Development of a Polymerase Chain Reaction Assay for Detection of Three Canola Transgenes

Tigst Demeke[a,*], Randal W. Giroux[b], Shari Reitmeier[a], and Sharon L. Simon[a]

[a]Canadian Grain Commission, Grain Research Laboratory, Winnipeg, Manitoba, R3C 3G8, Canada, and [b]Cargill Inc., Minneapolis, Minnesota 55440-5699

ABSTRACT: Many countries are developing or implementing regulatory requirements to monitor for the presence of genetically modified (GM) materials in seeds, grain, and derived food products using DNA and protein-based methods. There is no published report on the detection of different GM transgenes in canola, and this study is aimed at developing qualitative PCR methods for the three major GM transgenes commercially available in canola. Primer sequences were generated from GenBank and previously published information to develop a polymerase chain reaction (PCR) detection method for Roundup Ready (glyphosate tolerance, GT73 event), Liberty Link (glufosinate ammonium tolerance, HCN92 event), and BX (Bromoxynil tolerance, OXY235 event) canola varieties. On using PCR, two primer pairs for each of the GT73 and HCN92 and one primer pair for OXY235 amplified specific amplicons for the three GM transgenes. All three GM transgenes were detected simultaneously by multiplexing the five primer pairs in a single PCR reaction. Multiplexing of the five primer pairs for DNA prepared at 1% (one GM seed in 99 non-GM seeds) and 0.5% (one GM seed in 199 non-GM seeds) levels generated the expected DNA fragments for GT73, HCN92, and OXY235. This information will lay the groundwork for the development of a quantitative PCR assay for canola transgenic events.

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KEY WORDS: Bromoxynil, canola, glufosinate, glyphosate, GM detection, GT73, HCN92, multiplex PCR, OXY235, transgene.

Canola refers to oilseed rape varieties with <2% erucic acid in the oil and <30 μmol/g of the normally measured glucosinolates in the meal (1). Canola oil is used either pure or blended with other vegetable oils as salad oil or cooking oil and in the manufacture of margarine and shortenings. Canola meal, a co-product of the oil production process, is added to livestock feed rations. Canola was one of the first commercially released genetically modified (GM) crop plants in Canada and continues to be a target for further genetic engineering research and development. Genetically enhanced canola has provided major advantages to farmers for canola production, and over 60 GM canola varieties have been registered in Canada alone. The most commonly used canola GM traits[1] are glyphosate tolerance, glufosinate ammonium tolerance, bromoxynil tolerance, male sterility/fertility restoration, and improved oil quality (Canadian Food Inspection Agency; http://www.inspection.gc.ca). Glyphosate herbicide-resistant canola (GT73) produced by inserting a recombinant DNA construct containing the gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is the most common type of GM variety. Glyphosate specifically binds to and inactivates EPSPS, but transformed plants have a modified form of the enzyme from bacteria (CP4 EPSPS) that has a reduced binding affinity for glyphosate. The *pat* gene (HCN92 event[1]) encodes the enzyme phosphinothricin-*N*-acetyltransferase (PAT) and confers tolerance to glufosinate ammonium herbicide by inactivating glufosinate ammonium through acetylation. Glufosinate ammonium acts by inhibiting a key plant enzyme, glutamine synthetase, that detoxifies ammonia by incorporating it into glutamine. The oxynil family of herbicides (including bromoxynil and ioxynil) are active against dicotyledonous plants by blocking electron flow during the light reaction of photosynthesis. The nitrilase gene (OXY235 event) codes for a bacterial enzyme, nitrilase, which confers tolerance to the oxynil family of herbicides by hydrolyzing them into nonphytotoxic compounds (http://www.agbiosafety.unl.edu).

Genetically modified canola varieties containing the GT73, HCN92, and OXY235 events have been evaluated for food, feed, and environmental safety (most are approved in Canada, the US, and Japan) and are considered substantially equivalent to their traditional counterparts (http://www.canolainfo.org). Genetically modified canola has not yet received regulatory approval for use in other countries. Some of these countries are in the process of developing and/or implementing rules and regulations for the distribution, environmental release, food and feed uses, and product labeling of GM seeds, grain, and grain products. Because of a growing need to test and monitor for the presence of GM traits and/or events, many private and government research agencies are in the process of developing, comparing, and validating detection methods. Collaborative studies have been conducted on qualitative polymerase chain reaction (PCR) detection of GM varieties of soybean and maize (2). Quantitative PCR methods have also been developed for the detection of Roundup Ready GM soybean and maize

*To whom correspondence should be addressed at Canadian Grain Commission, Grain Research Laboratory, 1404-303 Main Street, Winnipeg, Manitoba, R3C 3G8, Canada. E-mail: tdemeke@grainscanada.gc.ca

[1]The use of the term "event" refers to the recombinant DNA insertion carrying a specific gene of interest; the term "trait" refers to the phenotype resulting from the expression of the event gene. For example, the GT73 event when expressed to produce EPSPS results in plants that are tolerant to glyphosate (Roundup Ready).

(3–6). To date, there are no published reports of a PCR assay for detection of GM canola.

The objectives of this research were (i) to develop suitable PCR primers for amplification of the three most common canola GM transgenes, GT73 (glyphosate tolerance), HCN92 (glufosinate ammonium tolerance), and OXY235 (bromoxynil tolerance); (ii) to test the efficiency of the primers for each transgene in GM canola varieties; (iii) to develop a multiplex PCR system for these transgenes; (iv) to determine a detection limit for canola GM transgenes based on multiplex PCR assay.

EXPERIMENTAL PROCEDURES

Seed sources. The list of canola varieties used for the study is presented in Table 1. The certified non-GM seeds (AC Parkland and Legend) were obtained from the Grain Research Laboratory's (GRL) Oil Seeds and Pulses group. Specific cultivars of harvest survey samples sent to GRL in 2001 were used for checking the presence of the three GM transgenes (Table 1). Breeder seed of Conquest was provided by Agricore.

DNA extraction and PCR. DNA was extracted from 20–25 seeds of each GM canola variety by the CTAB (cetyltrimethylammonium bromide) method (7) for specific varieties. To produce samples with varying GM levels, GM seeds of Conquest (GT73), Independence (HCN92), and Armor BX (OXY235) were mixed with certified seeds of non-GM AC Parkland at 1% (one GM seed in 99 non-GM seeds), 0.5% (one GM seed in 199 non-GM seeds), 0.25% (one GM seed in 399 non-GM seeds), and 0.1% (one GM seed in 999 non-GM seeds). DNA was extracted from the whole mix in duplicates using two independent subsamples. The CTAB extraction protocol was modified to include RNase A treatment (10 mg/mL stock) to digest RNA in the sample. The quality and amount of extracted DNA was estimated on agarose gels by comparison with 50 bp Gibco/BRL DNA standard marker (WB18009). Based on this estimation, 50.0 ng of DNA was used as the template for PCR reaction. Additional PCR reactions were included to control for cross-contamination and nonspecific PCR amplification. To control for false positives caused by reaction contamination, a sample containing all of the PCR components but no template DNA was included and is referred to as the negative control. In addition, a PCR reaction containing DNA extracted from non-GM soybeans was included in each sample set. Primer sequences suitable for different GM transgenes were developed from publicly available information (Table 2). PCR was performed in 96-well plates containing 50 μL of a reaction mixture consisting of 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 0.2 mM of each of the four dNTPs, 0.05 μM internal NAD primer, 0.25 μM of each oligonucleotide primer, 0.15 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster, CA). The NAD primer pair (Table 2), which gives consistent amplification, was used as internal control to monitor DNA quality and quantity in the PCR. DNA amplification was performed in an MJ Research PTC-200 Thermal Cycler using 5.0 min denaturation at 95°C initially, and then 35 cycles of 30 s at 95°C, 30 s at 60°C, and 1.0 min at 72°C. An additional 72°C final extension of the PCR products was performed for 3.0 min. The PCR conditions for multiplex PCR were the same as described above. Amplification products were separated by electrophoresis in 2.0% (wt/vol) agarose gels (2).

TABLE 1
List of Genetically Modified (GM) Canola Varieties Used for the Study and PCR Amplification Results[a]

			PCR amplification	
Cultivar name	GM event	Source	CAN2	4EPSPS
Conquest	GT73[a]	Agricore, MB (Breeder seed)	+	+
LG3235 (A)	GT73	Fairlight, SK	+	+
LG3235 (B)	GT73	Weyburn, SK	+	+
LG3345 (A)	GT73	NWT Unity, SK	+	+
LG3345 (B)	GT73	Starbuck, MB	+	+
Quest (A)	GT73	Edgerton, AB	+	+
Quest (B)	GT73	Vermilion, AB	+	+
SW Arrow (A)	GT73	Brandon North, MB	+	+
SW Arrow (B)	GT73	Foam Lake, SK	+	+
SW Rider (A)	GT73	Tribune, SK	+	+
SW Rider (B)	GT73	Fairlight, SK	+	+
			pNOS	AM35S
Exceed	HCN92; HCN28	Booth Siding, SK	+	+
Independence	HCN92[b]	Deloraine, MB	+	+
Innovator	HCN92	Perdue, SK	+	+
SW Flare LL	HCN92; HCN28	Amazon, SK	–	+
2273	Ms1/Rf2	Nokomig, SK	+	–
			BXN	
Armor BX	OXY235[c]	Carnduff, SK	+	
Cartier BX	OXY235	Alexander, MB	+	

[a]The superscripts "a," "b," and "c" indicate Roundup Ready, Liberty Link, and Bromoxynil traits, respectively. SK, Saskatchewan; MB, Manitoba; AB, Alberta; PCR, polymerase chain reaction.

TABLE 2
List of Primer Sequences, Sources, and Expected Amplicon Sizes for the PCR Products

Primer name	Transgene	Sequence (5′–3′)	Source	Amplicon size (bp)
CAN2 F	GT73	CCGTAAGGAAGGTGATACTTGGA	Genbank # A59871	385
CAN2 R		CATGATTGGCTCGATAACAGTGGT	Genbank # A59871	
4EPSPS F	GT73	CAACGCAAATCTCCCTTATCGG	Genbank # A59871	274
4EPSPS R		GACCTCCAAACATGAAGGACCT	Genbank # A59871	
pNOS F	HCN92	GGAACTGACAGAACCGCAACG	Refs. 10 and 11*	167
pNOS R		TGGAACGTCAGTGGAGCATTT	Refs. 10 and 11	
AM35S F	HCN92	AAGGGTCTTGCGAAGGATAG	Ref. 3	227
AM35S R		AGTGGAAAAGGAAGGTGGCT	Ref. 3	
BXN F	OXY235	ACTTTCAAAGCAGCCGCTGTT	Genbank #J03196	460
BXN R		CACCCGACCAACGCTAGTTT	Genbank #J03196	
NAD5 F	Internal primer	TAGCCCGACCGTAGTGATGTTAA	Ref. 12	813
NAD5 R	Internal primer	ATCACCGAACCTGCACTCAGGAA	Ref. 12	

*Also Genbank # V00087. PCR, polymerase chain reaction.

RESULTS AND DISCUSSION

PCR amplification condition for individual events. The CAN2 and 4EPSPS primers for the GT73 transgene (Table 2) amplified DNA fragments of 385 and 274 bp, respectively (Fig. 1). These primers produced amplification products consistently in all six Roundup Ready-tolerant varieties known to contain the GT73 transgene (Table 1). The two primer pairs that were used to detect the HCN92 transgene were derived from 35S and nopaline synthase promoter (pNOS) regions found in the DNA constructs introduced into canola during the production of the HCN92 event (Table 2). Based on data from our screen of varieties containing the HCN92 event, there appears to be multiple insertion of some distinguishing sequences. In the varieties Exceed, Independence, and Innovator the pNOS and AM35S primer pairs consistently produced PCR amplicons of 167 and 227 bp, respectively (Table 1, Fig. 1). In the variety SW Flare LL, only the AM35S product (227 bp) was detected (Table 2). The absence of the pNOS product in SW Flare LL compared to Exceed (both are listed as HCN92; HCN28) suggests that this variety may lack the kanamycin resistance marker. In the cultivar 2273, only the pNOS PCR product (167 bp) was detected (Table 2). The cultivar 2273 (Ms1/Rf2) contains the bar gene that confers resistance to glufosinate ammonium tolerance. However, the absence of the AM35S PCR product suggests that for 2273, promoters other than 35S may have been used in the gene construct.

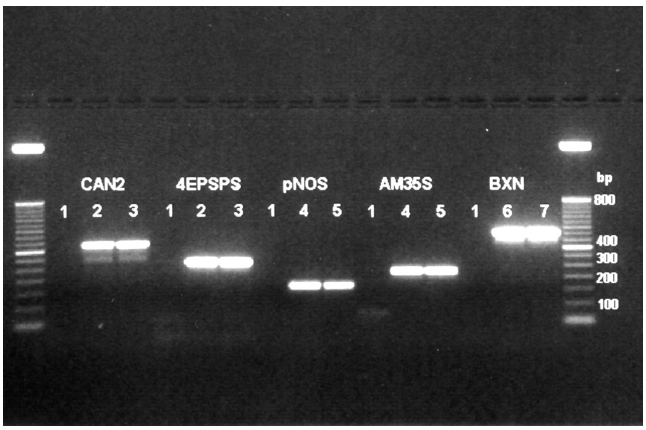

FIG. 1. Consistency of polymerase chain reaction amplification of individual transgene targets. 1 = Legend, non-genetically modified DNA control, 2 = LG3235, 3 = Conquest, 4 = Independence, 5 = Innovator, 6 = Armor BX, 7 = Cartier BX (see Table 1 for details). A 50-bp DNA ladder is shown in the first and last lanes.

For the OXY235 transgene, one primer set (BXN F and BXN R) designed for detection of the bromoxynil GM transgene (Table 2) amplified a PCR product of about 460 bp in Armor BX and Cartier BX varieties. Specificity is a critical parameter in the development of a PCR detection method for GM transgenes. Any primer set that produces false positive or ambiguous results is of limited utility. For all the primer sets described above, none produced an amplicon in any of the DNA samples extracted from non-GM varieties, thus indicating the specificity of the primers.

Multiplexing of PCR primers for detection of three GM events simultaneously. Currently, most PCR protocols for GM transgenes involve testing extracted DNA for each transgene individually in separate PCR reactions. Multiplex PCR refers to combining different primer sets in a single reaction for detection of multiple transgene specific amplicons at once. If developed correctly, the multiplexing of PCR primers for the detection of GM events can be advantageous since it can accelerate testing protocols, reduce sample loads, and conserve time and resources. Two primer pairs for GT73 (CAN2 and 4EPSPS), two primer pairs for HCN92 (pNOS and AM35S), and one primer pair for OXY235 (BXN) were combined in a single PCR for simultaneous amplification of the three canola GM transgenes. GT73 and OXY235 have at least one primer pair that targets a sequence within the functional transgene, namely, 4EPSPS and BXN, respectively, and thus have more specificity than HCN92. For HCN92, the primer pairs target pNOS and AM35S control element sequences. The AM35S sequence comes from cauliflower mosaic virus that can infect

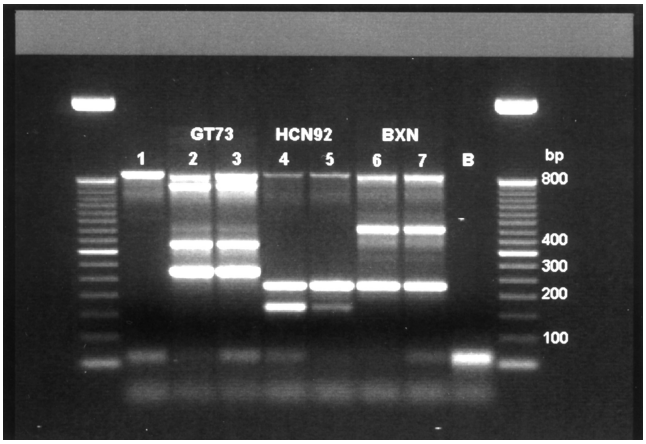

FIG. 2. Multiplexing of GT73 (CAN2, 385-bp; 4EPSPS, 274-bp), HCN92 (pNOS, 167-bp; AM35S, 227-bp), and BXN (460-bp) primers on polymerase chain reaction amplification. 1 = Parkland, non-genetically modified DNA control, 2 = LG3235, 3 = Conquest, 4 = Independence, 5 = Innovator, 6 = Armor BX, 7 = Cartier BX, B = blank. Note that the DNA of the genetically modified (GM) varieties is 100% GM organism. The ~775-bp product in GT73 results from the priming of 4EPSPS R and CAN2 F primers (they are in close proximity). The ~813-bp DNA fragment is the NAD internal primer product. A 50-bp DNA ladder is shown in the first and last lanes.

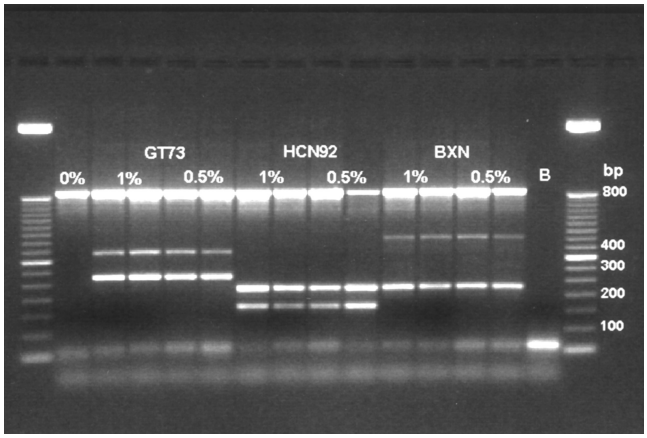

FIG. 4. The effect of multiplexing of five primer pairs on PCR amplification of 1 and 0.5% adulterated genetically modified polymerase chain reaction (GM) samples from two different DNA extractions. GM seeds of Conquest (GT73), Independence (HCN92), and Armor BX (OXY235) were mixed with certified seeds of non-GM Parkland at 1% (1 GM seed in 99 non-GM seeds) and 0.5% (1 GM seed in 199 non-GM seeds) for DNA extraction and PCR. All the expected DNA fragments are observed for the three transgenes. A 50-bp DNA ladder is shown in the first and last lanes.

Brassica species whereas the pNOS sequence comes from *Agrobacterium tumefaciens*, a soil-borne generic disease that affects dicotyledonous plants. Many of the GM organism detection methods use primers obtained from AM35S and pNOS, as there is a low probability of finding these sequences in nontransformed plants (especially in combination).

The development of the multiplex PCR reaction was considered successful since the expected DNA fragments were observed for all GM transgenes (Fig. 2). There was also amplification of the 35S promoter fragment (227 bp) in the OXY235 GM event (Fig. 2). We have verified from a literature survey that the gene construct contains a portion of the 35S promoter (8). The most common problems in multiplex PCR are nonspecific annealing and primer–dimer formation (9). None of these problems was encountered with the specific primer sets developed and used in this multiplexed PCR assay. A single tube multiplex PCR assay that can detect the three most common GM transgenes in canola should be a useful tool for qualitative PCR detection.

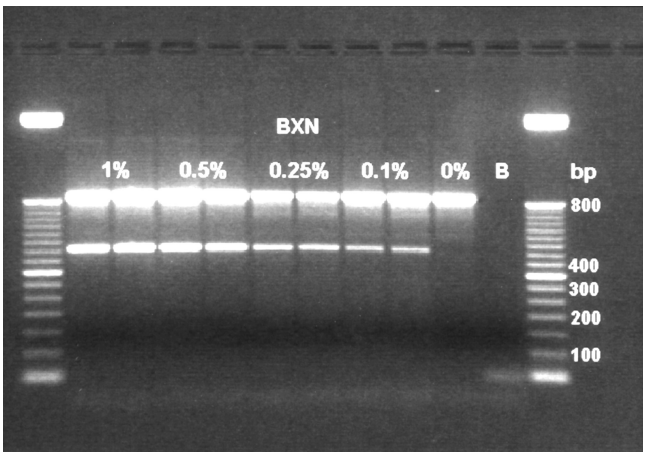

FIG. 3. DNA amplification from different genetically modified polymerase chain reaction (GM) concentrations. Seeds of the GM cultivar Armor BX were mixed with seeds of non-GM cultivar AC Parkland to attain GM ratios of 1% (1/100), 0.5% (1/200), 0.25% (1/400), and 0.1% (1/1000) and amplified with the primer BXN. Note the faint amplicons produced at the 0.25% and 0.1% levels. The ~813-bp DNA fragment is the NAD internal primer product. A 50-bp DNA ladder is shown in the first and last lanes.

Observation on detection limit. In order to understand the abilities of individual and multiplexed primer sets to detect mixed GM seeds, samples with specific levels of GM material were prepared and tested by PCR. GM seeds of Conquest (GT73), Independence (HCN92), and Armor BX (OXY235) were mixed with certified seeds of non-GM AC Parkland at specific ratios. These samples were prepared carefully and mixed thoroughly to obtain a homogeneous sample. In preliminary PCR tests, consistent PCR amplification of the GT73 transgene was achieved at 5 (5 GM seeds and 95 non-GM seeds), 2.5, 1.25, 0.8, 0.6, and 0.5% levels using the 4EPSPS and CAN2 primer sets (data not shown), and thus higher concentrations were not included for the followup study. For further study, duplicate DNA samples of canola mixed at 1, 0.5, 0.25, and 0.1% GM seeds were prepared and tested using PCR for the detection of the GT73, HCN92, and OXY235 transgenes. When individually used, the primer pairs 4EPSPS and CAN2 (GT73), pNOS and AM35S (HCN92), and BXN (OXY235) amplified the expected DNA fragments for 1, 0.5, 0.25, and 0.1% samples. A representative picture of PCR products amplified with the primer pair BXN for the four concentrations is depicted in Figure 3. Relatively faint PCR products were observed for 0.25 and 0.1% GM concentrations.

Multiplexing reaction containing the five primers was performed on the same samples to detect the three GM transgenes. The multiplex PCR did not produce all of the expected PCR products at the lower concentrations (0.25 and 0.1%, data not shown). However, we are encouraged by the expected PCR results for GT73, HCN92, and OXY235 at 1 and 0.5% levels (Fig. 4).

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