Evaluating Environmental Concentrations of Insecticidal Proteins for Ecological Risk Assessment of Conventionally Bred Transgenic Crops with Stacked Traits – A Non-Comparative Approach

by Justin McDonald

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Approved by advisory committee:
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BIOGRAPHY

Justin McDonald is a native of North Carolina, where he grew up in Winston-Salem and now lives in Raleigh with his wife and two children. Mr. McDonald earned a B.S degree in Agronomy with concentrations in Agronomic Business and Turfgrass management from North Carolina State University in 2003. During his undergraduate studies, he assisted several graduate students in the Crop Science Department with their research projects on turfgrass and tobacco transformation, and wheat breeding.

Mr. McDonald is a Regulatory Scientist with Syngenta Crop Protection, LLC, Research Triangle Park, NC. He has 11 years experience in the agriculture biotechnology industry specializing in product safety and regulatory affairs, agricultural field trials, biochemical analysis, data generation and interpretation, and project management. Mr. McDonald has managed over 30 scientific studies throughout his career that were aimed at characterizing the expression profile of insecticidal proteins produced by genetically-engineered crops. The data generated in those studies have been used to represent or calculate potential exposure to non-target organisms for ecological risk assessments.

The Environmental Assessment Master’s program at North Carolina State University has provided him with a better understanding of how to evaluate the risks that technological advancements pose to our environment.
ACKNOWLEDGEMENTS

I would like to thank Dr. Barry Goldfarb for his guidance as my advisor through the process of this project, and for his advice on how to present my ideas in the best way.

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The most important thanks goes to my wife, Laci and to my parents. There are no words that would express how much I appreciate your encouragement during all of my course work.
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ABSTRACT

Before approval and commercialization of a transgenic crop derived from a single transformation event, registrants must demonstrate to regulatory agencies that the transferred DNA has been stably integrated into the nuclear genome. With stable integration, breeding techniques may be used to create crops with multiple genetically-engineered (GE) traits just as with conventional traits. This conventional breeding technique used for combing multiple GE traits has been referred to as “trait stacking” and the resulting product as “breeding stacks” or “stacks”.

Many regulatory agencies require a comparison of transgenic protein concentrations in plant samples of a stack vs. those in corresponding component single event plants. To inform ecological risk assessment, this experiment is meant to test the hypothesis that transgenic protein concentrations in the stack are not significantly increased due to stacking (Raybould et al 2012). Corroboration of that hypothesis has been used to apply the Estimated Environmental Concentrations (EECs) used in risk assessment of the single events to a stack including corresponding component events (Raybould et al 2012).

The objective of this meta-analysis was to evaluate the results on the insecticidal transgenic proteins Cry1Ab, mCry3A, and Vip3Aa20 from several of these studies to assess the utility of the comparison (stack vs single event) for testing the stated hypothesis. Mean concentrations were also weighed against corresponding EECs previously set for the risk assessment of each single event relating to a specific insecticidal protein. Studies conducted on multiple combinations of the maize events: Bt11, MIR162, MIR604, and GA21 were used in this meta-analysis.
Results of the meta-analysis further confirms that transgenic protein expression was not affected by conventional breeding to combine the assessed traits. Although some significant differences were observed between stack and corresponding single event at alpha 0.05, most of those were not significant at alpha 0.01 and up to six (out of 111 comparisons) of those observations could have been solely due to chance associated with random sampling. Also, several mean transgenic protein concentrations of both stack and single events were higher (in most cases) than corresponding EECs, but many were also well below. This indicates that the EECs set for risk assessment of the single events remain appropriate for risk assessment of corresponding stacks.

Overall, the meta-analysis demonstrates the multitude of tests that corroborate the hypothesis of no significant increase in transgenic protein expression due to combining by conventional breeding. This assessment is good evidence to support the conclusion that future tests of the stated hypothesis on the insecticidal proteins Cry1Ab, mCry3A, and Vip3Aa20 would not be critically informative for ecological risk assessment of any combination of the maize events Bt11, MIR604, and MIR162. If concerns still exist, modifications to testing strategy for these particular proteins was proposed that eliminates re-testing of the hypothesis of no increase in expression due to stacking. Rather than re-testing the hypothesis, plant samples of the stack crop could be analyzed without a comparative field study that would include the component single event plants. The results from only stack samples could inform risk assessment by comparing the values to existing data on corresponding proteins such as those used in this meta-analysis. Prediction intervals with a margin of error that incorporates the variability from random influencing factors could be used with historical data to provide a range on which to compare results from future measurements.
INTRODUCTION/BACKGROUND

Insect pests in agricultural fields can cause immense damage to crops, lowering yields and decreasing grower income. Growers have many options to decrease insect pest populations in their crops including the application of chemical and biological insecticides (e.g. Bt microbial pesticides), and the use of seeds with insect-resistance traits introduced by gene engineering and plant transformation. Transgenic crops that have been engineered to express genes encoding proteins that are toxic to specific insect pests have been labeled as Plant Incorporated Protectants (PIP) by the United States Environmental Protection Agency (US EPA) (Matten 2012). These PIPs provide benefits to growers as protection to optimize yield and benefits to the environment as it may reduce and localize the need for application of insecticides (Carpenter et al 2002). This environmental benefit however, does not preclude transgenic crops with insecticidal traits from environmental and ecological risk assessment. Because these proteins have toxic activity to insect pests, it is important to evaluate the risk that these proteins may affect non-target organisms (NTOs) due to cultivation of transgenic crops in our environment (Carpenter et al 2002).

Ecological risk assessment is most informed when protection goals are clear and a case-by-case problem formulation is conducted to evaluate the overall risk hypothesis that no ecological harm will occur due to the cultivation of transgenic crops (Wolt et al 2010; Raybould 2007). Problem formulation is the first step in any risk assessment. Through problem formulation, pathways by which valued and potentially susceptible species may be exposed to the insecticidal proteins can be identified and testable hypotheses crafted to guide scientific study of those pathways to harm (Wolt et al 2010). This provides guidance for design of experiments needed to aid risk assessment of any new transgenic crop (Wolt et al 2010).
Many insecticidal traits have been introduced into commercial maize hybrids through genetic engineering and plant transformation since the first approval of such a product in 1995 (CERA 2012). Growers quickly realized that combining different insecticidal traits would be beneficial, because crops could be encountering multiple insect pest species. The additional protection offered by the expression of multiple insecticidal traits with activity against different insect pests is an obvious benefit. Another benefit is minimizing the potential for resistance by having a plant produce multiple insecticidal proteins that have different modes of action against the same insect pest (Storer 2012).

The combination of multiple desirable traits into one germplasm can be achieved through conventional breeding techniques, once the trait genes have been introduced into individual lines through transformation. The breeding techniques are then no different for traits that are native to the plant introduced from a different cultivar. For genetically modified crops, this has been referred to as “trait stacking” and the products have various names such as “stacks,” “breeding stacks,”, and “combined events products” (CLI 2011). The International Service for the Acquisition of Agri-Biotech Applications estimated that in 2014, 51 million ha were planted with stacks (James 2014).

Stacks are regulated to a lesser degree overall; however, some agencies apply more scrutiny to the safety evaluation than others. Regulatory agencies customarily recognize that the characterization studies to assess the allergenic and toxic potential of the encoded protein performed initially for risk assessment of the single event applies to the same protein produced by a stack. This is transferable if that stack has been confirmed to express the same genetic elements received from the parent plant expressing that transgene as a single event. However, uncertainty remains over the potential for interactions to occur between transgene products from
multiple single events. One concern related to this uncertainty was whether the production of a transgenic protein may increase due to the presence or production of another in the same plant (Raybould et al 2012). A comparative testing strategy has been used to evaluate this concern.

*Transgenic protein expression: stack vs single events*

Regulatory agencies in multiple countries require that the abundance of the transgenic proteins produced by a combined trait product be compared to that of the corresponding component single events. This type of experiment is required with differing conditions by regulatory agencies including the United States Environmental Protection Agency (US EPA), and the European Food Safety Authority (EFSA). From an ecological risk assessment perspective, this experiment is meant to test the hypothesis that the concentrations of transgenic proteins are similar between the stack and the corresponding component single events (Raybould et al 2012). Corroboration of this hypothesis has been used to provide confidence that the Estimated Environmental Concentrations (EECs) used for exposure purposes in risk assessment of the single event is applicable to a specific combined events product. In the Bt11 × MIR604 maize example, a few significant differences were observed in which the amount of the insecticidal proteins (Cry1Ab and mCry3A) were significantly higher in the stack than in Bt11 or MIR604 maize (Raybould et al 2012). In this situation, the relative difference of concentration between stack and single for these proteins was used to evaluate if extrapolation of the corresponding EECs were in reason (Raybould et al 2012). In this case, the relative increase was no greater than 1.5 fold and did not erode margins of exposure in any case (Raybould et al 2012). Therefore, the risk of adverse effects to NTOs from exposure to those proteins produced by Bt11 × MIR604 maize was deemed negligible; unchanged from that for each of the component single event maize Bt11 and MIR604.
The use of EECs in risk assessment of transgenic crops

Estimated Environmental Concentrations (EECs) have been set as the highest mean concentration of the transgenic protein in a plant tissue type most relevant to valued species of interest from a reasonable exposure scenario (Raybould 2007; Raybould 2010; US EPA 2010). These means that represent specific EECs were computed from concentrations measured in plant samples collected from field grown plants at multiple locations. These types of data are generated as part of a typical regulatory study conducted to establish the transgenic protein expression profile for any new genetically-engineered crop. The use of EECs in risk assessment of transgenic crops is similar to that for pesticides. Typically, exposure is computed using measured concentrations and other factors such as body weight and daily consumption when appropriate. The EECs are also refined in some cases to represent reasonable scenarios conforming to specific pathways of potential exposure. Toxicological experiments are conducted to determine the highest dose at which no effect is observed; referred to as a No Observed Effect Concentration (NOEC) or a No Observed Adverse Effect Concentration (NOAEC) (Raybould 2012). For the proteins in focus for this work, NOECs were determined based on one dose corresponding to a level many times higher than the EEC. The exposure (in some cases, the EEC) is then compared to the NOEC (NOEC ÷ exposure) to provide a quantitative value from which risk may be judged, referred to as a margin of exposure (MoE) (Raybould 2012). A MoE equal to or greater than one would indicate negligible risk from exposure in a field setting (Raybould 2012).

The framework for ecological risk assessment of a stack encourages extrapolation from risk assessment of component single events when reasonable and when proof of no change may be available. As stated in the previous section, corroboration of the hypothesis that protein
concentrations are not greater in the stack would provide reasonable justification for applying the EECs and MoEs from risk assessment of the single events to a stack.

**Increasing regulatory requirements: stack vs. single events**

Comparison of transgenic protein concentrations in stacked plants to those in component single event plants is theoretically a simple study to conduct. Plants of each component single event and the stack are grown together in a field trial with replication, and tissue samples are collected and analyzed to quantify the abundance of the transgenic proteins. Statistical comparisons test the null hypothesis that protein concentrations in the stack are no different than those of the component single event. Over the years, regulatory agencies have requested that this experiment include additional parameters, including: analysis of tissue types collected at multiple growth stages, and replication of the field experiment at multiple locations. Additionally, a high level of scrutiny has been applied by some regulatory agencies when comparisons of transgenic protein concentrations between a stack and the component single events result in statistical significance without considering relevance to risk assessment.

**Meta-analysis Objective**

The objective of this meta-analysis was to review the results from multiple studies conducted to compare transgenic protein concentrations between several stacks and component single events with an emphasis on statistical significance in regard to its utility in testing the hypothesis of no increase in EECs. Another objective was to evaluate the mean protein concentrations in relation to corresponding protein and tissue-specific EECs.
MATERIALS AND METHODS

Study-specific materials and methods

Samples for protein expression analysis were collected from plants in five replicate plots for each of the stack and the component single events; all arranged in a randomized complete block design within each field trial. Maize plants of the stack and the component single events grown for each field trial were of the same genetic background. Multiple tissue types at multiple growth stages were collected and analyzed for each study. The types of tissues collected were fairly consistent over all studies. The types of plant tissues collected overall included leaves, roots, pollen, and kernels at various stages in development (Table 1). The field trials were maintained according to normal agricultural practices for the region, including the use of pesticides necessary to maintain plant health.

Table 1. Summary of maize plant tissue samples collected across studies

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Growth stagea</th>
<th>Sample description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>whorl, V9-V12, R1, &amp; R6</td>
<td>All healthy leaves from one plant</td>
</tr>
<tr>
<td>Roots</td>
<td>whorl, V9-V12, R1, &amp; R6</td>
<td>All roots from one plant excluding above-ground brace roots</td>
</tr>
<tr>
<td>Pollen</td>
<td>R1</td>
<td>Pooled from multiple plants</td>
</tr>
<tr>
<td>Kernels</td>
<td>R6 &amp; senescence</td>
<td>All kernels from the primary ear of one plant</td>
</tr>
</tbody>
</table>

a Abendroth 2011

Each sample was put directly on dry ice immediately after removal from the maize plant and stored frozen. Frozen samples were ground using a commercial food processor with dry ice.

Proteins were extracted by homogenization in an appropriate buffer and each extract was analyzed by an Enzyme-Linked Immunosorbent Assay (ELISA) specific for a target protein. The concentration of each protein was interpolated from a standard curve and then converted to microgram (µg) of protein per gram (g) of sample. Data were available on the basis of both fresh weight and dry weight. Concentrations were converted between fresh weight and dry weight bases using the moisture content percentage for each sample. Analysis of variance was used
within each study to compare mean protein concentrations in the combined events product with the corresponding single event on a dry-weight basis for each tissue type and growth stage. In each analysis, the statistical significance of the stack/event effect was determined using a standard F-test at the customary alpha level of 0.05.

**Meta-analysis materials and methods**

Data used to perform this meta-analysis were sourced from 11 different studies with field trials in four different countries conducted on six different maize stacks. These maize stacks included various combinations of the transgene traits from plants derived from transformation events: Bt11, MIR604, MIR162, and GA21 (Table 2). These particular stacks were chosen for this meta-analysis to provide insight into trends that may be recognized with data related to multiple instances of specific trait combinations (Table 3).

Each stack was produced by conventional breeding of various combinations of maize lines derived from the individual transformation events: Bt11, MIR162, MIR604, and GA21 maize. Bt11 maize produces a truncated Cry1Ab insect-control protein, which has activity against certain lepidopteran pests, and a phosphinothricin acetyltransferase (PAT) protein, which confers tolerance to herbicide products containing glufosinate. MIR162 maize produces a Vip3Aa20 protein, for control of certain lepidopteran pests, and a phosphomannose isomerase (PMI) protein, which is a selectable marker enabling transformed plant cells to utilize mannose as a primary carbon source. MIR604 maize produces a modified Cry3A protein (mCry3A), which has activity against certain coleopteran pests, and a PMI protein. GA21 maize produces a double-mutated 5-enol pyruvylshikimate-3-phosphate synthase protein (mEPSPS), which confers tolerance to herbicide products containing glyphosate.
In accordance with a problem formulation regarding environmental and ecological risk assessment, only the data related to the insecticidal proteins (Cry1Ab, mCry3A, Vip3Aa20) were included in this meta-analysis. The mode of action of those insecticidal proteins results in toxicity to certain insects. Therefore, it is plausible to test the hypothesis of no effects to NTOs for those proteins. Therefore, across-study analysis of data for PAT, PMI, and mEPSPS proteins was not performed. Cry1Ab and mCry3A concentrations generated from the comparative protein expression studies for the stacks Bt11 × GA21 and MIR604 × GA21 were also included in the meta-analysis. These two stacks did not include a combination of two or more insecticidal traits. However, these were included in the meta-analysis for evaluation of potential interaction as some regulatory agencies require this test regardless of trait function (e.g., insecticidal, herbicide tolerance, etc.).

**Table 2. Summary of Comparative Protein Expression Studies**

<table>
<thead>
<tr>
<th>Stack</th>
<th>Field trial locations (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bt11 × MIR162 × MIR604 × GA21</td>
<td>Illinois (2006)</td>
</tr>
<tr>
<td>Bt11 × MIR162 × GA21</td>
<td>Illinois (2006)</td>
</tr>
<tr>
<td>Bt11 × MIR604</td>
<td>Illinois (2005)</td>
</tr>
<tr>
<td>Bt11 × GA21</td>
<td>Illinois (2005), Romania (2008), Spain (2008), RSA^ (2009)</td>
</tr>
<tr>
<td>MIR604 × GA21</td>
<td>Illinois (2005)</td>
</tr>
</tbody>
</table>

^RSA = Republic of South Africa
Table 3. Number of combined events products with combinations of specific insecticidal proteins available from data sourced for this meta-analysis

<table>
<thead>
<tr>
<th>Events</th>
<th>Possible combinations</th>
<th>No. of studies testing specific combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bt11, MIR162</td>
<td>Cry1Ab, Vip3Aa20</td>
<td>2</td>
</tr>
<tr>
<td>Bt11, MIR604</td>
<td>Cry1Ab, mCry3A</td>
<td>5</td>
</tr>
<tr>
<td>Bt11, GA21</td>
<td>Cry1Ab, mEPSPS*</td>
<td>9</td>
</tr>
<tr>
<td>Bt11, MIR162, MIR604</td>
<td>Cry1Ab, Vip3Aa20, mCry3A</td>
<td>1</td>
</tr>
<tr>
<td>MIR162, MIR604</td>
<td>Vip3Aa20, mCry3A</td>
<td>1</td>
</tr>
<tr>
<td>MIR162, GA21</td>
<td>Vip3Aa20, mEPSPS*</td>
<td>2</td>
</tr>
<tr>
<td>MIR604, GA21</td>
<td>mCry3A, mEPSPS*</td>
<td>5</td>
</tr>
<tr>
<td>Bt11, MIR162, MIR604, GA21</td>
<td>Cry1Ab, Vip3Aa20, mCry3A, mEPSPS*</td>
<td>1</td>
</tr>
</tbody>
</table>

* Concentrations of mEPSPS were not included in the meta-analysis as the focus was on insecticidal proteins.

Each of the field trials were conducted in a USA ‘Corn Belt’ location that satisfied regulatory requirements for cultivation approvals in the USA and in Canada, and to obtain import approvals from key maize grain importing countries. Similar studies were conducted for the two stacks, Bt11 × MIR604 × GA21 and Bt11 × GA21, in which the field trials were located in two separate European field trial sites and one located in The Republic of South Africa (Table 2).

Measured concentrations of each insecticidal protein in various maize tissues were compiled from all studies (Table 2). The EECs determined for the insecticidal protein produced by each of the Bt11 (US EPA 2010), MIR604 (Raybould et al 2007), and MIR162 (Raybould et al 2011) maize events were compared to the mean concentrations on a fresh-weight basis. The EECs were taken directly as are cited in those published papers with the exception of Cry1Ab (Bt11 maize). A published work from which to source tissue-specific Cry1Ab EECs was not available for this meta-analysis. Therefore, fresh-weight based Cry1Ab concentrations were sourced from an unpublished report submitted to the EPA to support the risk assessment of Bt11 maize (Privalle 2003). The highest mean concentration reported for each tissue type by Privalle...
2003 was used to represent each tissue-specific EEC for the Cry1Ab protein. The report by Privalle was cited in the Biosafety Registration Document (BRAD) for Cry proteins published by the US EPA in 2010 (Privalle 2003; MRID# 45879803 [US EPA 2010]). The Cry1Ab concentrations reported in this 2010 BRAD were sourced from one of the comparative studies (stack vs. single event) included in the meta-analysis; therefore, not appropriate to use as benchmarks in the meta-analysis.

RESULTS

Assessment of differences - stack vs. single event

This meta-analysis reviewed results from 11 separate studies in which transgenic protein concentrations in plant tissues of a maize stack were measured and compared to those of corresponding component single event maize. Over all these studies, 110 separate statistical comparisons were performed (Figures 1-11). The statistical tests were performed within each regulatory study (unpublished data); not within this meta-analysis.
Figure 1. Comparison of Cry1Ab concentrations in leaves of Bt11 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the Cry1Ab Estimated Environmental Concentration for Bt11 maize

**Upper graph:** Box plots within vertical gridlines represent summary statistics of Cry1Ab concentrations and comparison in leaves of Bt11 hybrid maize to those of the stack shown, each performed on a dry-weight basis (DW). Comparisons significantly different at $P < 0.05$ are indicated by orange shading of the interquartile ranges; from left to right: $P = 0.035, 0.037, 0.039, 0.05$.

**Lower graph:** Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for Cry1Ab in Bt11 maize leaves. The EEC is the maximum mean of Cry1Ab concentration (22.02 µg/g FW) in Bt11 maize leaves at a particular growth stage and location (Privalle 2003; unpublished [cited in US EPA 2010]).

**Growth stages:** V9–V12 = vegetative stage $k$: $k$th leaf collar visible; R1 = reproductive stage one: silking; R6 = reproductive stage six: physiological maturity (Abendroth 2011).
Figure 2. Comparison of Cry1Ab concentrations in roots of Bt11 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the Cry1Ab Estimated Environmental Concentration for Bt11 maize

**Upper graph:** Box plots within vertical gridlines represent summary statistics of Cry1Ab concentrations and comparison in roots of Bt11 hybrid maize to those of the stack shown; each performed on a dry-weight basis (DW). Comparisons significantly different at $P < 0.05$ are indicated by orange shading of the interquartile ranges; from left to right: $P = 0.016, 0.031, 0.034, 0.043, 0.005$.

**Lower graph:** Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for Cry1Ab in Bt11 maize roots. The EEC is the maximum mean of Cry1Ab concentration (4.15 µg/g FW) in Bt11 maize roots at a particular growth stage and (Privalle 2003; unpublished [cited in US EPA 2010]).

**Growth stages:** V9–V12 = vegetative stage $k$: $k$th leaf collar visible; R1 = reproductive stage one: silking; R6 = reproductive stage six: physiological maturity (Abendroth 2011).
Figure 3. Comparison of Cry1Ab concentrations in kernels of Bt11 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the Cry1Ab Estimated Environmental Concentration for Bt11 maize

**Upper graph:** Box plots within vertical gridlines represent summary statistics of Cry1Ab concentrations and comparison in kernels of Bt11 hybrid maize to those of the stack shown; each performed on a dry-weight basis (DW). Comparisons significantly different at $P < 0.05$ are indicated by orange shading of the interquartile ranges; from left to right: $P = 0.017$, $0.029$.

**Lower graph:** Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for Cry1Ab in Bt11 maize kernels. The EEC is the maximum mean of Cry1Ab concentration (1.56 µg/g FW) in Bt11 maize kernels at a particular growth stage and location (Privalle 2003; unpublished [cited in US EPA 2010]).

**Growth stages:** R6 = reproductive stage six: physiological maturity (Abendroth 2011).
Figure 4. Comparison of Cry1Ab concentrations in pollen of Bt11 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the Cry1Ab Estimated Environmental Concentration for Bt11 maize

Upper graph: Box plots within vertical gridlines represent summary statistics of Cry1Ab concentrations and comparison in kernels of Bt11 hybrid maize to those of the stack shown; each performed on a dry-weight basis (DW). None of these comparisons were not significantly different at $\alpha = 0.05$. ANOVA was not conducted for comparison of Cry1Ab in Bt11 × GA21 and Bt11 maize pollen because the sample size was too small. The horizontal lines for the Bt11 × GA21 and Bt11 maize comparison each represent the Cry1Ab concentration measured from a pollen sample pooled from 10 plants.

Lower graph: Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for Cry1Ab in Bt11 maize pollen. The EEC is the maximum mean of Cry1Ab concentration (0.08 µg/g FW) in Bt11 maize pollen at a particular location (Privalle 2003; unpublished [cited in US EPA 2010]). The vertical bars for the Bt11 × GA21 and Bt11 maize comparison each represent the Cry1Ab concentration measured from a pollen sample pooled from 10 plants.
Figure 5. Comparison of mCry3A concentrations in leaves of MIR604 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the mCry3A Estimated Environmental Concentration for MIR604 maize

Upper graph: Box plots within vertical gridlines represent summary statistics of mCry3A concentrations and comparison in leaves of MIR604 hybrid maize to those of the stack shown; each performed on a dry-weight basis (DW). Comparisons significantly different at $P < 0.05$ are indicated by orange shading of the interquartile ranges; from left to right: $P = <0.001$, 0.01, 0.021, 0.05.

Lower graph: Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for mCry3A in MIR604 maize leaves. The EEC is the maximum mean of mCry3A concentration (10.14 µg/g FW) in MIR604 maize leaves at a particular growth stage and location (Raybould 2007).

Growth stages: V9–V12 = vegetative stage $k$: $k$th leaf collar visible; R1 = reproductive stage one: silking; R6 = reproductive stage six: physiological maturity (Abendroth 2011).
Figure 6. Comparison of mCry3A concentrations in roots of MIR604 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the mCry3A Estimated Environmental Concentration for MIR604 maize

**Upper graph:** Box plots within vertical gridlines represent summary statistics of mCry3A concentrations and comparison in roots of MIR604 hybrid maize to those of the stack shown; each performed on a dry-weight basis (DW). Comparison significantly different at $P < 0.05$ is indicated by orange shading of the interquartile ranges; $P = 0.009$.

**Lower graph:** Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for mCry3A in MIR604 maize roots. The EEC is the maximum mean of mCry3A concentration (4.55 µg/g FW) in MIR604 maize roots at a particular growth stage and location (Raybould 2007).

**Growth stages:** V9–V12 = vegetative stage $k$: $k$th leaf collar visible; R1 = reproductive stage one: silking; R6 = reproductive stage six: physiological maturity (Abendroth 2011).
Figure 7. Comparison of mCry3A concentrations in kernels of MIR604 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the mCry3A Estimated Environmental Concentration for MIR604 maize

Upper graph: Box plots within vertical gridlines represent summary statistics of mCry3A concentrations and comparison in kernels of MIR604 hybrid maize to those of the stack shown; each performed on a dry-weight basis (DW). None of these comparisons were not significantly different at $\alpha = 0.05$.

Lower graph: Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for mCry3A in MIR604 maize kernels. The EEC is the maximum mean of mCry3A concentration (1.54 µg/g FW) in MIR604 maize kernels at a particular growth stage and location (Raybould 2007).
Figure 8. Comparison of Vip3Aa20 concentrations in leaves of MIR162 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the Vip3Aa20 Estimated Environmental Concentration for MIR162 maize

**Upper graph:** Box plots within vertical gridlines represent summary statistics of Vip3Aa20 concentrations and comparison in leaves of MIR162 hybrid maize to those of the stack shown; each performed on a dry-weight basis (DW). None of these comparisons were significantly different at $\alpha = 0.05$.

**Lower graph:** Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for Vip3Aa20 in MIR162 maize leaves. The EEC is the maximum mean of Vip3Aa20 concentration (56.56 $\mu$g/g FW) in MIR162 maize leaves at a particular growth stage and location (Raybould 2010).

**Growth stages:** R1 = reproductive stage one: silking; (Abendroth 2011).
Figure 9. Comparison of Vip3Aa20 concentrations in roots of MIR162 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the Vip3Aa20 Estimated Environmental Concentration for MIR162 maize

**Upper graph:** Box plots within vertical gridlines represent summary statistics of Vip3Aa20 concentrations and comparison in roots of MIR162 hybrid maize to those of the stack shown; each performed on a dry-weight basis (DW). None of these comparisons were significantly different at $\alpha = 0.05$.

**Lower graph:** Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for Vip3Aa20 in MIR162 maize roots. The EEC is the maximum mean of Vip3Aa20 concentration (6.2 $\mu$g/g FW) in MIR162 maize roots at a particular growth stage and location (Raybould 2010).
Figure 10. Comparison of Vip3Aa20 concentrations in kernels of MIR162 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the Vip3Aa20 Estimated Environmental Concentration for MIR162 maize

**Upper graph:** Box plots within vertical gridlines represent summary statistics of Vip3Aa20 concentrations and comparison in kernels of MIR162 hybrid maize to those of the stack shown; each performed on a dry-weight basis (DW). None of these comparisons were significantly different at $\alpha = 0.05$.

**Lower graph:** Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for Vip3Aa20 in MIR162 maize kernels. The EEC is the maximum mean of Vip3Aa20 concentration (30.9 $\mu$g/g FW) in MIR162 maize kernels at a particular growth stage and location (Raybould 2010).
Figure 11. Comparison of Vip3Aa20 concentrations in pollen of MIR162 maize and related stack maize over multiple studies and growth stages on a DW basis and mean concentrations on a FW basis compared to the Vip3Aa20 Estimated Environmental Concentration for MIR162 maize

Upper graph: Box plots within vertical gridlines represent summary statistics of Vip3Aa20 concentrations and comparison in pollen of MIR162 hybrid maize to those of the stack shown; each performed on a dry-weight basis (DW). Comparison significantly different at $P < 0.05$ is indicated by orange shading of the interquartile ranges; $P = 0.006$.

Lower graph: Mean concentrations on a FW basis are shown in relation to the Estimated Environmental Concentration (EEC) set for Vip3Aa20 in MIR162 maize pollen. The EEC is the maximum mean of Vip3Aa20 concentration (47.85 µg/g FW) in MIR162 maize pollen at a particular location (Raybould 2010).
Over all three proteins, 17 significant differences were observed out of 110 comparisons of concentrations between a stack maize and corresponding component single event maize. Approximately 71% of those significant differences (12 of 17) resulted from higher concentrations in the stack compared to those of the corresponding component single event. A large portion of the significant differences (12 of 17) were less than alpha at 0.05, but greater than alpha at 0.01 (Table 4). At the alpha level of 0.05 with 110 comparisons, five or six comparisons would be expected to show a significant difference due solely to random chance.

Although each study may have included a few significant differences at alpha 0.05 resulting from those statistical comparisons, none were consistently observed across proteins, tissues types, and growth stages.

### Table 4. Frequency of statistical comparisons with results significantly different between stack and corresponding component single event

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tissue type</th>
<th>No. of tests</th>
<th>No. of Significant Differences (α = 0.05)</th>
<th>No. of Significant Differences (α = 0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stack ≠ Single</td>
<td>Stack &gt; Single</td>
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<tr>
<td>Cry1Ab</td>
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<td>27</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>24</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Kernels</td>
<td>12</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Pollen</td>
<td>3</td>
<td>0</td>
<td>0</td>
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<tr>
<td>mCry3A</td>
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<td>4</td>
<td>3</td>
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<tr>
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<td>Roots</td>
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<td>1</td>
<td>1</td>
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<tr>
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<td>0</td>
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<tr>
<td></td>
<td>Roots</td>
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<td>0</td>
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<td></td>
<td>Pollen</td>
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<td>1</td>
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<tr>
<td>TOTALS</td>
<td></td>
<td>110</td>
<td>17</td>
<td>12</td>
</tr>
</tbody>
</table>
Transgenic protein concentrations in stack relative to EECs

Several mean concentrations on a fresh-weight basis were higher than the corresponding EEC set for risk assessment of the corresponding component single event. These mean concentrations were higher despite statistical comparisons (in some cases) between stack and single events indicating no significant difference at alpha 0.05 (bar graphs in Figures 1-11, except for Figures 7 and 8). Over all tissue types, only 20 out of 134 mean Cry1Ab concentrations (including both Bt11 and stacks) were greater than the EEC set for risk assessment of Bt11 maize. The greatest of those 20 means was for Cry1Ab in leaves, which was 14 µg/g higher than the corresponding EEC (22.02 µg/g FW [US EPA 2010]); less than a 2-fold increase (Figure 1). Fewer comparisons between a stack and component single event were available for mCry3A and Vip3Aa20. However, a portion of the means overall were higher than corresponding EECs: 21 out of 68 (31%; Figures 5-7) for mCry3A and ten out of 24 (42%; Figures 8-11) for Vip3Aa20. As observed for Cry1Ab, the magnitude of means greater than the corresponding EECs for both mCry3A and Vip3Aa20 were low. The greatest increase in means of mCry3A was around 1.5 fold higher than the corresponding EEC in MIR604 maize leaves (Figure 5). The greatest increase in means of Vip3Aa20 was close to 4-fold higher than the corresponding EEC in MIR162 maize kernels (Figure 10).

Further analyses of the concentrations against EECs were performed using prediction intervals. Prediction intervals were computed using the concentrations for each protein by tissue type and growth stage scenario. These prediction intervals were generated from a margin of error computed with 99% confidence to include the variability explained by environmental factors (field location and different genetics). Five hypothetical samples were included in the formula to match the replication used in typical protein expression studies for single event crops.
The EECs for each of the proteins were set as the highest mean from a sample size of five individual plants at a single location. Prediction intervals were computed to match how the EECs were set. Therefore, each result on a protein, tissue type, and growth stage represents an expectation that the average concentration from a sample size of five future observations should fall within the computed prediction interval. Data from single events and stacks were included in each computation of a prediction interval as the results from this meta-analysis concluded that concentrations in the stacks and the corresponding component single events were similar overall.

The equation for computation of the prediction intervals (SAS 2013) is shown below with an example using five future samples \( m \) on the mean \( \bar{x} \), sample size \( n \), and standard deviation \( s \) for Cry1Ab in whorl leaves (Table 5).

\[
\bar{x} \pm \frac{t_{\frac{1-\alpha}{2},n-1}}{\sqrt{n}} \sqrt{\frac{s^2}{m}}
\]

example: \( 18.2 \pm \frac{t_{1-0.01,136-1}}{\sqrt{10.1}} \sqrt{\frac{1}{5} + \frac{1}{136}} \)

Mean \( \bar{x} = 18.2 \), \( t = t \)-statistic, \( \alpha = 0.01 \) (99% confidence), \( n = 136 \), \( s \) (standard deviation) = 10.1 and, \( m \) (number of future observations) = 5. A result using this formula and a \( t \)-statistic from a published table of values for the student’s \( t \) distribution will not match exactly with those computed and reported in Table 5. The prediction intervals reported in Table 5 were computed using JMP version 11 statistical computer software in which the value of the \( t \)-statistic is more accurately achieved. Also, prediction intervals were generated with transformed values (log\(_{10}\)) because sampling distributions were not of the normal distribution in every case.

The growth stage for each tissue type with the highest mean was used to compare against the corresponding tissue-specific EEC for each protein. The EECs for Cry1Ab and mCry3A were close to the mean in most cases (except for mCry3A in kernels), and therefore, fell comfortably within prediction intervals. The Vip3Aa20 EEC was comfortably within the lower portion of the interval for roots. However, the Vip3Aa20 EEC was just barely in within the upper end of the interval for leaf, and outside the lower end of the interval for both kernels and pollen. These results for Vip3Aa20 are likely due to the smaller population of observations from which to compute a prediction interval as compared to those available for Cry1Ab and mCry3A.
Table 5. Prediction intervals for the mean of Cry1Ab, mCry3A, and Vip3Aa20 protein concentrations with 99% confidence using five future samples

<table>
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<tr>
<th>Protein</th>
<th>Tissue Type</th>
<th>Growth Stage</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>Log(_{10}) Lower</th>
<th>Log(_{10}) Upper</th>
<th>µg/g FW Lower</th>
<th>µg/g FW Upper</th>
<th>EEC</th>
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<td>7.66</td>
<td>31.4</td>
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<td></td>
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<td>V9-V12</td>
<td>20</td>
<td>8.04</td>
<td>1.64</td>
<td>0.37</td>
<td>0.770</td>
<td>1.024</td>
<td>5.89</td>
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<tr>
<td></td>
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<td>R1</td>
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</table>

The prediction interval (µg/g FW) for each protein and tissue type with the greatest upper end over growth stages is emphasized with bold italicized text.

All prediction intervals were generated with transformed values (log\(_{10}\)) because sampling distributions were not of the normal distribution in every case. The prediction intervals were transformed back (10\(^{x}\)) for concentrations in µg/g FW.

b Raybould 2007.
c Raybould 2010.

DISCUSSION

The conclusion within each of these studies was that the transgenic protein concentrations were generally similar between the stack and corresponding component single event, indicating a lack of interaction affecting the production of the transgenic proteins due to combination of the
traits by conventional breeding. The meta-analysis of the statistical comparisons further confirms the in-study conclusions for each of the proteins analyzed (upper graphs Figures 1-11).

However, mean protein concentrations were higher than corresponding EECs in several cases (lower graphs in Figures 1-11). In most of those cases, both means for the stack and for the corresponding single event were higher than the previously set EEC. This indicates that the increase was most likely not due to stacking and more likely due to differences in random influencing factors across studies; such as differences in genetic backgrounds and differences in environmental growing conditions.

Although many mean concentrations from these comparative protein expression studies were higher than EECs used for risk assessment of the single events, the majority were lower. Furthermore, only one mean was higher than a corresponding EEC by close to 4-fold, and none of the rest were higher by more than 2-fold. This provides confidence that those EECs used for single events are appropriate for use in ecological risk assessment of future products producing the insecticidal proteins encoded by any combination of those genes.

**Considerations to modify testing requirements**

When trait stacking by conventional breeding first began, the regulatory community was uncertain about the potential impacts on expression for insecticidal proteins. Now we have many tests of hypothesis that indicate effects on protein expression due to stacking are not likely. Therefore, the testing strategy for protein expression in stacks could be reconsidered.

This meta-analysis demonstrates a lack of an effect on protein production due to stacking for the Cry1Ab, mCry3A, and Vip3Aa20 proteins with multiple tests. Therefore, one may conclude that additional expression studies on a new stack with any combination of Bt11, MIR604, MIR162, and GA21 is no longer necessary to evaluate or determine EECs. These
results may also be useful in predicting whether that hypothesis is worth testing for combination of other genes encoding similar proteins.

If the concern does exist through problem formulation that production of transgenic proteins may increase due to interactions with other transgenic proteins combined through conventional breeding, testing could address uncertainty. The uncertainty should be related to risk assessment for which a possibility exists to erode a margin of exposure by a higher EEC. In this scenario, the hypothesis of “no increase in EECs” could be tested simply by analyzing only EEC-corresponding plant samples of the particular stack crop. Analysis of corresponding component single event crop samples to make comparisons to a corresponding stack would not be necessary. Measured concentrations of the transgenic proteins in stack plant samples may then be compared to historical data or a previously set corresponding EEC. Higher concentrations would not automatically result in a concerning outcome, as the next step would be to compare to a corresponding hazard-related dose or a refinement of exposure. Measured transgenic protein concentrations in stacks may be higher than previously set EECs; however, those EECs are likely several folds lower than corresponding NOECs related to valued and potentially susceptible species.

CONCLUSION

Across-study analysis of expression studies conducted to measure and compare transgenic protein concentrations in tissues of the various stacked maize lines to those in corresponding component single event maize lines reaffirm the hypothesis that transgenic protein concentrations do not increase due to stacking by conventional breeding. The multitude of tests that corroborate the stated hypothesis indicate that further tests would not provide new
information to inform risk assessment on stacks with any combination of the events Bt11, MIR604, MIR162, or GA21.

Additionally, this meta-analysis revealed that the EECs set to help define worst-case scenario exposure for risk assessment of single event crops were set at appropriate levels for risk assessment. This was confirmed by comparison to the protein corresponding measured concentrations sourced for this meta-analysis that incorporate variability associated with random influencing factors, such as different environmental growing conditions and genetic backgrounds. The presented prediction intervals computed for each EEC corresponding scenario provide an example of how to include that variability when considering future observations.
REFERENCES


