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## RESEARCH PAPER

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# Transgene-product expression levels in genetically engineered breeding stacks are equivalent to those of the single events

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**ABSTRACT.** Transgene product expression levels are measured in genetically engineered (GE) crops containing single transformation events and the measured expression levels are then utilized in food, feed, and environmental safety assessments as part of the requirements for de-regulation of the event. Many countries also require measurement of expression levels and safety assessments for GE breeding stacks, even though the breeding stacks are composed of single events that have been previously assessed. Transgene product expression levels were measured in tissues of maize, soybean, and cotton breeding stacks and each of their component single events. Expression levels in the breeding stacks were plotted against expression levels in the single events to quantify the ability of the single events to predict transgene product expression levels in the breeding stacks. These results indicate that transgene product expression levels in single events are a reliable indicator of expression levels in breeding stacks. Based on these results it is concluded that safety assessments for breeding stacks can be conducted using transgene product expression levels from single events.

**KEYWORDS.** feed and food environmental risk assessment; coefficient of identity; genetically engineered (GE) breeding stacks; genetically modified organisms (GMOs); safety assessment; Transgene expression

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## INTRODUCTION

Crops that are genetically engineered (GE) to contain insect protection and herbicide tolerance traits are invaluable management tools, and the use of GE crops is becoming increasingly prevalent in modern agriculture. In 2016, more than 185 million hectares of GE crops were produced in 26 countries, which represents a nearly 110 fold increase over the 1.7 million hectares that were produced in 1996 (the first year of GM crop commercialization).<sup>1</sup> GE crop technology can reduce the environmental impacts and therefore increase the sustainability of crop production. The use of chemical insecticides in crops has decreased as a result of the insect protection that is provided by GE insect traits, and the use of non-selective herbicides that control a vast array of weed species when applied to GE herbicide-tolerant crops has resulted in fewer herbicide applications and a reduced number of herbicide active ingredients applied.<sup>2,3</sup>

Combining two or more GE events using traditional plant breeding techniques produces a breeding stack that contains GE traits from each single event with no new recombinant DNA sequence insertion.<sup>4</sup> Breeding stacks are desirable to crop producers because they allow multiple traits to be contained within a single crop variety (e.g., insect protection and herbicide tolerance) and/or multiple forms of the same type of trait to be contained within a single crop variety (e.g., insect protection from multiple modes of action or tolerance to multiple herbicides).

Regulations currently require studies (referred to herein as “expression studies”) to be conducted where the GE crop is grown in replicate plots at multiple field sites and samples of various crop tissues are collected throughout the growing season. The expression of transgene products in the crop tissues is evaluated in laboratory assays, and the measured expression levels are then used to support a safety assessment. The fundamental principle of safety assessment is that risk is the product of hazard (toxicity) and exposure. To date, there are no known cases where a transgene product has posed any food or feed hazard. Because no hazard is present, there is

consequently no appreciable risk associated with currently commercialized GE crops in food and feed. Expression studies and safety assessments are required for breeding stacks in several geographies<sup>5</sup>, even when the breeding stacks consist of single events that have already been assessed and negligible risk has previously been concluded. When there is no scientific rationale to support a hypothesis that transgene expression would be altered in breeding stacks compared with single events, expression studies and safety assessments with breeding stacks are not scientifically justified.

The objective of this research was to determine if the expression of transgenes in the maize single events MON 89034, DAS-Ø15Ø 7-1, SYN-IR162-4, and NK603, the soybean single events DAS-444Ø6-6 (developed jointly by Dow AgroSciences and M.S. Technologies, LLC) and DAS-81419-2, and the cotton single events DAS-21Ø23-5, DAS-24236-5, SYN-IR1Ø2-7, and MON88913 are accurate predictors of transgene product expression in the breeding stacks MON 89034 × DAS-Ø15Ø 7-1 × SYN-IR162-4 × NK603 (maize), DAS-81419-2 × DAS-444Ø6-6 (soybean), and DAS-21Ø23-5 × DAS-24236-5 × SYN-IR1Ø2-7 × MON 88913 (cotton). Expression studies with the maize, soybean, and cotton breeding stacks and all single component events were conducted at field sites in Argentina (2014–2015), Brazil (2014–2015), and the US (2012), respectively. A graph was generated for each transgene product where the expression level of the product in each crop tissue from the single event was plotted on the  $x$  axis and the expression level in the breeding stack was plotted on the  $y$  axis; the ability of the single events to predict expression in the breeding stacks was then quantified using the coefficient of identity ( $I^2$ ), which is based on the percent of variation of the plotted points accounted for by the line of identity ( $y = x$ ). Because grain/seed is the predominant crop tissue that is used for human and animal consumption in food and feed and is the most important tissue for the safety assessment, a plot was also created for each crop where the expression levels of all transgene products in grain/seed in the single events are plotted against those of the breeding stacks. The data analysis methods used

here have been previously used to quantify the capability of expression in single events to predict expression in different breeding stacks in maize and cotton and in the soybean breeding stack DAS-81419-2 × DAS-444Ø6-6 from an expression study conducted in the US.<sup>6</sup> This statistical method has also been used to evaluate the compositional equivalence of maize breeding stacks containing event DAS-Ø15Ø7-1 and non-GE maize, compositional equivalence of soybean event DAS-444Ø6-6 and non-GE soybean, and endogenous allergen level equivalence of DAS-81419-2, DAS-444Ø6-6, DAS-81419-2 × DAS-444Ø6-6, and DAS-68416-4 × MON 89788 soybean and non-GE soybean.<sup>7-9</sup>

## MATERIALS AND METHODS

### GM Breeding Stacks

Maize (MON 89034 × DAS-Ø15Ø 7-1 × SYN-IR162-4 × NK603), soybean (DAS-81419-2 × DAS-444Ø6-6), and cotton (DAS-21Ø23-5 × DAS-24236-5 × SYN-IR1Ø2-7 × MON88913) breeding stacks were chosen to investigate the transgene product

expression levels in the breeding stacks compared with those of the single component events. The proteins expressed in each of these events and the traits they confer are provided in Table 1.

### Field Trials

Field trials with MON 89034 × DAS-Ø15Ø 7-1 × SYN-IR162-4 × NK603 maize and the four single events were conducted during the 2014–2015 growing season at six sites in Argentina (Berdier, El Crisol, Ines Indart, Los Indios, San Pedro, and Tacuari). Field trials with DAS-81419-2 × DAS-444Ø6-6 soybean and the two single events were conducted during the 2014–2015 season at three sites in Brazil (Indianópolis, MG; Montividiu, GO; and Cravinhos, SP). Field trials with DAS-21023-5 × DAS-24236-5 × SYN-IR102-7 × MON 88913 cotton were conducted during the 2012 growing season at six sites in the US (Tallassee, AL; Sycamore, GA; Washington, LA; Fisk, MO; Mebane, NC; East Bernard, TX). Trials for all crops were arranged in a randomized complete

TABLE 1. GE events, corresponding transgene products, and traits conferred.

Crop	GE Event	Transgene Product	Trait
Maize	MON 89034	Cry2Ab2 Cry1A.105	insect resistance
	DAS-Ø15Ø 7-1	Cry1F PAT	insect resistance herbicide tolerance
	SYN-IR162-4	Vip3Aa20 PMI	insect resistance selectable marker
	NK603	CP4 EPSPS	herbicide tolerance
Soybean	DAS-81419-2	Cry1F Cry1Ac PAT	insect resistance insect resistance selectable marker
	DAS-444Ø6-6	AAD-12 2M EPSPS PAT	herbicide tolerance
Cotton	DAS-21Ø23-5	Cry1Ac PAT	insect resistance selectable marker
	DAS-24236-5	Cry1F PAT	insect resistance selectable marker
	SYN-IR1Ø2-7	Vip3Aa19 APH4	insect resistance selectable marker
	MON88913	CP4 EPSPS	herbicide tolerance

block design; maize and cotton trials contained four replicate blocks at each field site and soybean trials contained three replicate blocks at each field site. Crop tissues that were sampled and analyzed and the growth stages at which samples were collected are provided in figure captions (Figs. 1–4).

### Analytical Methods

Proteins were quantified using ELISA methods validated following Good Laboratory Practices. CP4 EPSPS in maize and cotton and APH4 in cotton were quantified using validated Dow AgroSciences methods with kits

FIGURE 1. Expression of Cry1A.105, Cry2Ab2, Cry1F, and Vip3Aa19 (ng/mg dw) in MON 87427 × MON 89034 × DAS-Ø15Ø7-1 × MON 87411 × DAS-59122-7 × DAS-4Ø278-9 maize (y-axis) and single events (x-axis). The diagonal line represents the line of identity ( $y = x$ ) on both the  $\log_{10}$  transformed and natural scale plots. Tissue types are represented in the plots by the following symbols:  $\square$  = leaf V2-V4,  $\diamond$  = leaf V9,  $+$  = leaf R1,  $\triangle$  = root R1,  $\star$  = pollen R1,  $\circ$  = forage R5, and  $\Delta$  = grain R6.

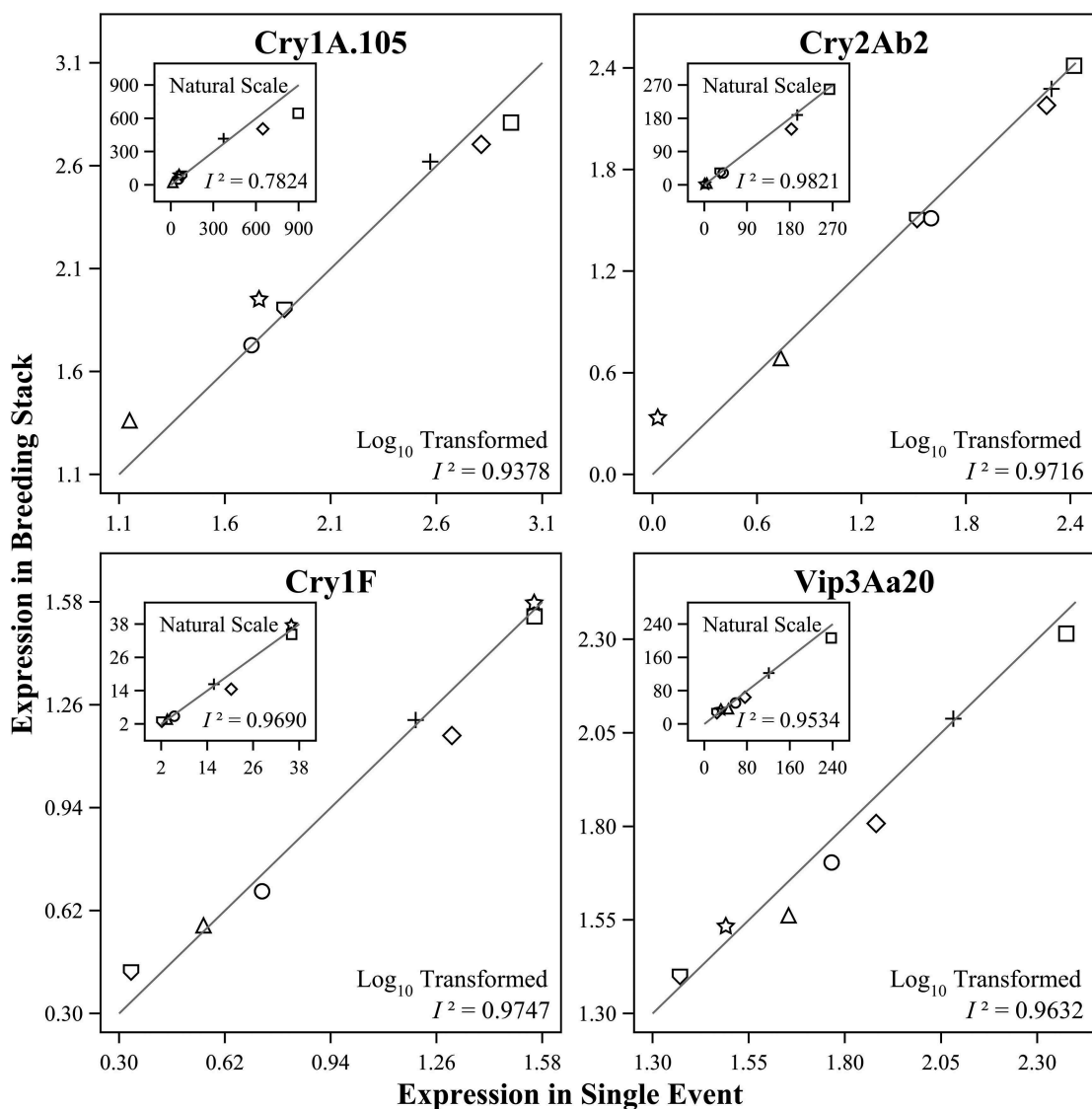
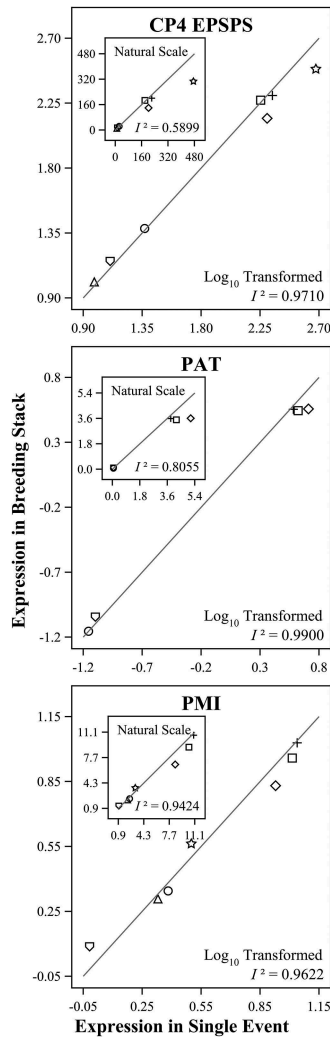
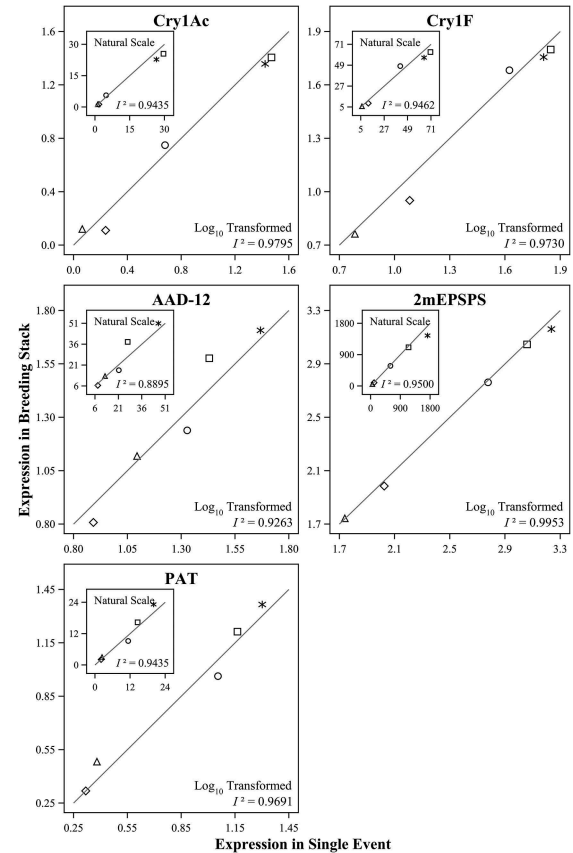


FIGURE 2. Expression of CP4 EPSPS, PAT, and PMI (ng/mg dw) in MON 87427 × MON 89034 × DAS-Ø15Ø7-1 × MON 87411 × DAS-59122-7 × DAS-4Ø278-9 maize (y-axis) and single events (x-axis). The diagonal line represents the line of identity ( $y = x$ ) on both the  $\log_{10}$  transformed and natural scale plots. Tissue types are represented in the plots by the following symbols:  $\square$  = leaf V2-V4,  $\diamond$  = leaf V9,  $+$  = leaf R1,  $\square$  = root R1,  $\star$  = pollen R1,  $\circ$  = forage R5, and  $\Delta$  = grain R6.



purchased from Acadia, LLC (Portland, ME). Cry2Ab2 and Cry1F in maize and PAT in maize, soybean and cotton were quantified using validated Dow AgroSciences methods with kits purchased from Envirologix, Inc

FIGURE 3. Expression of Cry1Ac, Cry1F, AAD-12, 2mEPSPS, and PAT (ng/mg dw) in DAS-81419-2 × DAS-444Ø6-6 soybean (y-axis) and single events (x-axis). The diagonal line represents the line of identity ( $y = x$ ) on both the  $\log_{10}$  transformed and natural scale plots. Tissue types are represented in plots by the following symbols:  $\square$  = leaf V3,  $\star$  = leaf R1,  $\circ$  = forage R3-R4,  $\diamond$  = root R3-R4, and  $\Delta$  = grain R8.



(Portland, ME). Cry1F in cotton and soybean, Vip3Aa19 in cotton, Vip3Aa20 and PMI in maize, and Cry1Ac in cotton and soybean were quantified using validated Dow AgroSciences methods with kits purchased from Romer Labs (Newark, DE). AAD-12 and 2mEPSPS in soybean were quantified using a validated method with ELISA kits produced collaboratively by Dow AgroSciences and M.S. Technologies LLC, West Point, Iowa. Cry1A.105 was quantified using a

FIGURE 4. Expression of Cry1Ac, Cry1F, Vip3Aa19, CP4 EPSPS, and PAT (ng/mg dw) in DAS-21Ø23-5 × DAS-24236-5 × SYN-IR1Ø2-7 × MON 88913 × DAS-8191Ø-7 cotton (y-axis) and single events (x-axis). The diagonal line represents the line of identity ( $y = x$ ) on both the  $\log_{10}$  transformed and natural scale plots. Tissue types are represented in plots by the following symbols: O = leaf, 4-leaf;  $\Delta$  = leaf, first white bloom;  $\star$  = squares, first white bloom;  $\square$  = pollen, early bloom;  $\diamond$  = flower, peak bloom;  $\star$  = bolls, peak bloom;  $\square$  = leaf, first open boll;  $\downarrow$  = root, maturity;  $\square$  = whole plant, maturity;  $\geq$  seed, maturity.

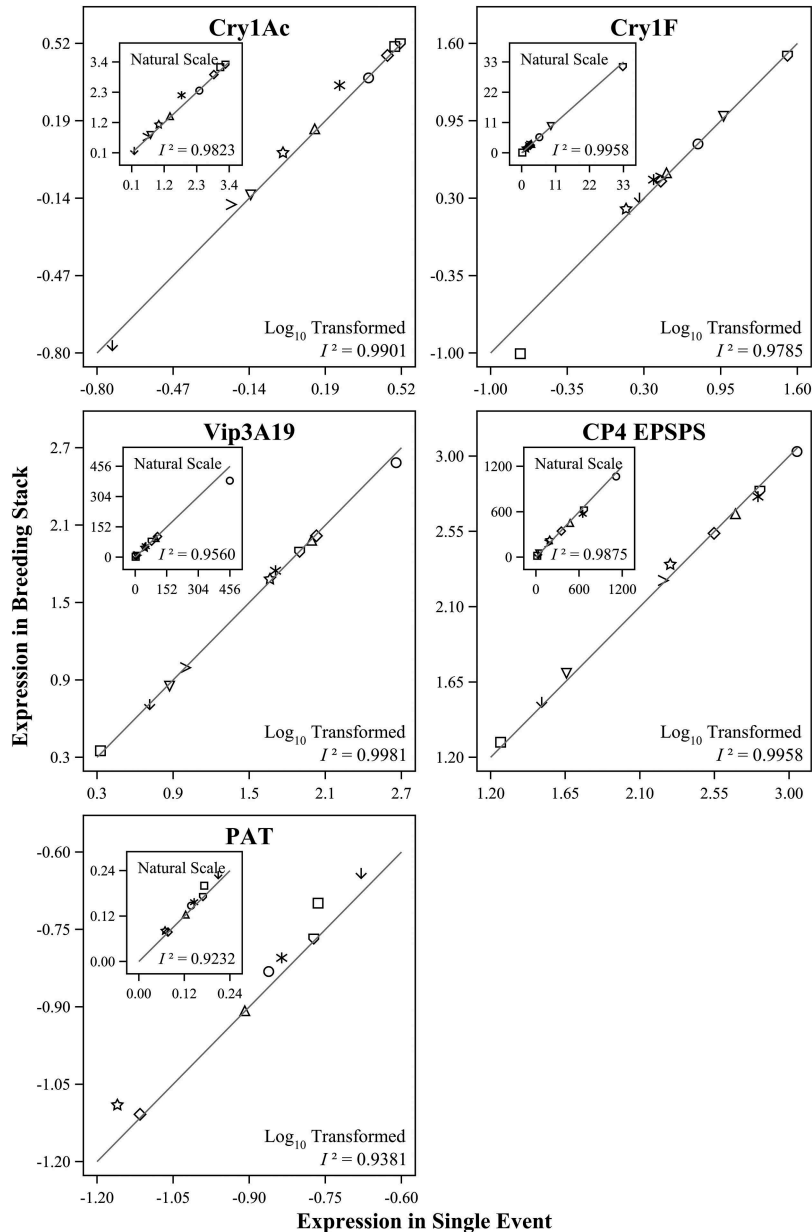
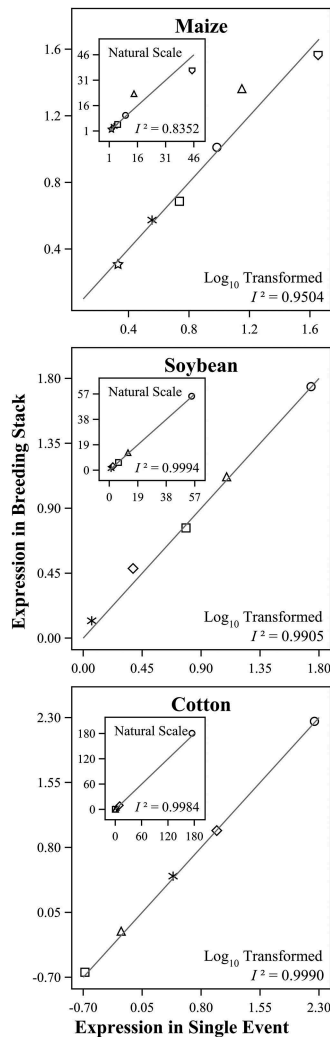


FIGURE 5. Expression of transgene products in grain/seed of maize, soybean, and cotton breeding stacks (y-axis) and single events (x-axis). The diagonal line represents the line of identity ( $y = x$ ) on both the  $\log_{10}$  transformed and natural scale plots. Transgene products in maize are represented in plots by the following symbols: O = CP4 EPSPS,  $\Delta$  = Cry1A.105,  $\star$  = Cry1F,  $\square$  = Cry2Ab2,  $\diamond$  = PAT,  $\star$  = PMI,  $\square$  = VIP3Aa20. Transgene products in soybean are represented by the following symbols: O = 2mEPSPS,  $\Delta$  = AAD-12,  $\star$  = Cry1Ac,  $\square$  = Cry1F,  $\diamond$  = PAT. Transgene products in cotton are represented by the following symbols: O = CP4 EPSPS,  $\Delta$  = Cry1Ac,  $\star$  = Cry1F,  $\square$  = PAT,  $\diamond$  = Vip3Aa20.



validated method following a protocol provided by Monsanto (St. Louis, MO) and kits produced at Dow AgroSciences.

### Data Analysis

For each transgene product in each crop, a plot was created where mean expression levels (expressed as ng/mg dry-weight of tissue) for each transgene product within each tissue in the GE single events were plotted ( $x$  axis) against expression levels in the corresponding breeding stack ( $y$  axis) for each transgene product. An additional plot was created for each crop in the same manner that included all transgene products in grain/seed only. Cotton events DAS-21023-5 and DAS-24236-5 both contain PAT. These events, however, were never commercialized separately; therefore, the two-event breeding stack of these events (DAS-21023-5  $\times$  DAS-24236-5) was treated as a single event for the purposes of this analysis. APH4 results were excluded from the analysis because it was not expressed at detectable levels in the majority of the crop tissues that were evaluated. Additionally, PAT was not expressed at detectable levels in pollen and grain (maize) and in root and whole plant (cotton).

The coefficient of identity ( $I^2$ ) was calculated to quantify the amount of variation in the data that is accounted for by the line of identity ( $y = x$ ).  $I^2$ , which is analogous to calculating the coefficient of determination ( $R^2$ ) for a regression line, is calculated as follows:

$$I^2 = 1 - \frac{\sum_{i=1}^N (y_i - x_i)^2}{\sum_{i=1}^N (y_i - \bar{y})^2}$$

It is noteworthy that  $I^2$  will never exceed  $R^2$  for any given dataset because, unlike a regression line that is fit to the data, the line of identity is fixed ( $y = x$ ). Analyses were conducted with data in the natural scale and with  $\log_{10}$  transformed data.  $\log_{10}$  transformation of the data weighted lower expression values more heavily and therefore helped reduce the skewedness of

the data sets for transgene products that contained large numbers of relatively low results.

## RESULTS

Plotted mean transgene product expression levels generally fell close to the line of identity for all transgene products, indicating similarity in expression between the single events and breeding stacks (Figs. 1–4).

$I^2$  values for transgene products in maize ranged from 0.9378 to 0.9900 ( $\log_{10}$  transformed) and from 0.5899 to 0.9821 (natural scale) (Figs. 1,2). The natural scale  $I^2$  values for CP4 EPSPS, Cry1A.105, and PAT in maize (0.5899, 0.7824, and 0.8055, respectively) were lower than for other comparisons. The predominant contributors to these lower  $I^2$  values were lower expression results in the breeding stack compared with that of the single events for CP4 EPSPS (pollen), cry1A.105 (leaf V2-V4 and leaf V9), and PAT (leaf V9.).

Soybean transgene product  $I^2$  values ranged from 0.9263 to 0.9953 ( $\log_{10}$  transformed) and from 0.8895 to 0.9500 (natural scale) (Fig. 3). One slightly low  $I^2$  value (0.8895) was observed for AAD-12, which can be attributed to higher expression in leaf V3 tissue in the breeding stack compared with the single event.  $I^2$  values for transgene products in cotton were all relatively high and ranged from 0.9381 to 0.9981 ( $\log_{10}$  transformed) and from 0.9323 to 0.9958 (natural scale) (Fig. 4).

When expression levels were plotted from grain/seed only across all transgene products within each crop (Fig. 5),  $\log_{10}$  transformed  $I^2$  values were 0.9504 (maize), 0.9905 (soybean), and 0.9990 (cotton) and natural scale  $I^2$  values were 0.8352 (maize), 0.9994 (soybean), and 0.9984 (cotton) (Fig. 5). The lowest  $I^2$  value that was observed in the natural scale results for maize was driven by Cry1A.105 (expression in breeding stack slightly higher than in single event) and Vip3Aa20 (expression in breeding stack slightly lower than single event).

For all cases where natural scale  $I^2$  values were slightly low, the values improved

substantially when data were  $\log_{10}$  transformed, which weighted the lower expression values more heavily. Differences that were observed that contributed to lower  $I^2$  values were not replicated across all transgene products within a particular tissue or across all tissues within a particular transgene product, and differences were not consistent across products (in some cases expression in the breeding stack was lower than that of the single event and vice versa). Furthermore, the magnitude of these differences in expression that were observed between the breeding stacks and single events is not sufficient to impact any safety assessment made based on single event expression.

## DISCUSSION

The similarity between transgene product expression levels in single events and breeding stacks indicates that expression of transgene products in single events is a reliable predictor of expression in breeding stacks. This was the expected outcome of the research because no scientific rationale exists to support a hypothesis that transgenes would interact with each other in any way to alter expression when multiple transgenic events are included in breeding stacks. This conclusion, which was drawn from this research with the maize, soybean, and cotton breeding stacks MON 89034  $\times$  DAS-Ø15Ø 7-1  $\times$  SYN-IR162-4  $\times$  NK603, DAS-81419-2  $\times$  DAS-444Ø6-6, and DAS-21Ø23-5  $\times$  DAS-24236-5  $\times$  SYN-IR1Ø2-7  $\times$  MON 88913, respectively, concurs with the conclusion of previously reported research that was conducted with the maize, soybean, and cotton breeding stacks MON 87427  $\times$  MON 89034  $\times$  DAS-Ø15Ø7-1  $\times$  MON 87411  $\times$  DAS-59122-7  $\times$  DAS-4Ø278-9, DAS-81419-2  $\times$  DAS-444Ø6-6, and DAS-21Ø23-5  $\times$  DAS-24236-5  $\times$  SYN-IR1Ø2-7  $\times$  MON 88913  $\times$  DAS-8191Ø-7, respectively.<sup>6</sup> It should be noted that the results we report here on the soybean breeding stack DAS-81419-2  $\times$  DAS-444Ø6-6 were generated from field studies conducted in Brazil, while the results reported by



others<sup>6</sup> was conducted in the US. Agreement of these results across geographies further supports the conclusion that transgene product expression in single events is a reliable predictor of expression in breeding stacks.

To date, safety assessments with transgene products have identified no appreciable risks associated with GE crops; however, assessments are still conducted with newly developed transgene products in single events, and in many countries they must be conducted with both single events and breeding stacks. The results reported here and the results reported by others<sup>6</sup> indicate that expression studies for transgene products and subsequent risk assessments are not necessary for breeding stacks because expression levels and safety assessment results from single events can be applied to breeding stacks.

### **DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors declare the following competing financial interests: DC, BF, and RH are employed by Corteva Agriscience™, Agriculture Division of DowDuPont™, which develops and markets transgenic seed.

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