\text{LOQ}(\frac{ng}{ml}) \times (\text{Volume of buffer [ml]})}{(\text{amount of tissue [g]}) \times 1000}$$

Limit of detection for Kernel. The limit of detection (LOD) for each matrix was evaluated by comparing the average optical density (OD) plus three standard deviations of an unspiked, nontransgenic sample extract with the average OD of nontransgenic sample extracts spiked with varying concentrations of eCry3.1Ab reference protein.

The theoretical LOD was the mean OD of the unspiked, nontransgenic sample extract plus three standard deviations of the mean. The measured LOD is the lowest spike concentration with an OD greater than the theoretical LOD.

The LOD (µg/g tissue) was calculated using the following formula:

$$\left(\frac{\text{Measured LOD } (\frac{ng}{ml}) \text{ x dilution factor x volume of extraction buffer (ml)}}{\text{amount of tissue extracted (g)}}\right) \div 1000$$

Limit of quantitation for Kernel. The limit of quantitation (LOQ) for each matrix was evaluated by spiking nontransgenic sample extracts with known concentrations of eCry3.1Ab reference protein, and measuring the percent recovery of eCry3.1Ab protein. The LOQ was the lowest spike concentration of eCry3.1Ab that recovered between 70% and 120% of nominal value and was greater than or equal to the LOD.

The percent recovery for each spiked sample was calculated using the following formula:

$$\frac{\text{mean protein concentration of spiked extract } (\frac{ng}{ml})}{\text{spiked protein concentration } (\frac{ng}{ml})} \text{ x } 100$$

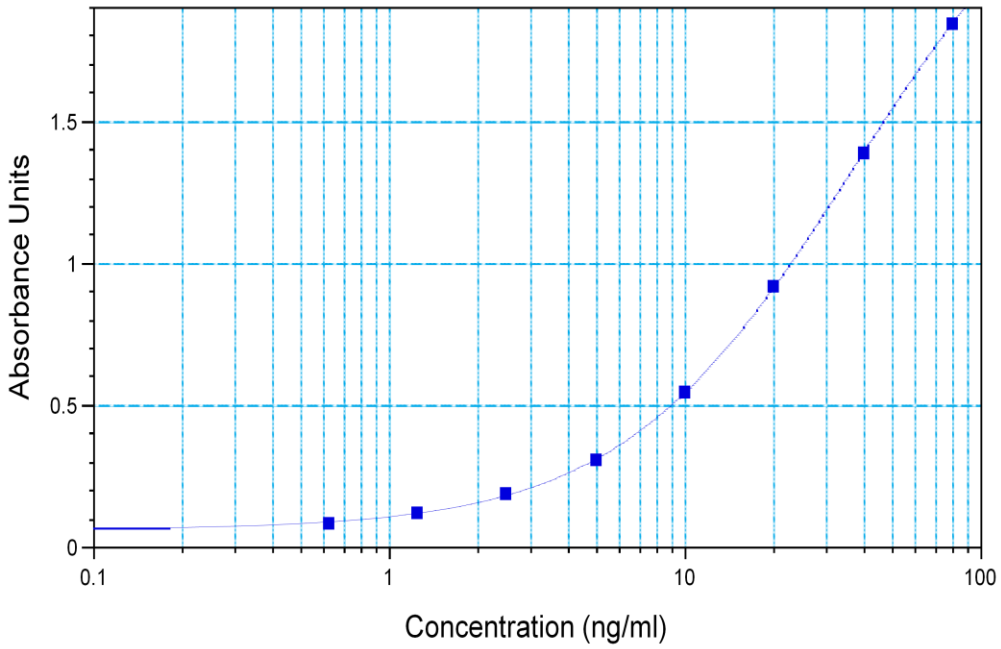
The LOQ (µg/g tissue) was calculated using the following formula:

$$\left(\frac{\text{LOQ } (\frac{ng}{ml}) \text{ x dilution factor x volume of extraction buffer (ml)}}{\text{amount of tissue extracted (g)}}\right) \div 1000$$

Minimum dilution factor, LOD and LOQ

Tissue Type	Minimum Dilution Factor	LOD (µg/g DW)	LOQ (µg/g DW)
Leaf	2	0.05	0.12
Root	2	0.04	0.08
Kernel	2	0.09	0.09
Pollen	1	0.10	0.10

Representative Standard Curve. Concentrations used to produce the ELISA standard curve: 80 ng/ml, 40 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, and 0.63 ng/ml. The representative standard curve for eCry3.1Ab is depicted below.


Absorbance Units
1.5
1
0.5
0
0.1
1
10
100
Concentration (ng/ml)

APPENDIX C: PMI QUANTIFICATION – EXTRACTION AND ELISA PROCEDURES

Buffers

The buffers used for extraction and enzyme-linked immunosorbent assay (ELISA) analysis of PMI are listed in the following table.

Name of buffer	Constituents
PBS	138 mM sodium chloride, 2.7 mM potassium chloride, 10.1 mM disodium phosphate, 1.8 mM potassium dihydrogen phosphate, pH 7.4
Blocking buffer	PBS, 1% powdered milk
Borate buffer	0.1 M sodium tetraborate decahydrate, 0.2% PVP-360, 7.69 mM sodium azide, 1.2% concentrated hydrochloric acid, 0.5% Tween 20; pH will be approximately 7.5. Complete Protease Inhibitor Cocktail (Roche Applied Science) added on day of extraction
Carbonate-bicarbonate buffer	34.9 mM sodium bicarbonate, 15.0 mM sodium carbonate, pH 9.5
Citrate-phosphate buffer	23.8 mM citric acid, 59.9 mM disodium phosphate, pH 5.0
Dilution buffer	PBS, 0.05% Tween 20, 1% powdered milk
Wash buffer	PBS, 0.05% Tween 20

PMI Extraction

Leaves, Roots, Whole Plants, and Kernels. A ratio of 3 ml of borate buffer, pH 7.5 was added to 100 mg of lyophilized tissue. The samples were mixed, placed on wet ice for at least 30 minutes, homogenized using an Omni-Prep Homogenizer, and centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at 2°C to 8°C until analysis. For root tissue, the pellet was retained and then processed through the aforementioned extraction procedure again. The supernatant removed from the second extraction was combined with that of the first extraction, mixed well and stored at 2°C to 8°C until analysis.

Pollen. For each sample, 25 mg of fresh, frozen pollen was weighed into a 2-ml Eppendorf tube containing three 4-mm glass beads and stored at -80°C ± 10°C for at least two hours. Each tube was then placed into a Four Station Titer Plate/Micro Tube Grinding Mill and set at ~3000 strokes per minute for approximately 45 seconds. The tubes were then placed on wet ice and 1.5 ml of borate buffer, pH 7.5 was added to each sample. The samples were mixed and set on wet ice for at least 20 minutes then centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at 2°C to 8°C until analysis.

PMI Quantification

Rabbit polyclonal anti-PMI antibody was diluted in carbonate-bicarbonate buffer and added to each well of a 96-well microtiter plate at a volume of 100 µl/well. The plates were stored overnight in a refrigerator set at 2°C to 8°C. The antibody solution was

removed and blocking buffer was added to the plate at a volume of 250 µl/well and then incubated at room temperature for at least 30 minutes. After blocking incubation, the plates were washed five times with wash buffer in a BioTek ELx405 microplate washer and dilutions of each tissue extract and appropriate serial dilutions of PMI reference protein (PMI-0105 [Nelson 2008b]) prepared in dilution buffer were applied to the plates at a total volume of 100 µl/well. The plates were incubated at 18°C to 22°C for 2 hours. After incubation, plates were washed five times as described above and a monoclonal anti-PMI antibody diluted in dilution buffer was added to the plate at a volume of 100 µl/well and incubated at 18°C to 22°C for one hour.

The plates were washed five times after incubation and a horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobin diluted in dilution buffer was added at a volume of 100 µl/well and incubated at 18°C to 22°C for one hour. After incubation, the plates were washed five times, and TMB substrate solution was added at a volume of 100 µl/well (1 tablet per 10 ml of citrate-phosphate buffer) and incubated at room temperature in the dark for 30 minutes. The reaction was stopped by addition of 3 M sulfuric acid at a volume of 50 µl/well, and the absorbance of the reaction was measured at 450 nm with a Tecan Sunrise microplate reader. The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2, using a four-parameter algorithm.

Validation of PMI ELISA Extraction Efficiency and Sensitivity

Protein extraction efficiency and minimum dilution factor were determined for each matrix outside the scope of this study (deFontes 2009). Method sensitivity data are summarized in below.

Minimum dilution factor. The minimum dilution factor for each matrix was determined by analyzing a dilution series of nontransgenic extracts spiked with a known quantity of PMI reference protein. The most concentrated dilution of spiked sample extract that yielded a percent recovery between 70% and 120%, and was followed by two subsequent dilutions with recoveries in the same range was selected as the minimum acceptable dilution factor.

Extraction efficiency. The efficiency of the PMI protein extraction method was evaluated in each matrix through exhaustive extractions of PMI protein from transgenic samples. Each extraction was analyzed by ELISA to determine the concentration of PMI protein present. The extraction efficiencies (%) were calculated using the following formula:

$$\frac{\text{Amount of PMI (ng/ml) from 1st extraction}}{\text{Total PMI (ng/ml) from all extractions}} \times 100$$

For matrices that required pooling supernatants from two extraction iterations to achieve ≥ 70% efficiency, the extraction efficiency was calculated using the following formula:

$$\frac{\text{Amount of PMI (ng/ml) from } 1^{st} + 2^{nd} \text{ extraction}}{\text{Total PMI (ng/ml) from all extractions}} \times 100$$

Limit of detection. The limit of detection (LOD) for each matrix was estimated using the lowest concentration of PMI reference protein used to produce the standard curve (ng/ml) and the minimum dilution factor.

The LOD (µg/g tissue) was calculated using the following formula:

$$\left(\frac{\text{LOD } (\frac{ng}{ml}) \text{ x dilution factor x volume of extraction buffer (ml)}}{\text{amount of tissue extracted (g)}}\right) \div 1000$$

Limit of quantitation. The limit of quantitation (LOQ) for each matrix was estimated based on the average PMI reference protein concentration corresponding to the lowest point on the linear portion of the standard curve (ng/ml) and the minimum dilution factor.

The LOQ (µg/g tissue) was calculated using the following formula:

$$\left(\frac{\text{LOQ } (\frac{ng}{ml}) \text{ x dilution factor x volume of extraction buffer (ml)}}{\text{amount of tissue extracted (g)}}\right) \div 1000$$

Minimum dilution factor, LOD and LOQ for at whorl stage

Tissue Type	Minimum Dilution Factor	LOD (µg/g DW)	LOQ (µg/g DW)
Leaf	1	0.01	0.06
Root	1	0.02	0.10

Minimum dilution factor, LOD and LOQ at anthesis stage

Tissue Type	Minimum Dilution Factor	LOD (µg/g DW)	LOQ (µg/g DW)
Leaf	1	0.01	0.03
Root	1	0.02	0.09
Kernel	1	0.01	0.06
Pollen	1	0.003	0.012

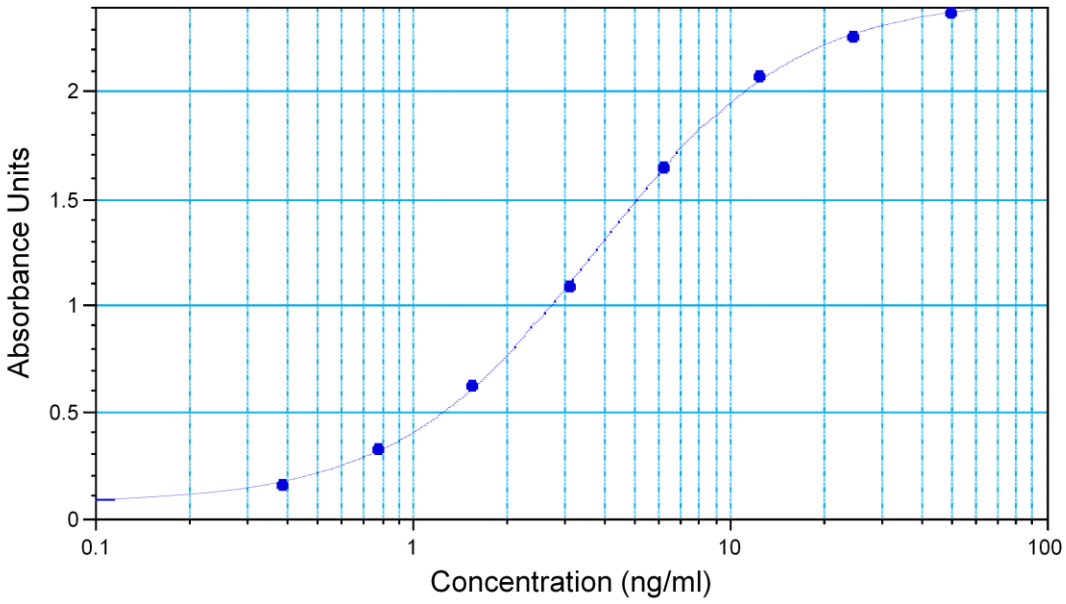
Minimum dilution factor, LOD and LOQ for at physical maturity stage

Tissue Type	Minimum Dilution Factor	LOD (µg/g DW)	LOQ (µg/g DW)
Leaf	1	0.01	0.06
Root	1	0.02	0.11
Kernel	1	0.01	0.06

Minimum dilution factor, LOD and LOQ for each matrix at senescence stage

Tissue Type	Minimum Dilution Factor	LOD (µg/g DW)	LOQ (µg/g DW)
Leaf	1	0.01	0.05
Root	1	0.02	0.10
Kernel	1	0.01	0.05

Representative PMI ELISA standard curve. Concentrations used to produce the ELISA standard curve: 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml, 3.125 ng/ml, 1.563 ng/ml, 0.781 ng/ml, and 0.391 ng/ml



APPENDIX D: CONCENTRATIONS OF eCry3.1Ab IN INDIVIDUAL SAMPLES

Table D1. Concentrations of eCry3.1Ab measured in leaves from each of the replicate plants of 5307 maize by growth stage on a dry-weight (DW) and a fresh-weight (FW) basis

	Whorl		Anthesis		Maturity		Senescence	
Location	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW
Shirley, IL	68.34	12.96	58.24	14.37	81.41	38.69	8.25	6.09
	98.04	18.89	64.15	16.58	74.49	35.07	3.47	2.65
	103.10	19.66	62.19	15.60	75.32	54.90	2.42	1.87
	75.19	13.79	49.41	11.68	56.74	37.78	20.43	15.65
	81.14	14.90	53.94	13.48	78.84	32.99	11.14	7.62
Sadorus, IL	86.84	14.92	74.24	18.55	49.40	24.36	1.16	0.86
	100.50	17.70	78.33	18.19	40.43	21.03	6.33	4.59
	110.74	17.83	75.97	17.83	20.11	12.72	1.56	1.11
	109.39	18.92	77.87	21.27	14.31	5.59	<LOQ[a]	<LOQ[a]
	109.19	18.92	71.53	17.01	40.15	16.92	2.39	1.66
Bloomington, IL	77.54	16.48	86.82	19.76	1.12	0.69	2.87	2.30
	83.38	20.64	57.59	13.43	10.48	6.35	7.06	5.62
	80.75	17.20	60.00	14.05	29.90	14.26	<LOQ[a]	<LOQ[a]
	84.47	16.91	61.52	14.22	46.01	21.89	2.55	1.96
	83.75	17.59	54.89	12.25	48.55	21.34	3.93	3.09
Stanton, MN	157.76	18.32	47.31	10.66	22.24	11.48	2.59	1.38
	150.64	19.93	48.99	11.14	24.17	11.55	1.68	1.22
	150.96	20.89	69.50	17.07	13.54	6.76	1.75	1.02
	215.69	26.06	79.65	19.40	8.83	5.46	5.87	3.55
	176.78	23.62	68.11	15.27	20.01	10.69	1.58	1.01

[a] LOQ = 0.10 µg/g DW and 0.02 µg/g FW

Values have not been adjusted for extraction efficiency.

Table D2. Concentrations of eCry3.1Ab measured in roots from each of the replicate plants of 5307 maize by growth stage on a dry-weight (DW) and a fresh-weight (FW) basis

	Whorl		Anthesis		Maturity		Senescence	
Location	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW
Shirley, IL	25.60	3.53	10.03	1.75	16.61	4.46	16.23	3.97
	32.98	3.34	11.02	2.08	11.43	3.19	11.84	4.04
	35.29	3.57	12.51	2.06	12.68	3.69	11.40	5.83
	29.89	2.74	10.49	1.77	9.56	2.83	11.59	4.23
	32.53	4.34	9.83	1.86	11.95	2.54	11.08	2.70
Sadorus, IL	33.99	4.91	11.51	1.83	6.27	1.34	2.49	0.47
	22.75	2.82	14.42	2.49	5.70	1.15	5.36	0.89
	27.92	2.98	16.84	2.79	5.22	0.96	3.98	0.62
	24.60	3.85	13.36	2.16	4.18	0.76	1.72	0.30
	30.12	4.06	15.47	2.68	5.66	1.36	6.16	1.13
Bloomington, IL	25.06	3.84	11.86	2.10	8.47	2.15	8.46	2.74
	31.29	4.35	10.84	1.81	6.99	1.55	10.33	2.99
	38.00	4.22	11.23	2.01	13.16	4.04	9.21	2.15
	31.99	5.04	9.51	1.41	9.64	3.03	7.77	2.33
	34.09	4.97	9.25	1.58	10.62	2.69	8.90	3.23
Stanton, MN	32.06	3.72	16.87	2.95	11.25	2.18	1.05	0.33
	30.74	4.35	18.80	3.48	2.38	0.90	1.04	0.38
	33.30	5.10	22.10	4.47	9.04	1.65	2.68	0.82
	33.69	4.08	15.93	3.94	6.54	1.22	3.49	1.21
	52.01	6.93	19.81	3.62	11.22	2.81	1.48	0.43

Values have not been adjusted for extraction efficiency.

Table D3. Concentrations of eCry3.1Ab measured in replicate whole plants of 5307 maize by growth stage on a dry-weight (DW) and a fresh-weight (FW) basis

	Whorl		Anthesis		Maturity		Senescence	
Location	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW
Shirley, IL	89.83	10.64	32.93	7.95	5.00	2.64	10.04	4.24
	73.52	9.31	37.13	6.52	7.45	3.16	9.42	3.81
	72.71	9.60	43.65	8.08	16.09	7.78	11.70	4.10
	73.96	8.95	43.40	7.83	13.44	8.22	6.95	2.96
	87.66	12.69	40.20	7.71	30.53	17.22	10.18	4.04
Sadorus, IL	70.45	9.49	27.76	7.26	10.64	5.14	3.35	1.90
	67.42	11.09	34.41	10.28	15.71	10.13	5.23	2.38
	87.97	22.46	36.18	8.77	19.78	11.37	7.65	3.81
	86.05	14.35	25.84	7.09	25.96	16.49	4.42	1.74
	71.73	15.62	23.14	6.34	13.37	8.72	3.97	1.88
Bloomington, IL	58.93	10.57	26.55	4.77	9.36	4.57	4.51	2.32
	59.32	10.27	26.48	5.04	9.79	5.98	3.08	1.55
	63.64	12.92	24.92	4.64	15.66	10.44	4.93	2.36
	62.25	12.69	26.33	4.67	10.92	6.70	3.91	1.92
	63.36	10.49	31.91	5.66	11.91	5.65	7.37	2.91
Stanton, MN	139.74	13.02	22.73	4.88	6.77	2.82	2.73	1.41
	131.76	17.05	30.06	6.29	6.25	2.80	3.58	1.74
	130.04	12.00	25.97	5.39	6.50	2.63	3.93	1.75
	129.70	11.98	27.35	5.58	8.91	3.76	2.67	1.33
	121.83	12.18	11.12	2.43	7.27	2.72	19.96	8.35

Values have not been adjusted for extraction efficiency.

Table D4. Concentrations of eCry3.1Ab measured in kernels from each of the replicate plants of 5307 maize at two stages on a dry-weight (DW) and a fresh-weight (FW) basis

	Maturity		Senescence	
Location	**µg/g DW**	**µg/g FW**	**µg/g DW**	**µg/g FW**
Shirley, IL	6.16	4.63	3.32	2.50
	5.45	4.30	2.66	2.10
	8.48	6.41	3.12	2.36
	4.40	3.09	3.89	2.72
	6.28	4.69	3.20	2.39
Sadorus, IL	6.16	4.38	4.79	3.41
	7.21	4.97	5.95	4.10
	7.02	4.77	5.02	3.41
	7.39	5.35	3.59	2.60
	7.44	4.85	3.90	2.55
Bloomington, IL	3.71	3.05	3.17	2.61
	6.05	4.79	3.19	2.53
	5.90	4.96	2.57	2.16
	4.18	3.38	4.18	3.38
	7.16	5.64	3.61	2.85
Stanton, MN	6.12	3.86	5.16	3.26
	2.09	1.41	5.64	3.79
	2.58	2.02	3.89	3.05
	3.03	2.16	3.37	2.41
	2.17	1.52	4.05	2.84

Values have not been adjusted for extraction efficiency.

Table D5. Concentrations of eCry3.1Ab measured in pollen samples[a] from plants of 5307 maize at anthesis on a dry-weight (DW) and a fresh-weight (FW) basis

Location	µg/g DW	µg/g FW
Shirley, IL	0.10	0.09
Sadorus, IL	0.10	0.09
Bloomington, IL	<LOQ[b]	<LOQ[b]
Stanton, MN	ND[c]	ND[c]

[a] one composite sample collected from each location
[b] LOQ = 0.10 µg/g and 0.08 µg/g FW
[c] ND = Not Determined, sample unavailable
Values have not been adjusted for extraction efficiency.

APPENDIX E: CONCENTRATIONS OF PMI IN INDIVIDUAL SAMPLES

Table E1. Concentrations of PMI measured in leaves from each of the replicate plants of 5307 maize by growth stage on a dry-weight (DW) and a fresh-weight (FW) basis

	Whorl		Anthesis		Maturity		Senescence	
Location	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW
Shirley, IL	2.66	0.50	3.73	0.92	2.51	1.19	0.09	0.07
	3.09	0.60	1.48	0.38	1.61	0.76	<LOQ[b]	<LOQ[b]
	3.30	0.63	1.46	0.37	1.43	1.04	<LOD[b]	<LOD[b]
	2.51	0.46	1.42	0.34	0.75	0.50	0.39	0.30
	2.78	0.51	1.25	0.31	1.90	0.79	0.13	0.09
Sadorus, IL	3.75	0.65	2.36	0.59	0.56	0.28	<LOD[b]	<LOD[b]
	3.93	0.69	2.21	0.51	0.34	0.18	<LOQ[b]	<LOQ[b]
	3.74	0.60	2.32	0.54	0.17	0.11	<LOD[b]	<LOD[b]
	3.64	0.63	2.40	0.66	0.11	0.04	<LOD[b]	<LOD[b]
	4.68	0.81	2.58	0.61	0.43	0.18	<LOD[b]	<LOD[b]
Bloomington, IL	2.78	0.59	2.08	0.47	<LOQ[a]	<LOQ[a]	<LOQ[b]	<LOQ[b]
	2.28	0.57	1.87	0.44	<LOQ[a]	<LOQ[a]	0.08	0.07
	2.40	0.51	1.87	0.44	0.65	0.31	<LOD[b]	<LOD[b]
	2.13	0.43	1.90	0.44	0.59	0.28	<LOD[b]	<LOD[b]
	2.18	0.46	1.89	0.42	0.71	0.31	<LOQ[b]	<LOQ[b]
Stanton, MN	4.27	0.50	2.06	0.46	0.29	0.15	<LOD[b]	<LOD[b]
	5.97	0.79	1.82	0.41	0.10	0.05	<LOD[b]	<LOD[b]
	4.35	0.60	2.50	0.61	0.07	0.03	<LOD[b]	<LOD[b]
	4.52	0.55	2.44	0.59	0.07	0.05	0.07	0.04
	4.33	0.58	2.07	0.47	0.30	0.16	<LOD[b]	<LOD[b]

[a] LOQ = 0.06 µg/g DW and 0.03 µg/g FW at maturity

[b] LOQ = 0.05 µg/g DW and 0.03 µg/g FW; LOD = 0.01 µg/g DW and FW at senescence

Values have not been adjusted for extraction efficiency.

Table E2. Concentrations of PMI measured in roots from each of the replicate plants of 5307 maize by growth stage on a dry-weight (DW) and a fresh-weight (FW) basis

	Whorl		Anthesis		Maturity		Senescence	
Location	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW
Shirley, IL	1.60	0.22	1.47	0.26	1.91	0.51	2.72	0.67
	1.92	0.19	1.08	0.20	1.88	0.53	2.28	0.78
	1.74	0.18	0.87	0.14	1.89	0.55	1.89	0.97
	1.54	0.14	0.91	0.15	1.41	0.42	2.42	0.88
	2.04	0.27	1.08	0.20	2.25	0.48	0.86	0.21
Sadorus, IL	2.52	0.36	1.40	0.22	1.29	0.28	0.16	0.03
	1.17	0.14	1.29	0.22	1.45	0.29	1.28	0.21
	1.79	0.19	1.38	0.23	1.44	0.27	0.67	0.11
	1.96	0.31	1.35	0.22	1.04	0.19	<LOQ[a]	<LOQ[a]
	1.96	0.26	1.55	0.27	2.38	0.57	1.67	0.31
Bloomington, IL	1.44	0.22	1.27	0.22	1.87	0.47	1.07	0.35
	1.31	0.18	1.12	0.19	1.07	0.24	1.70	0.49
	1.25	0.14	1.30	0.23	1.55	0.47	0.61	0.14
	1.24	0.20	1.29	0.19	1.45	0.45	0.81	0.24
	1.38	0.20	1.35	0.23	1.71	0.43	1.04	0.38
Stanton, MN	2.36	0.27	1.88	0.33	2.59	0.50	0.33	0.10
	2.17	0.31	1.43	0.26	0.87	0.33	0.12	0.05
	1.85	0.28	1.69	0.34	2.18	0.40	0.66	0.20
	1.24	0.15	1.25	0.31	0.83	0.16	1.79	0.62
	1.54	0.20	1.97	0.36	2.32	0.58	0.34	0.10

[a] LOQ = 0.10 µg/g DW and 0.03 µg/g FW at senescence
Values have not been adjusted for extraction efficiency.

Table E3. Concentrations of PMI in replicate whole plants of 5307 maize by growth stage on a dry-weight (DW) and a fresh-weight (FW) basis

	Whorl		Anthesis		Maturity		Senescence	
Location	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW
Shirley, IL	2.19	0.26	6.63	1.60	0.83	0.44	1.14	0.48
	1.99	0.25	6.02	1.06	0.92	0.39	0.92	0.37
	2.48	0.33	5.75	1.06	0.92	0.44	1.52	0.53
	2.38	0.29	5.67	1.02	0.51	0.31	0.67	0.29
	2.34	0.34	5.89	1.13	0.96	0.54	0.94	0.38
Sadorus, IL	3.79	0.51	2.61	0.68	1.22	0.59	0.44	0.25
	3.84	0.63	2.87	0.86	1.82	1.18	0.37	0.17
	3.33	0.85	3.27	0.79	1.78	1.02	0.39	0.20
	3.62	0.60	2.49	0.68	1.60	1.02	0.30	0.12
	3.78	0.82	2.39	0.65	1.64	1.07	0.65	0.31
Bloomington, IL	2.45	0.44	2.11	0.38	1.22	0.59	1.03	0.53
	2.96	0.51	2.52	0.48	1.92	1.17	0.86	0.43
	2.32	0.47	1.98	0.37	1.31	0.87	0.97	0.46
	2.09	0.43	2.16	0.38	1.44	0.89	0.87	0.43
	1.94	0.32	2.66	0.47	1.05	0.50	0.64	0.25
Stanton, MN	5.77	0.54	2.14	0.46	1.85	0.77	0.57	0.30
	3.89	0.50	3.08	0.64	1.76	0.79	0.37	0.18
	4.08	0.38	1.60	0.33	1.43	0.58	0.34	0.15
	4.06	0.38	2.46	0.50	1.72	0.73	0.32	0.16
	4.14	0.41	1.50	0.33	1.48	0.56	1.16	0.49

Values have not been adjusted for extraction efficiency.

Table E4. Concentrations of PMI measured in kernels from each of the replicate plants of 5307 maize at two stages on a dry-weight (DW) and a fresh-weight (FW) basis

	Maturity		Senescence	
Location	**µg/g DW**	**µg/g FW**	**µg/g DW**	**µg/g FW**
Shirley, IL	1.46	1.10	0.90	0.68
	2.09	1.43	0.94	0.74
	2.34	1.64	0.53	0.40
	1.29	0.93	0.90	0.63
	1.54	1.16	0.87	0.65
Sadorus, IL	1.87	1.13	0.88	0.63
	1.57	0.95	0.95	0.66
	1.62	0.99	0.95	0.64
	1.09	0.66	0.77	0.56
	1.66	0.97	0.96	0.63
Bloomington, IL	1.14	0.84	0.90	0.74
	0.83	0.59	1.01	0.80
	1.26	0.86	0.56	0.47
	1.46	1.02	0.76	0.62
	1.27	0.92	1.29	1.02
Stanton, MN	2.72	1.70	1.09	0.69
	1.58	0.97	0.92	0.62
	1.73	1.07	0.76	0.59
	1.54	0.92	0.89	0.64
	3.05	1.90	0.94	0.66

Values have not been adjusted for extraction efficiency.

Table E5. Concentrations of PMI in pollen samples[a] from plants of 5307 maize at anthesis on a dry-weight (DW) and a fresh-weight (FW) basis

Location	µg/g DW	µg/g FW
Shirley, IL	6.86	5.75
Sadorus, IL	5.49	4.93
Bloomington, IL	6.19	4.89
Stanton, MN	ND[b]	ND[b]

[a] one composite sample collected from each location

[b] ND = Not Determined, sample unavailable

Values have not been adjusted for extraction efficiency.

APPENDIX F: ESTIMATES OF EXTRACTION EFFICIENCIES

The apparent extraction efficiency for eCry3.1Ab was greater than 70% in leaves, roots, and whole plants, which indicates that the procedure was well optimized for the extraction of the eCry3.1Ab protein from these maize tissues. The extraction efficiency for eCry3.1Ab in leaves and roots was determined in the matrix validation study (Murray 2008). The extraction efficiency for eCry3.1Ab in kernels was determined in the matrix validation study (Schwartz 2010). The extraction efficiency for eCry3.1Ab in whole plant was determined within the context of this study.

Table F1. Extraction efficiency of eCry3.1Ab from each maize tissue type analyzed

Tissue Type	Extraction Efficiency (%)
Leaves	77.09
Roots	74.66
Whole Plants	78.41
Pollen	–
Kernels	88

– = protein not quantifiable

The apparent extraction efficiency for PMI was greater than 70% in each tissue type, which indicates that the procedure was well optimized for the extraction of the PMI protein from these maize tissues. The extraction efficiency for PMI in leaves, roots, and kernels was determined in a separate study (deFontes 2009). The extraction efficiency for PMI in whole plant and pollen was determined within the context of this study.

Table F2. Extraction efficiency of PMI from each maize tissue type analyzed

Tissue Type	Extraction Efficiency (%)
Leaves	71.70
Roots	90.09[a]
Whole Plants	75.04
Pollen	94.89
Kernels	79.80

[a] extraction efficiency of 62.80% was used for whorl and anthesis roots for Stanton, MN