AgBioForum, 17(1): 75-83. ©2014 AgBioForum.

Analysis of the Prevalence of CDC Triffid Transgenic Flax in Canadian Grain Stocks

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CDC Triffid transgenic flax was deregistered in 2001 due to concerns about the effect of its production on offshore markets. A decade after removal of CDC Triffid from the commercial system in Canada, it was detected in grain shipments to Europe. This event resulted in a disruption of trade between Canada and the EU. To demonstrate compliance, the industry in Canada adopted a testing protocol involving testing grain samples (post-harvest) using a RT-PCR test for the construct found in CDC Triffid. This study re-evaluates GM presence in Canadian grain stocks for the updated data set of 2009-2013 using a previously described simulation model to estimate low-level GM presence. The test results were broken down by category (i.e., seed or grain) as well as crop year. For each category of seed or grain, it was determined whether the observed positives deviated from the expected number of false positives (i.e., due to chance alone). In most cases, the number of positive tests was significantly higher than expected due to chance alone. For these categories of seed or grain, a simulation model was applied to the test results to estimate GM contamination levels. The number of positive tests showed a downward trend, indicating removal of transgenic flax from the commercial system. However, low-level GM presence persists in grain stocks. A way forward for the Canadian grain industry is presented, including renewal of seed stocks with reconstituted GM-free varieties.

Key words: CDC Triffid, *Linum usitatissimum*, GMO, seed purity analysis, seed testing, statistical methods, transgenic seed.

Introduction

In April 2009, transgenic flax seeds identified as the genetically modified (GM) flax variety CDC Triffid (McHughen, Rowland, Holm, Bhatty, & Kenaschuk, 1997) were found in two 5,000 tonne shipments of flax grain during preprocessing in Europe. Further, shipments of Canadian flax have tested positive for a transgene in Japan and Brazil (Flax Council of Canada, 2010). CDC Triffid was deregistered in 2001 due to concerns about the effect of production of transgenic flax on export markets. Approximately 4,000 hectares of Triffid were grown by Canadian seed growers across Western Canada and about 5,500 tonnes of Triffid seed were collected during the recall (Ryan & Smyth, 2012). Offshore markets for Canadian flax have not approved GM flax and have no tolerance for its detection in shipments (Flax Council of Canada, 2009). The Flax Council of Canada (2009) has reported detection of widespread, low-level presence of GM flax in commercial flax stocks. Since this detection has been reported, extensive flax seed testing has been instituted in Canada: prior to planting, post-harvest, at initial receptor sites (elevators, railcars), and at grain terminals prior to export. Previous reports (Booker & Lamb, 2012; Lamb & Booker, 2011) have examined the prevalence of CDC Triffid in Canadian flax stocks.

There was interest in re-evaluating GM seed presence for an updated dataset of 2009-2013. This study updates results presented in Booker and Lamb (2012) with more recent testing data provided by the Flax Council of Canada.

Background

Testing of adventitious presence, a critical element of regulatory compliance, is confounded by the practical level of detection of real time PCR assays (0.01% or 1 GM seed in 9,999 conventional seeds) and the large sources of error inherent in taking representative and random samples in large seed lots (Begg, Cullen, Iannetta, & Squire, 2007; Lamb & Booker, 2011). The current testing protocol requires the collection of a 2kg sample of any flax entering the handling system and testing of four 60g subsamples (4×60) for the presence of GM flax (Canadian Grain Commission, 2010). The sampling protocol presumably gives a 95% probability (or 5% error) of detecting 1 GM in 9,999 non-GM flax

Table 1. Seed lots tested including the number of tests and the number of positive tests. The estimated mean presence is the proportion of seeds in that category estimated to be positive for CDC Triffid using the simulation tool. The 95% confidence intervals indicate the amount of uncertainty around the estimate. The p-value is the probability of observing a number of positive tests equal to or greater than the observed number of positives tests simply from false positives. P-values less than 0.05 indicate a high degree of confidence that the reported number of tests include true positive results, and are not simply the result of testing uncertainty.

Type	Year	Type of test	# of lots tested	# positive tests	Estimated mean Triffid presence	Lower 95% CI	Upper 95% CI	p
Farm saved	2009_2010	1×60	14	1	1.76E-05	1.55E-05	1.98E-05	0.0808
Farm saved	2009_2010	4×60	821	117	4.26E-06	4.18E-06	4.34E-06	<0.0001
Farm saved	2010_2011	4×60	318	14	1.24E-06	1.20E-06	1.27E-06	0.0216
Farm saved	2011_2012	4×60	421	19	1.22E-06	1.18E-06	1.26E-06	0.0067
Farm saved	2012_2013	4×60	403	18	1.20E-06	1.16E-06	1.25E-06	0.0089
Pedigreed	2009_2010	1×60	112	10	1.11E-05	1.04E-05	1.19E-05	<0.0001
Pedigreed	2009_2010	4×60	193	13	1.96E-06	1.86E-06	2.06E-06	0.0008
Pedigreed	2010_2011	4×60	239	5	4.98E-07	4.94E-07	5.03E-07	0.6741
Pedigreed	2011_2012	4×60	72	0	NA	NA	NA	1.0000
Pedigreed	2012_2013	4×60	68	0	NA	NA	NA	1.0000
Production	2009_2010	1×60	6016	248	4.15E-05	4.11E-05	4.19E-05	<0.0001
Production	2010_2011	4×60	4400	305	1.89E-06	1.87E-06	1.92E-06	<0.0001
Production	2011_2012	4×60	2158	76	8.81E-07	8.60E-07	9.03E-07	0.0007
Production	2012_2013	4×60	2478	102	1.02E-06	1.00E-06	1.05E-06	<0.0001

seeds (Remund, Dixon, Wright, & Holden, 2001; Whitaker, Freese, Giesbrecht, & Slate, 2001). However, Lamb and Booker (2011) demonstrated that low levels of presumed contamination (less than 1 in 9,999) are indistinguishable from the number of positive tests expected from a clean seed lot given the observed rates of false positives. This finding has significant implications for the testing of flax seed lots for GM presence and the whole notion of zero tolerance in the grain industry. Continued testing will be required for the foreseeable future to reduce the risk of product rejection.

Why transgenic flax was found in Canadian flax after removal of CDC Triffid from the commercial system is not known. There are many potential sources of seed-mediated gene flow, including crop volunteers, mixtures during seed multiplication, transport, planting, harvest, post-harvest transport, and handling by intermediates and end-users (Wilkinson, 2011). Seed-mediated gene flow in flax as a result of harvest loss, seed bank longevity, and the emergence and persistence of volunteer flax in subsequent crops has been reported by Dexter et al. (2010). Another situation where the GM flax may have been introduced into the commercial seed stocks is via cross pollination (out-crossing). However, the rate of gene flow in flax is low and is estimated to be between 0.0013 and 0.00003, at 3 and 35m, respectively (Jhala, Bhatt, Topinka, & Hall, 2011). Introduction of GM flax into the commercial seed stocks is most likely to have occurred through seed carry over from farm machinery, storage facilities, and mixtures during seed multiplication.

Methods

Observed Canadian Test Results

Data from the Flax Council of Canada were obtained detailing the number of tests carried out to detect the GM construct found in CDC Triffid between January 2009 and March 2013. Test results on farm-saved sowing seed, pedigreed seed, and production or grain were obtained and summarized according to type, year, and variety (Tables 1-3). In total, data on 52,426 individual tests on 17,713 seed lots were obtained. Initially, the industry testing protocol only required a 1×60g subsample for each 2kg composite be tested for GM. This protocol was later updated in September 2010 to require 4×60g subsample for each 2kg composite to be tested for GM. Here we report only the number of positive and negative test results. Some labs report detection below the 0.01% level (less than 1 GM seed in 9,999 seeds) as "trace," however there was no consistency between labs or years on how trace results were reported. In particular, prior to December 2010, trace results obtained in the lab were reported as negative. Here we have treated all "trace" reports as negative.

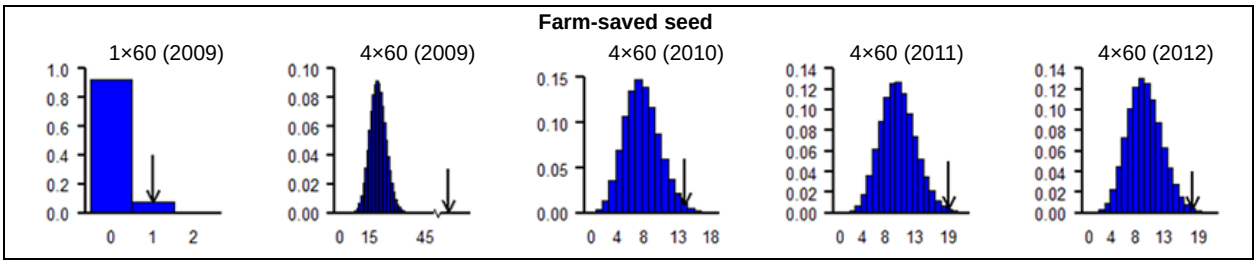

Figure 1. The expected distribution of false positive tests for each series of tests completed on farm-saved data. The arrows indicate the observed number of positive tests.

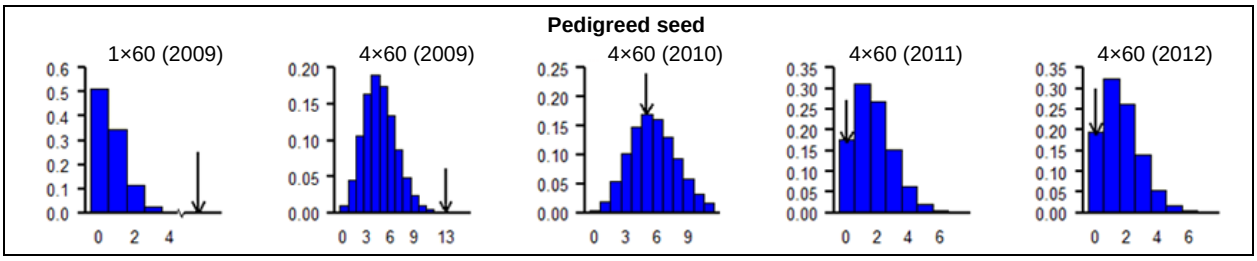

Figure 2. The expected distribution of false positive tests for each series of tests completed on pedigreed data. The arrows indicate the observed number of positive tests.

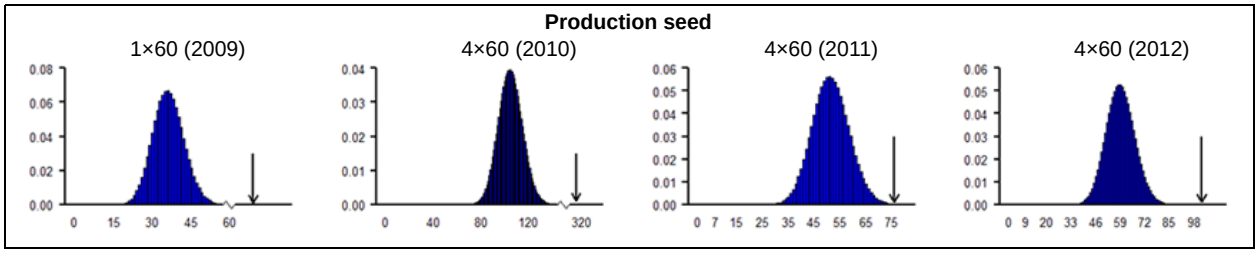

Figure 3. The expected distribution of false positive tests for each series of tests completed on production data. The arrows indicate the observed number of positive tests.

Expected Levels of Positive Tests

The test of GM construct has a specificity of 0.006, indicating that a false positive result can be expected in 0.6% of individual tests (Booker & Lamb, 2012; Lamb & Booker, 2011). This low rate of false positives, however, can result in substantial numbers of positive results in tests of clean seed. It is critical to determine if the number of positive tests observed deviates from the expected number of false positives given the observed false positive rate. This question was evaluated by first estimating the probability that the observed or a larger number of positive results could have arisen given the rate of false positives. A probability of ≥ 0.05 indicates that the number of observed results is not significantly different than that expected due to false positives (due to chance). This probability was calculated using the dbinom function in the R statistical package (R Development Core Team, 2011). The probability was estimated for a particular number of positive tests that could arise given the false positive rate with the total number of tests and the false positive rate as arguments. In cases where only a single test of 10,000 seeds was reported per lot (1×60g tests), a false positive rate of 0.006 was used. In cases where four tests per lot were performed (4×60g tests), a false positive rate of 0.0238 was used, since a false positive rate of 0.006 per test means that on average 2.4% of clean lots will have at least one false positive test out of 4 ($0.0238=1-0.994^4$). Summing the results of the dbinom function across all numbers of positive tests $\geq$ the observed number of positives gives the probability that the observed or a larger number of positive results could have arisen given the rate of false positives. In addition, the expected distribution of false positive results was plotted using the dbinom function.

Table 2. Summary of tests sorted by variety.

Variety	0 positives	1 positive	2 positives	3 positives	4 positives	Total positive tests	Total tests
AC Carnduff	5	1	0	0	0	1	6
AC Emerson	12	1	0	0	0	1	13
AC Linora	0	0	1	0	0	1	1
AC McDuff	5	0	0	0	0	0	5
AC Watson	115	3	0	1	0	4	119
CDC Arras	108	13	0	3	1	17	125
CDC Arras	0	1	0	0	0	1	1
CDC Bethune	3,792	274	45	5	11	335	4,127
CDC Gold	7	0	0	0	0	0	7
CDC Mons	7	0	1	0	0	1	8
CDC Normandy	3	7	6	3	3	19	22
CDC Sanctuary	3	1	0	0	0	1	4
CDC Sorrel	2,215	29	6	0	1	36	2,251
CDC Valour	11	1	0	0	0	1	12
Flanders	59	3	0	0	0	3	62
FP2141	1	0	0	0	0	0	1
Hanley	203	5	1	0	0	6	209
Lightning	89	0	0	0	0	0	89
Macbeth	14	0	0	0	0	0	14
McGregor	10	4	0	1	1	6	16
Missing	9,052	393	33	13	15	454	9,506
NorLin	45	1	2	2	0	5	50
Nulin 50	168	0	0	0	0	0	168
Prairie Blue	44	0	0	0	0	0	44
Prairie Grande	12	1	0	0	0	1	13
Prairie Thunder	70	0	0	2	0	2	72
Prairie Sapphire	3	0	0	0	0	0	3
Somme	15	1	1	2	0	4	19
Taurus	158	1	1	0	0	2	160
Vimy	559	23	2	0	2	27	586

Estimation of GM Prevalence in Contaminated Seed or Grain Lots

In most cases, the number of positive tests was much higher than what was expected given the false positive rates. We used a simulation model to estimate the prevalence of GM contamination for these lots (Booker & Lamb, 2012; Lamb & Booker, 2011). This simulation model generates the range of GM prevalence expected to arise, given the number of positive tests observed and the total number of tests done. The model incorporates the rates of false positive and false negative results expected to occur during the testing process and the number of individual seeds used in each test. The simulation was written using the open-source R statistical package (R Development Core Team, 2011); for a full description of the simulation and all code required to produce the results described here, see Lamb and Booker (2011). The simulation was used to estimate the mean level of GM contamination and 95% confidence intervals in each type of seed (Table 1). We estimated the contamination level separately for each seed type and year. We also produced separate estimates for the cases where 1×60g and 4×60g tests were carried out on the same seed type. This was done because many of the 4×60g tests reported only aggregate results (positive if one or more of the four tests was positive) and not the results of the four individual tests.

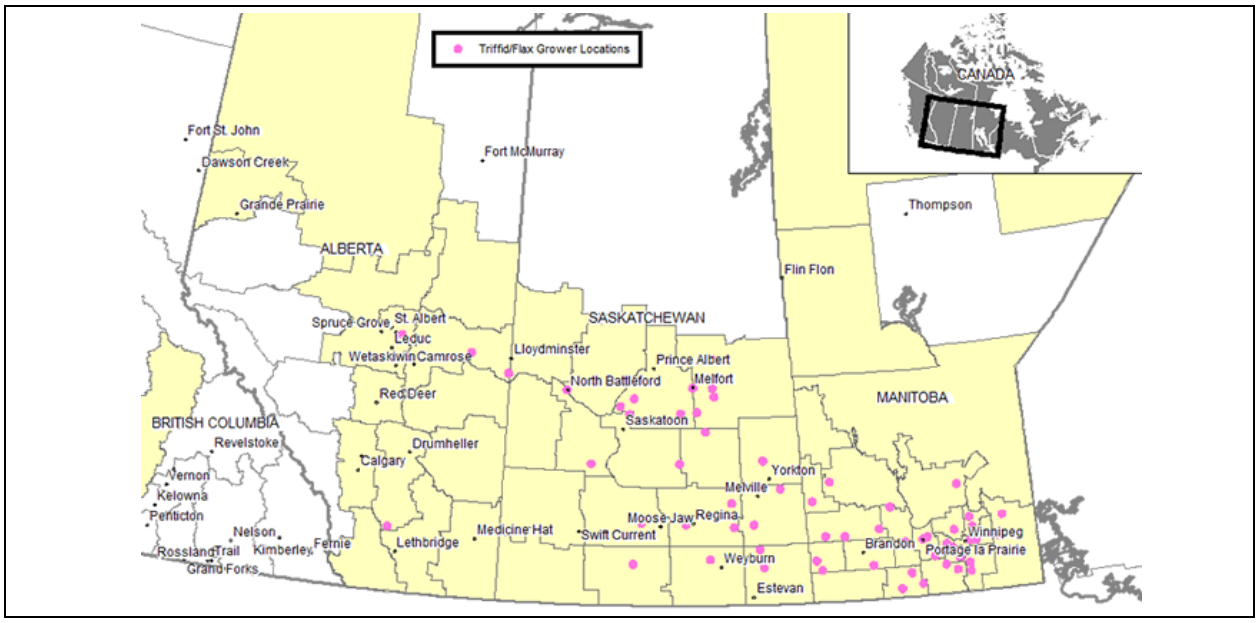

Figure 4. Triffid flax seed production (1997-1999); locations denoted by pink circles.

Regional Analysis of Canadian Test Results

Data Cleaning and Export. The dataset with blank fields in the postal code data was separated and postal codes found using the website "http://postal-code.org/postal_code_search" and entering the name of the town/city. If there was a spelling error in the town/city name it was corrected, if possible. Unsuccessful corrections were left blank. If a postal code could not be found then the cell was left blank. Once the missing postal codes were added, any other typos of existing postal codes were fixed, if possible. A pivot table was created to aggregate the data by unique postal code and sum the values in the column 'total positive tests' for each unique postal code. The resulting table contained 1,603 unique postal codes. The starting dataset had 2,057 rows of data. The pivot table was exported as a text file for use in the GIS software. The data included the postal code and the summation of the 'total positive tests.'

Mapping the Canadian Test Results. ArcMap software (published by ESRI) was used to construct the final map. The map (Figure 4) shows Canadian Census Divisions and postal codes, which are represented by dots. The postal code GIS layer contains the 6-character postal code that is also found in the spreadsheet data. The spreadsheet data was imported into ArcMap. The data was joined to the GIS data using the JOIN function by matching postal codes from the GIS data to the spreadsheet data. After the JOIN operation, there were 1,485 points. The spreadsheet data had 1,603 postal codes. That means that 118 postal codes (1,603−1,485=118) were not matched. The most likely reason for the discrepancy is errors in the postal codes in the spreadsheet data. The 1,485 points on the map were colored using a blue square for points where 'total positive tests'=0 and a red triangle for the remaining points were 'total positive tests'>0. The map was completed by adding other geographic layers to represent the Census Divisions in which the postal codes reside.

Mapping of Triffid Seed Grower Locations. The data for the mapping of Triffid seed growers' locations in Western Canada from 1997 to 1999 was provided by the Canadian Seed Growers Association. The above postal codes were plotted with a different symbol colored pink to denote the location of Triffid/flax seed growers.

Results and Discussion

Between 2009 and 2012 a total of 17,713 seed or grain lots were tested, with 928 lots testing positive (Table 1).

No positive results were reported from pedigreed seed in 2011 or 2012, though the testing rate was low. The number of positives observed from pedigreed seed in 2010 and the farm-saved seed from 1×60 tests in 2009 was not significantly different than expected from the false positive rate (Figure 1 and 2). Rates of positive tests were significantly higher than expected by chance in all other categories of seed or grain (Table 1; Figures

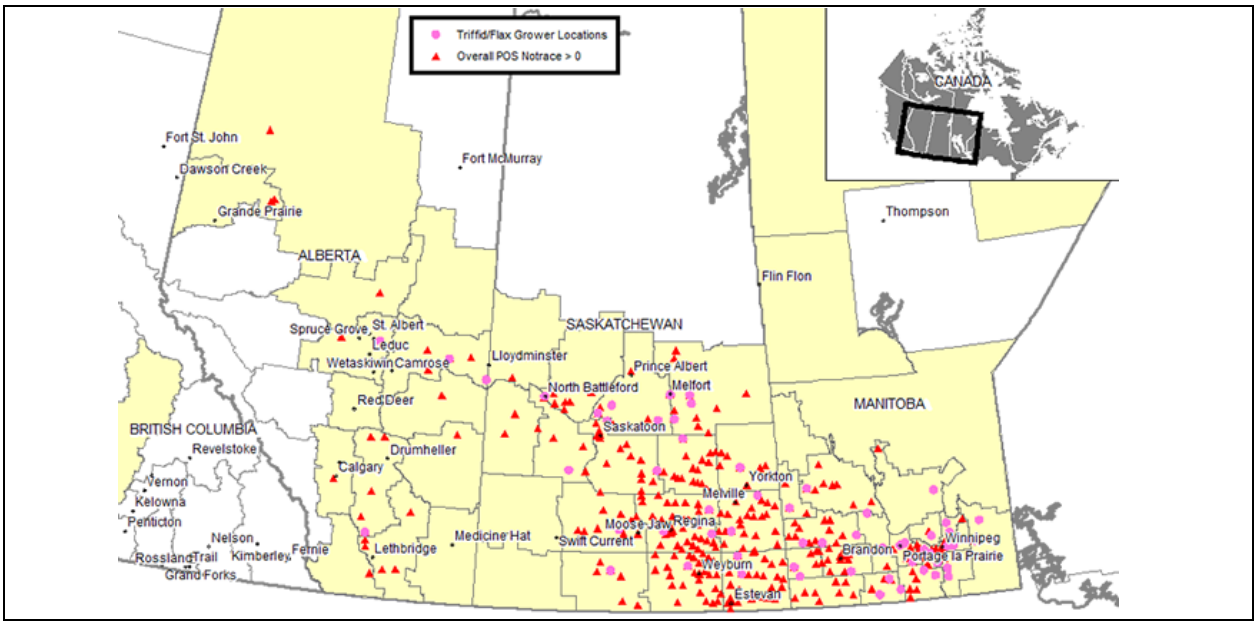

Figure 5. A regional analysis of Canadian test results (2009-2013) showing a red triangle for points where 'total positive tests' > 0. Pink circles mark the location of Triffid flax seed production (1997-1999) in Western Canada.

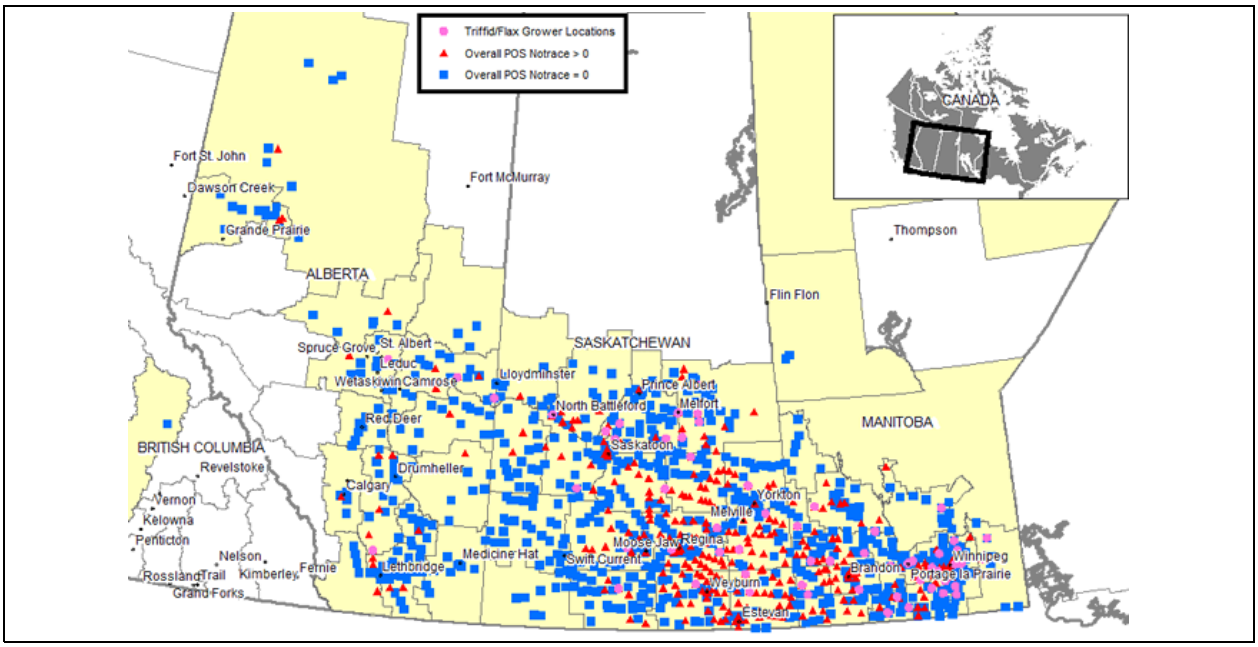

Figure 6. A regional analysis of Canadian test results (2009-2013) showing blue squares for points where 'total positive tests' = 0 and a red triangle for the remaining points were 'total positive tests' > 0. Pink circles mark the location of Triffid flax seed production (1997-1999) in Western Canada.

1-3). Rates of GM prevalence in contaminated seed or grain categories were evaluated; the results indicate similar trends as in Booker and Lamb (2012; see Table 1). Table 2 shows test results separated by variety.

CDC Triffid (McHughen et al., 1997) received registration from the Canadian Food Inspection Agency (CFIA) for production in Canada in 1994 and, although never released commercially, was produced by seed growers in the three Prairie Provinces (Alberta, Saskatchewan, and Manitoba) from 1997 to 1999 before being recalled in 2000 (Figure 4). The regional analysis (Figure 5) shows the occurrence of positive tests for the

Table 3. Summary of tests on CDC Bethune and CDC Sorrel sorted by seed type and year.

Variety	Type	Year	0 positives	1 positive	2 positives	3 positives	4 positives	Total positive tests	Total tests
CDC bethune	Farm saved	2009_2010	367	63	0	0	0	63	430
		2010_2011	118	6	0	0	0	6	124
		2011_2012	143	7	1	0	0	8	151
		2012_2013	147	6	2	0	0	8	155
	Pedigreed	2009_2010	83	9	0	0	0	9	92
		2010_2011	36	3	0	0	1	4	40
		2011_2012	16	0	0	0	0	0	16
		2012_2013	12	0	0	0	0	0	12
	Production	2010_2011	1493	120	25	4	5	154	1647
		2011_2012	652	25	10	0	5	40	692
		2012_2013	725	35	7	1	0	43	768
CDC sorrel	Farm saved	2009_2010	85	6	0	0	0	6	91
		2010_2011	53	0	0	0	0	0	53
		2011_2012	74	0	0	0	0	0	74
		2012_2013	91	0	0	0	0	0	91
	Pedigreed	2009_2010	82	3	0	0	0	3	85
		2010_2011	59	0	0	0	0	0	59
		2011_2012	24	0	0	0	0	0	24
		2012_2013	20	0	0	0	0	0	20
	Production	2010_2011	743	16	3	0	1	20	763
		2011_2012	454	1	1	0	0	2	456
		2012_2013	530	3	2	0	0	5	535

construct found in CDC Triffid overlaid onto the map of Triffid/flax seed growers locations. The occurrence of transgenic flax was widespread across the three Prairie Provinces, consistent with expected admixtures due to crop volunteers, mixtures during seed multiplication, transport, planting, harvest, post-harvest, and storage (Wilkinson, 2011).

Per the Canadian Grain Commission's Flax Harvest Surveys (2009-2012), the CDC flax varieties accounted for greater than 80% of the seeded flax acreage in Western Canada (B. Siemens, Canadian Grain Commission, personal communication). Popularly grown varieties CDC Bethune (Rowland, Hormis, & Rashid, 2002) and CDC Sorrel received registration by the CFIA for production in Canada in 1998 and 2005, respectively. These widely grown varieties were sorted by seed type and year (Table 3). Not surprisingly, for production in crop years 2010 to 2013, grain derived from variety CDC Bethune—in commercial production longer—had a higher proportion of positive tests (9.4%, 5.8%, and 5.6%) than CDC Sorrel (2.6%, 0.4%, and 0.9%; see Table 3). This marked difference in GM prevalence between two widely grown varieties may be due to the greater chance for admixtures to have occurred with variety CDC Bethune, which is owed to the longevity and widespread production of this variety in Western Canada. Additionally, seed production of CDC Triffid (1997-1999) coincided with that of variety CDC Bethune (1998-2000), thus increasing the likelihood of admixtures during seed multiplication.

Traditionally, the majority of the flax crop has commonly been sown with farm-saved seed, with less than 25% planted from pedigreed seed (T. Hyra, Western Canada SeCan, personal communication, January 11, 2011). Importantly, the pedigreed seed of all Canadian flax varieties has tested negative for the construct found in CDC Triffid for the past two years (Table 1). Moreover, the Crop Development Centre, University of Saskatchewan has developed and applied a protocol to reconstitute commercially important flax varieties, including CDC Bethune, CDC Sorrel, and new varieties CDC Sanctuary and CDC Glas. Pedigreed seed of these re-constituted CDC varieties is available to farmers in the Fall of 2013 (T. Hyra, Western Canada SeCan, personnel communication, July 25, 2013). The intention of the Canadian flax industry is to strongly encourage

renewal of farmers' seed stocks with pedigreed seed in the 2013-2014 crop year, thereby further diluting commercial flax stocks of remaining transgenic flaxseed.

Summary Points

This study re-evaluates GM presence in Canadian grain stocks for the updated dataset of 2009-2013 using a previously described simulation model to estimate low-level GM presence. The test results were broken down by category (i.e., seed or grain), as well as crop year. For each category seed or grain, it was determined whether the observed positives deviated from the expected number of false positives (i.e., due to chance alone). In most cases, the number of positive tests was significantly higher than expected due to chance alone. That is, the arrow indicating the number of positive tests lies at the far right tail end or outside the probability distribution (Figures 1-3).

For these categories of seed or grain, a simulation model was applied to the test results to estimate GM contamination levels (Table 1). The number of positive tests over time showed a downward trend, indicating removal of transgenic flax from the commercial system (production) from a high of approximately 4 GM seeds in 100,000 (0.004%) to 1 GM seed in one million (0.0001%; see Table 1). Pedigreed seed has shown no positive tests since the 2011 crop year (Table 1).

The test results were separated by variety (Table 2); the majority of flax varieties surveyed tested positive for the construct found in CDC Triffid. Test results on two widely grown flax varieties (CDC Bethune and CDC Sorrel) were shown in Table 3. CDC Bethune was registered in 1998 and showed a larger proportion of positive tests than CDC Sorrel, which was registered for production in Canada in 2005.

A map of Western Canada (Figure 4) shows the location of Triffid flax seed production from 1997 to 1999. Transgenic flaxseed was recalled in 2000 by the Canadian industry and crushed in North America. A regional analysis of the Canadian test results (2009-2013) showed the widespread prevalence of transgenic flax in the production areas of Western Canada (Figures 5, 6) a decade after the recall.

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Acknowledgments

The Flax Council of Canada kindly provided data on the testing carried out to detect GM flax. Quantum Biosciences (Saskatoon, SK, Canada) provided data on the construct specific PCR assay error rates. The Canadian Seed Growers Association provided data for maps of Triffid seed production locations in Western Canada. Dr. Kofi Agblor generously provided comments on drafts of this manuscript.