Evaluation of Transgenic Protein Levels in Multiple Generations of Plants Derived from Transformation Event 5307 Maize

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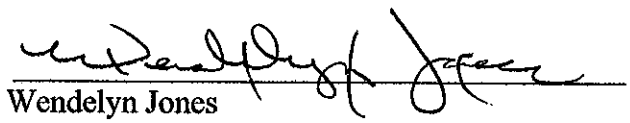

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Nov 10, 2008
Date

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES

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

1. The production of the plant material was performed outside the scope of this study.
2. The characterization reports of protein reference substances ECRY3.1AB-0208 and PMI-0105 were not finalized prior to use in this study. Although, analytical work for each reference substance was conducted using GLP standards.
3. Characterization of the test and control substances was not conducted according to GLP.
4. TaqMan PCR used to verify the presence or absence of the intended transgenes was not performed according to GLP.

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QUALITY ASSURANCE STATEMENT

Study Title: Evaluation of Transgenic Protein Levels in Multiple Generations of Plants Derived from Transformation Event 5307 Maize

Study Director: Mark Bednarcik

Study Number: 5307-08-04 and amendment(s)

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 Date
Audit Protocol	March 31, 2008	March 31, 2008
In-Progress Inspection	September 3, 2008	September 4, 2008
Initial Final Report	November 6, 2008	November 7, 2008
Second Final Report	November 10, 2008	November 10, 2008

Prepared by: Kimberly W. Hill Date: Nov. 10, 2008

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DW	dry weight
eCry3.1Ab	engineered chimera of modified Cry3A and Cry1Ab
ELISA	enzyme-linked immunosorbent assay
PMI	phosphomannose isomerase
FW	fresh weight
GLP	Good Laboratory Practice
LOD	limit of detection
LOQ	limit of quantitation
OD	optical density
PBS	phosphate-buffered saline
R1	reproductive stage – silking and pollen shed
SBI	Syngenta Biotechnology, Inc.
TMB	3,3′,5,5′-tetramethylbenzidine
Tween 20	polyoxyethylene (20) sorbitan monolaurate
VT	vegetative stage - tasseling

SUMMARY

The purpose of this study was to measure the concentration of proteins eCry3.1Ab and PMI in tissues of four generations 5307 maize plants.

Plants of four generations (F1, NP2171 x BC5F3, BC6, and BC7) of 5307 maize were grown under standard greenhouse conditions at Syngenta Biotechnology, Inc. Leaf, root, and pollen samples were collected at vegetative growth stage VT-R1 from five plants that were hemizygous for the transgenes, and the concentrations of eCry3.1Ab and PMI in these samples were measured by enzyme-linked immunosorbent assay (ELISA). Samples from a corresponding nontransgenic, near-isogenic maize plant were analyzed to identify background effects of the plant extract on the ELISA

The mean concentrations of eCry3.1Ab measured in leaves from the F1, NP2171 x BC5F3, BC6, and BC7 generations ranged from 83.40 to 93.67 µg/g DW. The mean eCry3.1Ab concentrations measured in roots from the F1, NP2171 x BC5F3, BC6, and BC7 generations ranged from 23.88 to 35.39 µg/g DW. The eCry3.1Ab concentrations measured in pollen from the F1, NP2171 x BC5F3, and BC7 generations were below the limit of detection (0.08 µg/g DW). The eCry3.1Ab concentrations measured in pollen from BC6 ranged from below the limit of detection to 0.15 µg/g DW.

The mean concentrations of PMI measured in leaves from the F1, NP2171 x BC5F3, BC6, and BC7 generations ranged from 1.77 to 1.95 µg/g DW. The mean PMI concentrations measured in roots from the F1, NP2171 x BC5F3, BC6, and BC7 generations ranged from 1.05 to 1.19 µg/g DW. The mean PMI concentrations measured in pollen from the F1, NP2171 x BC5F3, BC6, and BC7 generations ranged from 18.96 to 25.58 µg/g DW.

The concentrations of eCry3.1Ab and PMI measured across four generations of 5307 maize at VT-R1 were comparable, indicating consistency of expression of these proteins across multiple generations.

INTRODUCTION

Using the techniques of modern molecular biology, Syngenta has transformed maize (*Zea mays*) to produce Event 5307 maize, a new cultivar that has insecticidal activity against certain corn rootworm (*Diabrotica*) 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 purpose of this study was to measure the concentration of proteins eCry3.1Ab and PMI in tissues of four generations 5307 maize plants. The concentrations of both these proteins in leaf, root, and pollen were measured by enzyme-linked immunosorbent assay (ELISA), to evaluate consistency of expression across generations.

MATERIALS AND METHODS

Test and Control Substances

The test materials for this study were the four generations of 5307 maize shown below. The control material was the nontransgenic, near-isogenic counterpart (from one generation) to the test material used to asses the matrix effects and in the ELISA. Seed lots of the test and control materials were subject to the appropriate Stewardship Quality Control testing to confirm plant genotype according to the current Syngenta standards.

Table 1. Hybrid backgrounds of the maize plants used in this study

Generation	Event 5307 Hybrids	Nontransgenic, Near-Isogenic Hybrids
F1	NP2460//NP2222/(5307)	NP2171 /NP2460
NP2171 X BC5F3	NP2171 /(NP2460*//NP2222/(5307)	NP2171 /NP2460
BC6	(NP2460*//NP2222//(5307)	NP2171 /NP2460
BC7	(NP2460*//NP2222/(5307)	NP2171 /NP2460

A pedigree chart for the hybrids and generations shown in Table 1 is presented in Appendix A.

Verification of Plant Genotypes

TaqMan PCR (Ingham *et al*., 2001) was used to verify the presence or absence of the intended *ecry3.1Ab* and *manA* transgenes in individual plants prior to sampling.

Plant Material

Leaf, root, and pollen samples were collected at VT-R1 from five transgenic plants per generation grown in a greenhouse at Syngenta Biotechnology, Inc. according to standard greenhouse practices. Tissues from a single generation of nontransgenic plants were grown and collected in the same manner. At anthesis, pollen was manually collected. Following collection of the pollen, all healthy leaves were collected. Following removal of the leaf tissue, the plant was manually cut at the soil level to allow for root sampling. The roots were cleaned with water to remove dirt and dried with a towel to remove any moisture. Each plant's leaf, root, and pollen tissue was held separate from others and represented one sample. Samples were stored at –80ºC ± 10ºC after collection.

Plant Tissue Processing

Following processing the individual leaf and root samples were weighed to determine fresh weight. Processed samples were stored at –80 ± 10ºC until lyophilization. Following lyophilization, the samples were reweighed individually to determine the dry weight. The two weight measurements allowed for determination of the % Dry Weight. Pollen collection was performed by greenhouse personnel. Subsequently, the pollen was stored at –80ºC ± 10ºC until analysis. The pollen was sieved to remove non-pollen debris (*e.g.*, anthers and aphids). Following analysis, the pollen was weighed to determine fresh weight, lyophilized, and reweighed individually to determine dry weight.

A percent dry weight value for each sample was calculated as follows:

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

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

Protein Extraction and ELISA Analysis

Extracts of the lyophilized tissue samples (except pollen) were prepared for eCry3.1Ab and PMI analysis. For each subsample analyzed, lyophilized material was transferred into a 15-ml polypropylene tube, suspended in extraction buffer and homogenized. The homogenate was incubated on wet ice for 30 minutes, and centrifuged. The supernatants were stored at 2°C to 8°C until analysis. The supernatants were used for eCry3.1Ab and PMI quantification by ELISA. Details of the extraction procedures are described in Appendices B and C.

Pollen extracts were prepared by transferring pollen into a 2-ml Eppendorf tube containing glass beads and stored at -80°C for at least 2 hours. Each tube was then placed into a grinding mill set at ~3000 strokes per minute for 45 seconds. Each tube contained only the pollen from one individual plant. The tubes were then set on wet ice and Borate buffer, pH 7.5 was added to each sample. The samples were mixed and incubated on wet ice. The samples were then centrifuged and the supernatants were stored at 2°C to 8°C until analysis. The supernatants were used for eCry3.1Ab and PMI quantification by ELISA. Details of the extraction procedures are described in Appendix B and C.

The eCry3.1Ab and PMI proteins were extracted from the samples and the extracts were analyzed by ELISA (Tijssen 1985) to quantify the amount of eCry3.1Ab and PMI in each sample. Sample extracts were analyzed in triplicate, and standard curves were generated with known amounts of the corresponding microbially produced reference proteins, ECRY3.1AB-0208 (Nelson 2008a) and PMI-0105 (Nelson 2008b). 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 mean absorbance for each sample was plotted against the standard curve to obtain the concentrations of eCry3.1Ab and PMI in the extract (ng/ml). The concentrations of eCry3.1Ab and PMI in each sample (µg/g) were calculated as follows:

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

LOQ and LOD Determination

The limit of quantitation (LOQ) of each ELISA 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) of each ELISA sample type was based on the average OD values of nontransgenic extracts and the lowest concentration of reference protein used to produce the standard curve.

The LOQ and LOD for eCry3.1Ab in each sample type were determined in ongoing study number ECRY3.1ABVAL-08-02. The limits of quantitation and limits of detection for PMI in each sample type were determined in the context of this study.

Statistical Methods

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

RESULTS AND DISCUSSION

Analysis of eCry3.1Ab in Four Generations of 5307 Maize

It is important to note that no circumstances occurred during the conduct of this study that would have adversely affected the quality or integrity of the data generated.

The mean eCry3.1Ab concentrations in leaf, root and pollen from the F1, NP2171 x BC5F3, BC6, and BC7 generations of 5307 maize are shown in Table 2. A graphical representation of relative concentrations in each tissue type across generations is shown in Figure 1. Appendix D contains all the concentrations of eCry3.1Ab in tissues from

each plant of the four generations of 5307 maize on a dry-weight (DW) and a fresh-weight (FW) basis.

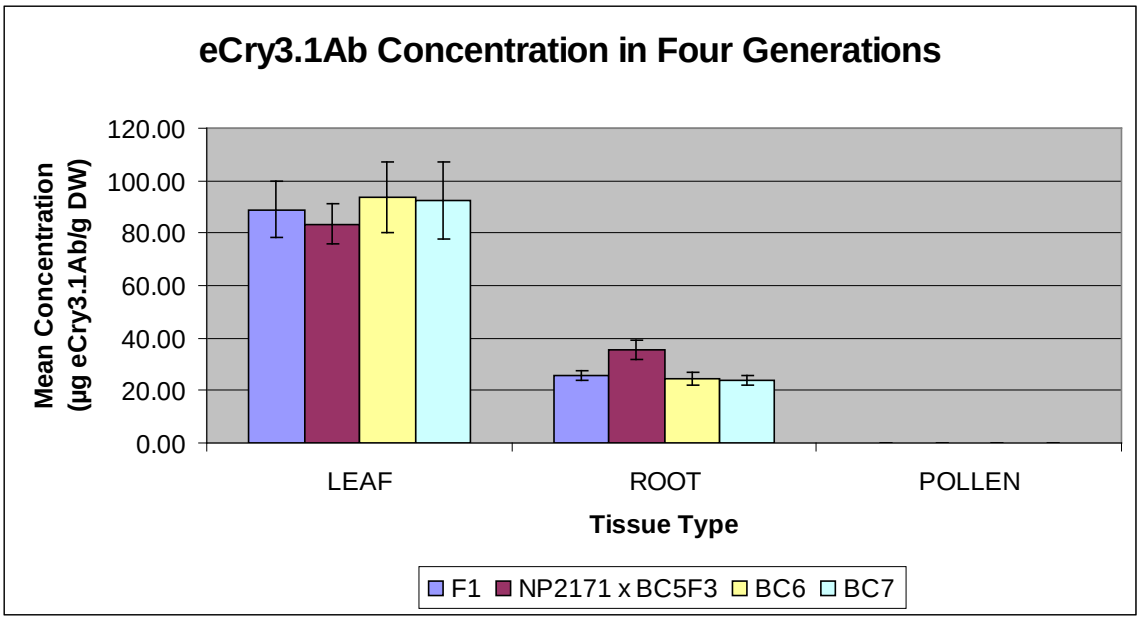
Table 2. Concentrations of eCry3.1Ab in four generations of 5307 maize on a dry-weight (DW) and a fresh-weight (FW) basis

Protein concentration units are µg/g DW and µg/g FW. *N* = 5

		µg/g DW		µg/g FW	
Tissue	**Generation**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Leaf	F1	89.07 ± 10.52	75.17–101.72	21.72 ± 2.74	18.04–-25.33
	NP2171 x BC5F3	83.40 ± 7.67	77.02–96.54	20.39 ± 2.24	18.81–-24.28
	BC6	93.67 ± 13.27	77.45–105.80	23.16 ± 3.17	19.46–-26.83
	BC7	92.42 ± 14.75	78.24–113.06	23.71 ± 4.10	19.69–-29.41
Root	F1	25.89 ± 1.93	24.42–-29.12	2.82 ± 0.31	2.36–3.17
	NP2171 x BC5F3	35.39 ± 3.78	31.38–-41.21	3.79 ± 0.33	3.35–4.12
	BC6	24.43 ± 2.24	21.92–-27.71	2.83 ± 0.44	2.28–3.47
	BC7	23.88 ± 2.10	21.50–-26.51	2.58 ± 0.53	1.81–3.12
Pollen	F1	–-	< LOD	–-	< LOD
	NP2171 x BC5F3	–-	< LOD	–-	< LOD
	BC6	–-	< LOD–-0.15	–-	< LOD–0.15
	BC7	–-	< LOD	–-	< LOD

–- = not applicable. The LOD for pollen was 0.08µg/g DW. (Ongoing study No. ECRY3.1ABVAL-08-02). It was not possible to calculate the means for pollen as some or all values were below LOD.

Figure 1. Concentrations of eCry3.1Ab in four generations of tissues on a dry-weight (DW) basis. The bars indicate the standard deviation of the values per tissue type by generation.



Analysis of PMI in Four Generations of 5307 Maize

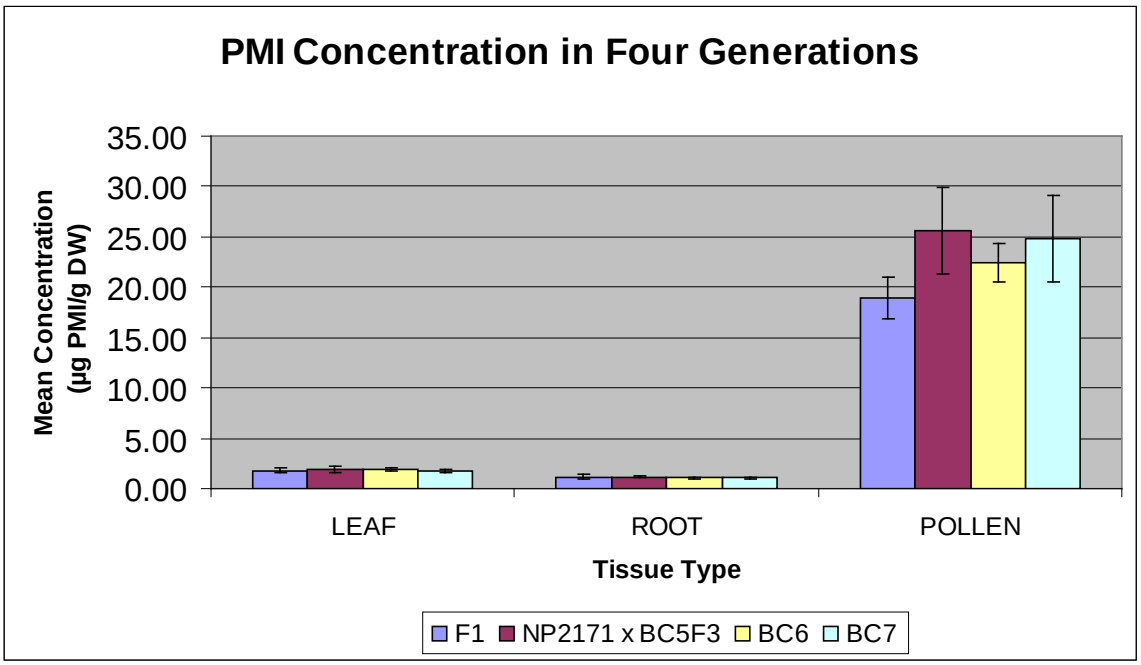
The mean PMI concentrations in leaf, root, and pollen from the F1, NP2171 x BC5F3, BC6, and BC7 generations of 5307 maize are shown in Table 3. Graphical representations of relative concentrations in each tissue type across generations are shown in Figure 2. Appendix E contains all the concentrations of eCry3.1Ab in tissues from each plant of the four generations of 5307 maize on a dry-weight (DW) and a fresh-weight (FW) basis.

Table 3. Concentrations of PMI in four generations of 5307 maize on a dry-weight (DW) and a fresh-weight (FW) basis

Protein concentration units are µg/g DW and µg/g FW. *N* = 5

		µg/g DW		µg/g FW	
Tissue	**Generation**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Leaves	F1	1.82 ± 0.23	1.48–2.04	0.44 ± 0.05	0.37–0.50
	NP2171 x BC5F3	1.91 ± 0.24	1.55–2.15	0.47 ± 0.07	0.38–0.53
	BC6	1.95 ± 0.19	1.79–2.28	0.48 ± 0.04	0.45–0.55
	BC7	1.77 ± 0.19	1.55–2.05	0.45 ± 0.05	0.38–0.52
Root	F1	1.19 ± 0.17	1.02–1.45	0.13 ± 0.03	0.11–0.18
	NP2171 x BC5F3	1.17 ± 0.06	1.08–1.25	0.13 ± 0.02	0.10–0.14
	BC6	1.05 ± 0.11	0.86–1.15	0.12 ± 0.02	0.09–0.14
	BC7	1.10 ± 0.08	1.07–1.23	0.12 ± 0.02	0.09–0.14
Pollen	F1	18.96 ± 2.09	17.09 –21.97	18.46 ± 2.03	16.69–21.46
	NP2171 x BC5F3	25.58 ± 4.30	19.63–31.01	25.18 ± 4.17	19.51–30.49
	BC6	22.47 ± 1.88	20.63–25.29	22.22 ± 1.84	20.44–24.98
	BC7	24.87 ± 4.27	19.99–31.12	24.58 ± 4.15	19.81–30.61

Figure 2. **Concentrations of PMI in four generations of tissues on a dry-weight (DW) basis**. The bars indicate the standard deviation of the values per tissue type by generation.



CONCLUSIONS

The concentrations of eCry3.1Ab and PMI measured across four generations of 5307 maize at VT-R1 were comparable, indicating consistency of expression of these proteins across multiple generations.

RECORDS RETENTION

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

CONTRIBUTING SCIENTISTS

All analytical work was conducted by Mark Bednarcik, Neha Upadhyay, and Jim Branson at the Regulatory Science Laboratory, Research Triangle Park, NC, USA.

Reported by: Mark J. Bednarcik November 11, 2008

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Approved by: Scott Rabe November 11, 2008

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Group Leader, Regulatory Science
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Date

CRITICAL DATES

Study initiation date:	April 2, 2008
Experimental start date:	May 7, 2008
Experimental end date:	September 19, 2008

REFERENCES

ECRY3.1ABVAL-08-02**:** *Validation of a Quantitative eCry3.1Ab ELISA for Analysis of Maize Leaf, Root, Kernel, and Pollen Extracts.* (Ongoing study).

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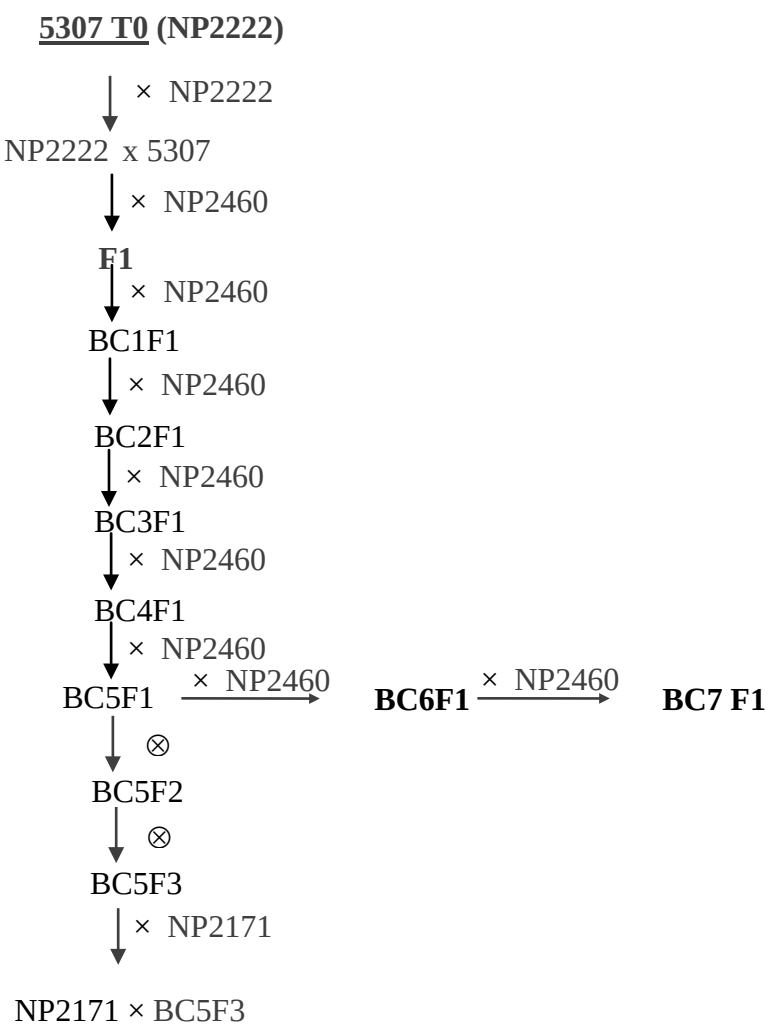
APPENDIX A: PEDIGREE CHART FOR 5307 SEED



The generations tested in this study are highlighted in bold.

APPENDIX B: PMI QUANTIFICATION PROCEDURE

Buffers

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

Name of buffer	Constituents
Phosphate-buffered saline (PBS)	138 mM sodium chloride, 2.7 mM potassium chloride, 10.14 mM disodium phosphate, 1.8 mM potassium phosphate, pH 7.4
Blocking buffer	PBS, 1% non-fat powdered milk
Borate buffer	0.1 M Sodium tetraborate decahydrate, 0.2% PVP-40, 7.69 mM Sodium azide, 1.2% Concentrated hydrochloric acid, 0.5% Tween 20, Complete Protease Inhibitor Cocktail (Roche Applied Science Cat. No. 11697498001), pH 7.5
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% non-fat powdered milk
Wash buffer	PBS, 0.05% Tween 20

PMI Extraction

Leaf and Root. A ratio of 3 ml of Borate buffer pH 7.5 was added to 100 mg of lyophilized tissue. The samples were vortexed and homogenized in an Omni-Prep Homogenizer (Omni International) incubated on wet ice for 30 minutes, and centrifuged for 15 minutes at 10,000 × *g* at 2°C to 8°C. The supernatants were stored at 2°C to 8°C until analysis.

Pollen. For each sample, 25 mg of fresh pollen was weighed into a 2-ml Eppendorf tube containing three 4-mm glass beads and stored at -80°C for at least 2 hours. Each tube was then placed into a Four Station Titer Plate/Micro Tube Grinding Mill (KLECO; Model 4-96) and set at ~3000 strokes per minute for 60 seconds. The tubes were then set on ice and 1.5 ml of Borate buffer, pH 7.5 was added to each sample. The samples were mixed briefly and incubated on wet ice for 30 minutes then centrifuged at 13,200 × *g* for 15 minutes at 2°C to 8°C. The supernatants were stored at 2°C to 8°C until analysis.

PMI Quantification

Rabbit anti-MIR 604 PMI antibody was diluted to 2 µg/ml in carbonate-bicarbonate buffer and used to coat Nunc MaxiSorp 96-well plates (ThermoFisher Scientific Cat. No. 12-565-276) at a volume of 100 µl/well. The plates were incubated overnight at 2°C to 8°C. The antibody solution was manually flicked into a sink, and the plates were tapped on paper towels to remove residual solution. The plates were then incubated with blocking buffer at a volume of 250 µl/well for at least 30 minutes at room temperature (20°C ± 8°C). The plates were washed five times with wash buffer in a BioTek ELx405

Microplate Washer. Dilutions of each tissue extract and appropriate serial dilutions of microbially produced PMI reference protein prepared in dilution buffer were applied to the plates at a total volume of 100 µl/well. The plates were incubated for 2 hours at 18°C to 20°C and washed five times as described above. After washing, the plates were incubated with mouse anti-MIR604 PMI monoclonal antibody diluted to 1 µg/ml in dilution buffer, at a volume of 100 µl/well, for 1 hour at 18°C to 20°C.

The plates were washed five times and incubated for 1 hour at 18°C to 20°C with horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobin G (Sigma-Aldrich Cat. No. A9044) diluted to 1:20,000 in dilution buffer. The plates were then washed five times, and TMB substrate solution (Sigma-Aldrich Cat. No. T3405) was added at a volume of 100 µl/well (1 tablet per 10 ml of citrate-phosphate buffer). The plates were incubated for 30 minutes in the dark at room temperature. The reaction was stopped by addition of 3 M sulfuric acid (50 µl per well), and absorbance at 450 nm was measured 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.

APPENDIX C: eCry3.1Ab QUANTIFICATION PROCEDURE

Buffers

The buffers used for extraction and enzyme-linked immunosorbent assay (ELISA) analyses 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, 1.81 mM sodium phosphate monobasic, pH 6.75
Borate buffer (pollen)	0.1 M Sodium tetraborate decahydrate, 0.2% PVP-40, 7.69 mM Sodium azide, 1.2% Concentrated hydrochloric acid, 0.5% Tween 20, Complete Protease Inhibitor Cocktail (Roche Applied Science Cat. No. 11697498001), pH 7.5
Borate buffer (leaf and root)	0.1 M Sodium tetraborate decahydrate, 0.2% PVP-40, 7.69 mM Sodium azide, 0.5% Tween 20, Complete Protease Inhibitor Cocktail (Roche Applied Science Cat. No. 11697498001), titrate to pH 10.0
Dilution buffer	PBS, 0.05% Tween 20, 1% BSA, 0.02% sodium azide
Wash buffer	10mM Tris, 0.05% Tween 20, 0.02% sodium azide

eCry3.1Ab Extraction

Leaf and Root. A ratio of 3 ml of Borate buffer pH 10.0 was added to 100 mg of lyophilized tissue. The samples were vortexed and homogenized in an Omni-Prep Homogenizer (Omni International) incubated on wet ice for 30 minutes, and centrifuged for 15 minutes at 10,000 × *g* at 2°C to 8°C. The supernatants were stored at 2°C to 8°C until analysis.

Pollen. For each sample, 25 mg of fresh pollen was weighed into a 2-ml Eppendorf tube containing three 4-mm glass beads and stored at -80°C for at least 2 hours. Each tube was then placed into a Four Station Titer Plate/Micro Tube Grinding Mill (KLECO; Model 4-96) and set at ~3000 strokes per minute for 45 seconds. The tubes were then set on wet ice and 1.5 ml of Borate buffer, pH 7.5 was added to each sample. The samples were mixed and incubated on wet ice for 30 minutes then centrifuged at 13,000 × *g* for 15 minutes at 2°C to 8°C. The supernatants were stored at 2°C to 8°C until analysis.

eCry3.1Ab Quantification

The eCry3.1Ab ELISA kit is 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 is diluted to 3 µg/ml and is used to coat each well of the 96-well microtiter plate. The plate was then blocked using a proprietary method. Dilutions of each tissue extract and appropriate serial dilutions of microbially produced eCry3.1Ab reference protein, 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 titer 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 titer plate shaker at 400 rpm for 1 hour and washed five times as described above.

After the plates were washed, a tertiary, donkey anti-rabbit antibody conjugated with alkaline phosphatase (Jackson ImmunoResearch Labs, Cat # 711-055-152) diluted to 0.5 µg/ml in dilution buffer was added to each of the wells (100 µl/well) and incubated at room temperature on a titer plate shaker at 400 rpm for 1 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 titer plate shaker at 400 rpm. The reaction was stopped by the addition of 3N sodium hydroxide (100 µl per well), and absorbance of the wells at a dual wavelength 405-492 nm was measured 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.

APPENDIX D: CONCENTRATIONS OF eCry3.1Ab PER SAMPLE

Table D1. Concentrations of eCry3.1Ab in tissues from each plant of the four generations of 5307 maize on a dry-weight (DW) and a fresh-weight (FW) basis

	Leaf		Roots		Pollen	
Generation	**µg/g DW**	**µg/g FW**	**µg/g DW**	**µg/g FW**	**µg/g DW**	**µg/g FW**
F1	101.72	25.33	25.13	3.17	<LOD	<LOD
	82.10	20.21	26.16	2.68	<LOD	<LOD
	91.29	22.42	24.63	2.36	<LOD	<LOD
	95.06	22.59	29.12	2.97	<LOD	<LOD
	75.17	18.04	24.42	2.92	<LOD	<LOD
NP2171 x BC5F3	79.27	19.56	33.29	3.35	<LOD	<LOD
	82.81	20.20	36.72	4.10	<LOD	<LOD
	81.35	18.81	41.21	3.60	<LOD	<LOD
	96.54	24.28	31.38	4.12	<LOD	<LOD
	77.02	19.09	34.33	3.78	<LOD	<LOD
BC6	77.45	19.46	25.43	2.87	0.09	0.09
	105.52	26.83	23.12	2.93	<LOD	<LOD
	105.80	25.36	27.71	3.47	<LOD	<LOD
	81.91	20.35	21.92	2.28	<LOD	<LOD
	97.69	23.79	23.96	2.62	0.15	0.15
BC7	78.24	19.69	22.04	2.30	<LOD	<LOD
	79.01	20.51	21.50	1.81	<LOD	<LOD
	113.06	29.41	25.16	2.99	<LOD	<LOD
	100.32	26.37	26.51	3.12	<LOD	<LOD
	91.48	22.58	24.20	2.66	<LOD	<LOD

The LOD for pollen was 0.08/µg/g DW. The LOQ for pollen was 0.08/µg/g DW. (Ongoing study No. ECRY3.1ABVAL-08-02)

APPENDIX E: CONCENTRATIONS OF PMI PER SAMPLE

Table E1. Concentrations of PMI in tissues from each plant of the four generations of 5307 maize on a dry-weight (DW) and a fresh-weight (FW) basis

	Leaf		Roots		Pollen	
Generation	**µg/g DW**	**µg/g FW**	**µg/g DW**	**µg/g FW**	**µg/g DW**	**µg/g FW**
F1	1.81	0.45	1.45	0.18	20.28	19.64
	1.48	0.37	1.10	0.11	17.49	17.05
	2.02	0.50	1.15	0.11	21.97	21.46
	2.04	0.49	1.25	0.13	17.09	16.69
	1.73	0.42	1.02	0.12	17.97	17.48
NP2171 x BC5F3	2.15	0.53	1.05	0.11	27.31	26.88
	2.04	0.50	1.25	0.14	23.31	22.85
	1.78	0.41	1.19	0.10	19.63	19.51
	2.03	0.51	1.08	0.14	26.64	26.17
	1.55	0.38	1.13	0.12	31.01	30.49
BC6	1.90	0.48	1.11	0.12	22.19	21.97
	1.79	0.45	1.07	0.14	23.23	22.93
	2.28	0.55	1.15	0.14	20.63	20.44
	1.85	0.46	0.86	0.09	21.00	20.75
	1.94	0.47	1.04	0.11	25.29	24.98
BC7	2.05	0.52	1.03	0.11	31.12	30.61
	1.82	0.47	1.10	0.09	22.67	22.38
	1.67	0.44	1.07	0.13	26.86	26.57
	1.75	0.46	1.23	0.14	19.99	19.81
	1.55	0.38	1.08	0.12	23.72	23.55