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Detection of Genetically Modified Canola Using Multiplex PCR Coupled with Oligonucleotide Microarray Hybridization

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A rapid method was developed for concurrent screening of transgenic elements in GM canola. This method utilizes a single multiplex PCR coupled with an oligonucleotide DNA array capable of simultaneously detecting the 12 approved GM canola lines in Canada. The assay includes construct-specific elements for identification of approved lines, common elements (e.g., CaMV 35S promoter, *Agrobacterium tumefaciens* nos terminator, or *npt*II gene) for screening of approved or unapproved lines, a canola-specific endogenous gene, and endogenous genes from heterologous crops to serve as additional controls. Oligonucleotide probes were validated individually for functionality and specificity by amplification of specific transgene sequences from appropriate GM canola lines corresponding to each probe sequence, and hybridization of amplicons to the array. Each target sequence hybridized to its corresponding oligonucleotide probe and no significant cross-hybridization was observed. The limit of detection was examined for the GM lines GT73, T45, and MS8/RF3, and was determined to be 0.1%, 0.1%, and 0.5%, respectively, well within the European food and feed labeling threshold level of 0.9% for approved GM product. Practically, the method was demonstrated to be effective for the detection of GM canola in several types of animal feed, as well as in commercial canola meal.

KEYWORDS: Canola; *Brassica napus*; genetically modified; multiplex PCR; oligonucleotide microarray

INTRODUCTION

Canola makes up 13% of the world's oilseed supply, second only to soybean in global oilseed production (*1*). Although canola seeds are crushed for their oil, the solids (meal) are also important and used as a protein supplement in animal feeds. Canola is grown extensively in Canada, Europe, China, India, Australia, and, to a limited extent, the United States. It is one of four principal biotech crops grown worldwide, occupying 6% of the total global biotech area. In Canada, 77% of the canola hectarage is planted with genetically modified (GM) varieties, establishing canola as the dominant GM crop in this country (*2*). Nations in the European Union (EU), as well as Japan, Australia, New Zealand, Thailand and China have implemented mandatory labeling for foods and feed derived from transgenic plants, and several other countries are considering voluntary or mandatory labeling proposals. Although no mandatory labeling legislation has been implemented in Canada to date, nearly 75% of the canola seed, oil and meal produced in Canada is being exported (*3*), in some cases to destinations where mandatory labeling regulations exist (EU, Japan, and China) or where cultivation of GM canola is not permitted (EU). Therefore, the development of reliable methods to detect transgenic events has become increasingly important for government regulators, international trade organizations and industries utilizing these products.

PCR is among the most accepted and useful techniques for the detection and identification of transgenic crops and food/feed products derived from these crops, primarily due to the stable nature of the DNA molecule, as well as the extreme sensitivity of the PCR technology *(4)*. Detection of genetically modified organisms (GMOs) by conventional (end-point) and real-time PCR has been shown to be highly sensitive and specific; however, the evaluation of samples with unknown GM content demands the analysis of multiple targets. Multiplex PCR is a popular means to detect multiple targets in a single assay (*5*). Common problems associated with multiplex PCR, particularly when expanding the number of targets in the reaction, are decreased sensitivity and increased nonspecific amplification products. Additionally, agarose gel electrophoresis is typically used to evaluate the results of end-point multiplex PCR; however, this method is restricted by the number of amplified products that can be adequately resolved on a gel (*6*). Alternatively, probe- or SYBR Green-based real-time multiplex PCR is either instrument-limited by the number of fluorophores that can be simultaneously detected in a single reaction in the case of probe-based, or melting temperature (T_m)-limited in the case of SYBR Green-based.

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Table 1. Microarray Oligonucleotide Probe Sequences and Position on Array

probe	sequence (5′−3′)	position
oc-cru-2	AAGGAGAGCTTCGCGGTGCTAAAGAAAGAGGGCAAGTAGAGACCCTTAGACTCGATGATGTAACGTACAAA	A1, E1, E6
oc-tRNleu-2	TGGAAGTTACCACTTAGCAGGTTTCCATACCAAGGCTCAATACAATCAAGTCCGTAGCGTCTACCGATTTC	B1
oc-papain	ACCCGTAGCTACGTCTGTCGCAGTCAAGCAGTTCTTGCTCTGAGTATTCATTTAAGTTCCCAGTTCTAATC	C1
oc-patatin	GCCGGAATGATTCCCTTAATTCCACCTCCATCAATACTAAGAACAGTCACCATTTCTCCCAACGTAGCACA	D1
og-EPSPS	ATACGAGTTTCACCGCTAGCGAGACCTCCAAACATGAAGGACCTGTGGGAGATAGACTTGTCACCTGGAATA	A2
og-bxn-2	TCTTTTTATTAGGAATGTCCGCAATAACGGGGTAGAACCCCTATTGCCGTAGAGATTACGACCTCACGATC	B2
og-pat-2	ATGTGGATCCTAGGCCCAACCTTTGATGCCTATGTGACACGTAAACAGTACTCTCAACTGTCCAATCGTAA	C2
og-bar-2	GATGACAGCGACCACGCTCTTGAAGCCCTGTGCCTCCAGGGACTTCAGCAGGTGGGTGTAGAGCGTGGAG	D2
og-bar-3	GATGACAGCGACCACGCTCTTGAAGCCCTGTGCCTCCAGGGACTTCAGCA	E2
og-nptII	AGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGA	A3
og-nptII-2	CGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTG	B3
og-gox	AACAAGAGGGTCACGAGCCCTTGCGGATTAATCGTGTGACCGTTCTCTTCTATAAGAATGCCCTTGGTAAA	C3
og-gox-2	CCAAGGACTTGATCAAAGGCACAGTGGACTTGATGAGGTTACGGAGTGCCTTAGCTTGCTCCTTCACCTT	D3
op-FMV34S-2	ATATAGAGGGAATTCTTTTGTGGTCGTCACTGCGTTCGTCATACGCATTAGTGAGTGGGCTGTCAGGACA	A4
op-35S	ATATAGAGGAAGGGTCTTGCGAAGGATAGTGGGATTGTGCGTCATCCCTTACGTCAGTGGAGATATCACAT	B4
op-TA29	AAACCGGATAGTGACAAAGTCACATATCCATCAAACTTCTGGTGCTCGTGGCTAAGTTCTGATCGACATGG	C4
op-SsuAra-2	TGAGGTTAATTTTACTTGGTAACGGCCACAAAGGCCTAAGGAGAGGTGTTGAGACCCTTATCGGCTTGAA	D4
op-NOS	TTGCTAGCTGATAGTGACCTTAGGCGACTTTTGAACGCGCAATAATGGTTTCTGACGTATGTGCTTAGCTC	E4
ot-35S	TCTTATATGCTCAACACATGAGCGAAACCCTATAAGAACCCTAATTCCCTTATCTGGGAACTACTCACA	A5
ot-E9T	TGAGAATGAACAAAAGGACCATATCATTCATTAACTCTTCTCCATCCATTTCCATTTCACAGTTCGATAGC	C5
ot-NOS-4	ACATGCTTAACGTAATTCAACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGATTCAATCT	B5
ot-Tr7-2	GCAAGTTTAAATTCAGAAATATTTCAATAACTGATTATATCAGCTGGTACATTGCCGTAGATGAAAGACTGAGTGCG	D5
ot-OCS	GGACCGGGTTGAATGGTGCCCGTAACTTTCGGTAGAGCGGACGGCCAATA	E5
or-CTP-a	CACCATTGCAGATTCTGCTAACTTGCGCCATCGGAGAAACTTCAATTGAAGCAATCGAAGAATGGGGATTG	A6
or-CTP-b	ACTTGGGGTTTATGGAAATTGGAATTGGGATTAAGGGTTTGTATCCCTTGAGCCATGTTGTTAATTTGTGC	B6
or-CTP-c	TCTCAAACTTCTTCTTTCCAATCGGAGGCCACACCTGCATGCAGTTAACTCTTCCGCCGTTGCTTGTGAT	C6

One possible means of overcoming these limitations is hybridization of labeled PCR products to a DNA microarray. Because of its capacity to accommodate hundreds to thousands of individual elements, an array allows the simultaneous detection of potentially any amplifiable GM sequence present in a sample. The combination of nucleic acid amplification strategies used with the extensive screening capability of microarray technology results in a high degree of sensitivity, specificity and throughput capacity. One approach for coupling nucleic acid amplification and microarray technology is to use multiplex PCR to amplify a number of discreet targets that are subsequently detected using a DNA microarray. This approach takes advantage of the inherent sensitivity of PCR, while eliminating the need to visualize the amplification products on a gel. Furthermore, interference from nonspecific amplified products is minimized, and products that are too faint to be readily detected on a gel are easily detectable on an array, improving the sensitivity and allowing for more flexibility with an increased number of primers in the multiplex reaction.

Several DNA microarray approaches have been developed for use in combination with multiplex PCR for the detection of GMOs: a multiplex quantitative DNA array-based PCR method for the detection and quantification of seven different transgenic maize events (*7*); a ligation detection reaction combined with a universal array approach for the detection of Bt176 transgenic maize (*8*) and subsequently for the detection of four transgenic maize, Roundup Ready soybean, and two endogenous control genes (*9, 10*); a peptide nucleic acid array platform, also for the detection of four transgenic maize, Roundup Ready soybean, and two endogenous control genes (*11*); a method involving two separate multiplex reactions coupled with an oligonucleotide array for the detection of Roundup Ready soybean and transgenic maize (*12*); multiplex PCR coupled with an oligonucleotide array platform capable of detecting 21 unique GM elements or endogenous sequences between three distinct arrays (*13*); a method which utilizes a limited numbers of PCR reactions combined with an array containing 20 different capture probes for the detection of nine specific GM events, five plant species, and three GMO screening elements (*14*); as well as a multiplex-array method for the detection of specific integration junction sequences for one GM soybean and six GM corn events (*15*).

Here we describe a method utilizing a single multiplex PCR coupled with an oligonucleotide DNA array capable of simultaneously detecting the 12 approved GM canola lines in Canada. The assay includes construct-specific elements for identification of approved lines, common elements (e.g., CaMV 35S promoter, *Agrobacterium tumefaciens* nos terminator, or *npt*II gene) for screening of unidentified or unapproved lines, a canola-specific endogenous control, and endogenous genes from heterologous crops to serve as additional controls in mixed crop species samples.

MATERIALS AND METHODS

Materials. Certified seed of non-GM canola (*B. napus* 'Westar', 'Hyola 401', 'AC Excel') and the GM canola lines Oxy235, MS8/RF3, MS1/RF2 (PGS2), MS1/RF1 (PGS1), HCN92 (synonym Topas19/2), and T45 (synonym HCN28; Bayer Crop Science, formerly Aventis) were obtained from the commercial seed market. Transgenic Roundup Ready (GT73; synonym RT73) and non-GM canola (*B. napus* 'Sponsor') seeds were provided by Monsanto Co. (St. Louis, MO). Canola meal was obtained from an industrial canola oil processing plant. Commercial animal feed (proprietary blend) was purchased at a local market.

DNA Extraction. Genomic DNA was extracted and purified from all samples using a modified Wizard (Promega) protocol (*16*). DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Montchanin, DE), and sample purity was determined by measuring the A_{260}/A_{280} ratio. Extracts (200 ng) were further analyzed by electrophoresis on a 0.8% agarose gel containing 0.1 μg/mL ethidium bromide.

Oligonucleotide Primers and Probes. PCR primers and probes were designed using PrimerSelect software (DNASTAR, Inc., Madison, WI). Primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA), and probes were purchased from Operon Biotechnologies, Inc. (Huntsville, AL) or Integrated DNA Technologies, Inc. The 70-mer oligonucleotide probes (**Table 1**) were designed based on a narrow melting temperature (T_m) range across all probes (approximately 69.5 °C), uniqueness of nucleotide sequences, and sufficient nucleotide complexity with approximately 45% GC content. Occasion-

Table 2. Multiplex PCR Primer Sequences and Targets

target	amplicon size (bp)	primer	orientation	sequence (5′−3′)
FMV 34S promoter	145	pFMV-F2	forward	CTAGTACAAGTGGGGAACAAAATAACG
		pFMV-R2	reverse	ATCTGATGATCCTTCAAATGGGAATGA
cp4-epsps	190	EPSPSs-F1	forward	AGCCGTCCAGCAACTGCTCGTAAG
		EPSPS-R1	reverse	TTCTGGCACCCATAGCTTGCATAG
oxy	207	Oxy-F2	forward	CAACGGCAGCCTGCGGTGTCA
		Oxy-R2	reverse	CTCTGGGCTATATTTTCTGCGTCTGGA
nos terminator[a]	225	Nos-F2	forward	CGTTCAAACATTTGGCAATAAAGT
		Tnos-R1	reverse	GCGCGATAATTTATCCTAGTTTG
bar	288	Bar-Fmp2	forward	GCACGCAACGCCTACGACTGGAC
		Bar-R2	reverse	GGGCGGTACCGGCAGGCTGAA
nptII	233	nptII-F1	forward	GGGAAGGGACTGGCTGCTATT
		nptII-R1	reverse	GCCCCTGATGCTCTTCGTC
pat	238	Pat-Fmp2	forward	GAGGTTGAGGGTGTTGTGGCTGGTATT
		Pat-Rmp2	reverse	ATCCCAAAGCCTCATGCAACCTAACAG
CaMV 35S promoter	123	p35S-cf3	forward	CCACGTCTTCAAAGCAAGTGG
		p35S-cr4	reverse	TCCTCTCCAAATGAAATGAACTTCC
CaMV 35S terminator	207	t35S-F1	forward	AGGCATGCCCGCTGAAAT
		35STR1	reverse	TACCCCTGGATTTTGGTTTTAG
cruciferin	190	Cru-F2	forward	GGTCGCATCGAGGTGTGG
		Cru-R2	reverse	CACGAATTTGAATCTCGATACTCA

[a] From *Agrobacterium tumefaciens*.

ally, oligonucleotide length was adjusted to account for variations in GC content, and to normalize T_m values. The primers (**Table 2**) targeted nucleotide sequences flanking the respective oligonucleotide probes. Because PCR products were not intended to be resolved on a gel, amplicon sizes were designed to be <300 bp for more efficient amplification. Potential primer and probe sequences were analyzed for specificity by comparison with known gene sequences using the BLAST N search program provided by the National Center for Biotechnology Information.

Multiplex PCR and Cy3-ULS Labeling. Multiplex PCR assays were carried out using a GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA). A total reaction volume of 50 μL contained 100 ng of genomic DNA, 20 mM Tris HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.3 mM dNTP, 2 units FastStart *Taq* DNA polymerase (Roche Applied Science, Laval, Canada) and the following primer concentrations: 0.2 μM Bar-Fmp2, Bar-R2, nptII-F1, nptII-R1, Pat-Fmp2, Pat-Rmp2; 0.3 μM Cruc-F2, Cruc-R2, EPSPSs-F1, EPSPS-R1; 0.4 μM p35S-cf3, p35S-cr4, pFMV-F2, pFMV-R2; 0.5 μM Oxy-F2, Oxy-R2; 0.7 nosF2, tnos-R1; 0.8 μM t35S-F1, 35STR1. Duplicate 50 μL reactions for each template type were run. Reactions ran under the following conditions: initial denaturation at 94 °C for 5 min; 35 cycles of 30 s at 94 °C, 30 s at 54 °C, 40 s at 72 °C; and a final extension at 72 °C for 7 min. A portion of the amplified products (10 μL) was analyzed visually by electrophoresis on a 2% (wt/vol) agarose gel containing 0.1 μg/mL ethidium bromide.

Following electrophoresis, the remainder of the amplified products, pooled from both reactions, was purified using the Qiagen MinElute PCR purification kit (Mississauga, Canada), according to manufacturer's instructions. The DNA was eluted with 12 μL sterile, UV-treated water. Labeling of purified PCR products was performed using either the Amersham Biosciences Cy3-ULS labeling kit (Québec, Canada), or the Kreatech ULS aRNA Fluorescent Labeling Kit (Cy3-ULS, Kreatech Biotechnology, Amsterdam, The Netherlands). In both cases, 0.5 U of Cy3-ULS was used to label 500 ng input cDNA in a total reaction volume of 20 μL (Amersham kit) or 10 μL (Kreatech kit). The labeling reaction was incubated at 85 °C for 15 min, followed by removal of unincorporated dye by purification using the Qiagen MinElute PCR purification kit, as above, or Krea*pure* columns (Kreatech Biotechnology). Label incorporation was quantified using a NanoDrop ND-1000 spectrophotometer.

GM Canola Mixture Composition. A mixture of GM canola template which included all multiplex PCR targets was made by combining 25 ng each of the GM canola lines GT73, Oxy235, MS8/RF3, and HCN92, for a total of 100 ng DNA in a 50 μL reaction volume. Multiplex PCR, Cy3 labeling, and purifications were carried out as above.

Singleplex PCR. To verify the amplification of specific product from each primer pair in the multiplex PCR, singleplex reactions were carried out using a GeneAmp PCR System 9700 under the same conditions as the multiplex PCR. A total reaction volume of 50 μL contained 100 ng of genomic DNA; buffer, $MgCl_2$, dNTP, and *Taq* polymerase at concentrations as above; and a primer concentration of 0.3 μM. Cycling conditions were the same as for the multiplex PCR.

Real-Time PCR. Real-time PCR for the detection and quantification of GT73 canola was carried out using an Applied Biosystems 7500 Real Time PCR system (Foster City, CA) according to the procedure recommended by Monsanto Co. (St. Louis, MO) (*17*), except a total reaction volume of 25 μL was used.

Microarray Construction. Aminosilane coated slides (Nexterion Slide A+, Schott North America Inc., Louisville, KY) were used as solid supports to which oligonucleotide probes were linked. Oligonucleotide probes were diluted to a final concentration of 10 − 50 μM in spotting solution (50% DMSO and 1% CHAPS) and transferred to a sterile 384-well polystyrene plate (Nalge Nunc International, Rochester, NY) for printing. Probes were printed in triplicate onto slides using an *ArrayIt* SpotBot personal microarrayer (TeleChem International, Inc., Sunnyvale, CA). Printed slides were stored in containers overnight, followed by probe immobilization by cross-linking at 150 mJ using a Stratagene Stratalinker 1800 UV cross-linker (La Jolla, CA). Cross-linked slides were stored in slide boxes at room temperature until use.

Sample Hybridization. Printed slides were prehybridized at 42 °C for 45 − 60 min in preheated buffer (5× SSC, 1% BSA, 0.1% SDS). Purified Cy3-ULS labeled amplicons (20 pmol) were brought to a volume of 50 μL with hybridization buffer (5× SSC, 0.1% SDS, 50% formamide), heat denatured at 95 °C for 3 min, and hybridized in a 42 °C water bath overnight in sealed hybridization chambers (Die-Tech, San Jose, CA). Following hybridization, slides were washed sequentially, with agitation, in solutions of increasing stringency: once with wash 1 (2× SSC, 0.1% SDS) for 5 min at hybridization temperature; twice with wash 2 (0.1× SSC, 0.1% SDS) for 3 min each at room temperature; and twice with wash 3 (0.1× SSC) for 3 min each at room temperature. Slides were then spin-dried for 5 min at 1000 rpm. All posthybridization steps were performed in a dark environment to prevent degradation of the Cy3 fluorophore used to label the target amplicons.

Signal Detection. The slides were scanned using a GeneFocus DNAscope LM+ scanner (Biomedical Photometrics, Inc., Waterloo, Canada) and visualized with MACROview software (Biomedical Photometrics, Inc.). Quantitative estimates based on fluorescence intensities were made with the Amersham Bioscience ImageQuant TL software (GE Healthcare, Pittsburgh, PA) using the spot edge average

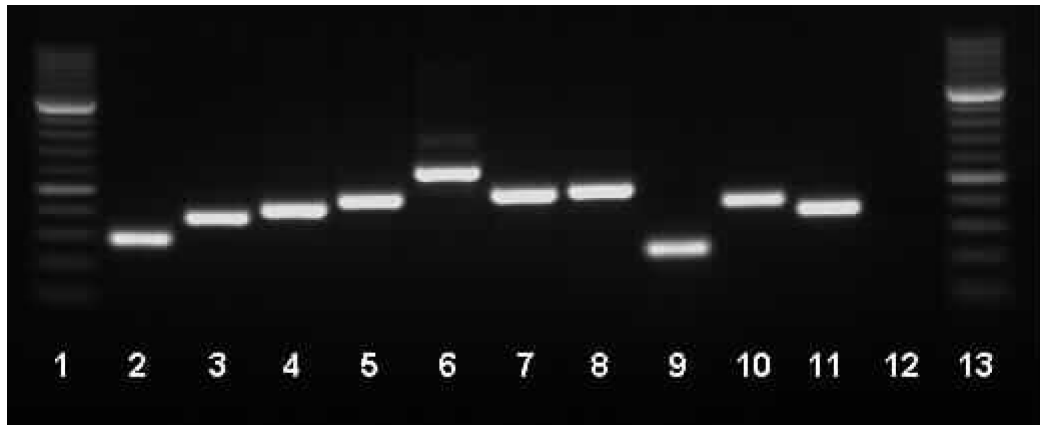

Figure 1. Singleplex PCR amplifications with the 10 primer pairs used in the canola array multiplex. Lanes 1 and 13, 50 bp ladder; lane 2, pFMV-F2/R2; lane 3, EPSPSs-F1/EPSPS-R1; lane 4, Oxy-F2/R2; lane 5, nosF2/tnos-R1; lane 6, Bar-Fmp2/Bar-R2; lane 7, nptII-F1/R1; lane 8, Pat-Fmp2/Rmp2; lane 9, p35S-cf3/cr4; lane 10, t35S-F1/35STR1; lane 11, Cruc-F2/R2; lane 12, no template control (NTC).

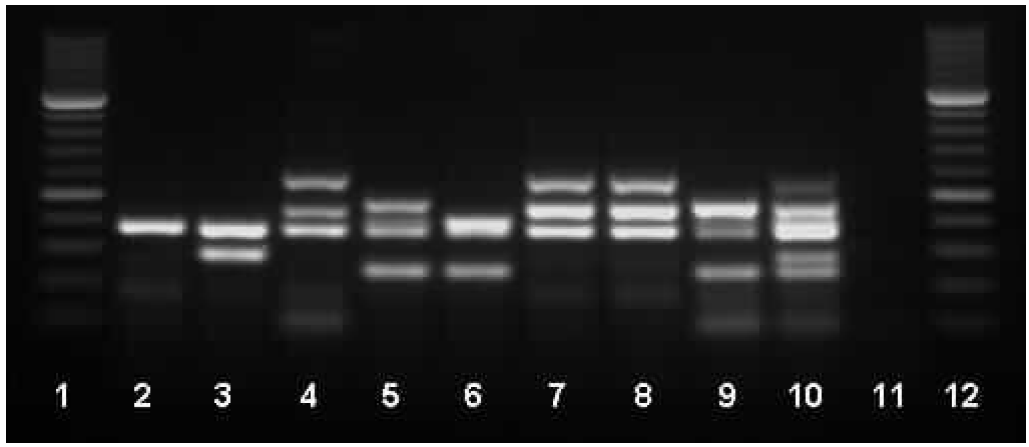

Figure 2. Canola array multiplex PCR amplification profiles of various templates. Lanes 1 and 12, 50 bp ladder; lane 2, non-GM canola; lane 3, GT73; lane 4, MS8/RF3; lane 5, T45; lane 6, Oxy235; lane 7, MS1/RF2; lane 8, MS1/RF1; lane 9, HCN92; lane 10, GM mixture (GT73, MS8/RF3, Oxy235, HCN92); lane 11, NTC.

background subtraction method. The mean value from the set of triplicate spots with the highest signal intensity was arbitrarily set to 100%. The background was calculated from the mean of the six replicates per array spotted with spotting solution only. Results were considered positive when the average signal intensity of the triplicate spots from the elements on the array was at least 10-fold greater than the background signal intensity or, in situations where the background intensity was particularly low, a minimum relative intensity of 2% was required for an initial positive score.

Array Specificity and Assay Sensitivity. Oligonucleotide probes were individually validated for functionality and specificity by amplification of specific transgene sequences from appropriate GM canola lines corresponding to each probe sequence, and hybridization of amplicons to the array as described above. Assay sensitivity was assessed using decreasing concentrations of GM canola target DNA diluted in non-GM canola DNA. Concentrations of 5, 1, 0.5, 0.1 and 0% GM canola were tested, in accordance with food labeling thresholds imposed by the EU of 0.9% for 'adventitious or technically unavoidable' traces of GMOs in food and feed (Regulations EC 1829/2003, 1830/2003). Sensitivity limits were established for the three Canadian registered GM canola lines GT73, T45, and MS8/RF3, and were determined as the lowest concentration giving signal for *every* transgene of the particular GM line tested with its specific array probe.

RESULTS

Multiplex PCR Development. The approach used in this work relies on multiplex PCR amplification of specific targets followed by hybridization of the labeled amplicons to an oligonucleotide microarray for identification. For this purpose, we developed a single multiplex PCR consisting of 10 primer pairs (**Table 2**) targeting construct-specific GM elements, common GM elements, and a canola-specific gene. The multiplex assay was designed to simultaneously detect at least two elements from each of the 12 approved GM canola lines in Canada. As an initial step in the multiplex strategy, specific primers with similar T_m values were designed, based on the nucleotide sequences flanking the respective oligonucleotide probes, and tested individually for specificity and good amplification under the same conditions ultimately used for the multiplex PCR (**Figure 1**). Acceptable primer pairs were systematically added to the multiplex reaction, and the resulting products were monitored and evaluated by separation on an agarose gel as well as by hybridization to the array. Several primer pairs amplified products of similar size, resulting in poor resolution of these amplicons on agarose gels; however, the amplification profile of each template type gave an indication of reaction efficacy before continuing with array hybridizations. Typical agarose gel profiles from the amplification of various canola templates using the final canola multiplex reaction are shown in **Figure 2**.

Oligonucleotide Array Development. The canola-specific array contains 26 different elements (**Table 1**) which can be categorized into six classes, including those targeting: (1) plant-specific genes; (2) transgenes; (3) marker genes; (4) promoters; (5) terminators; and (6) transit peptides. Prospective oligonucleotide probes were independently validated for functionality and

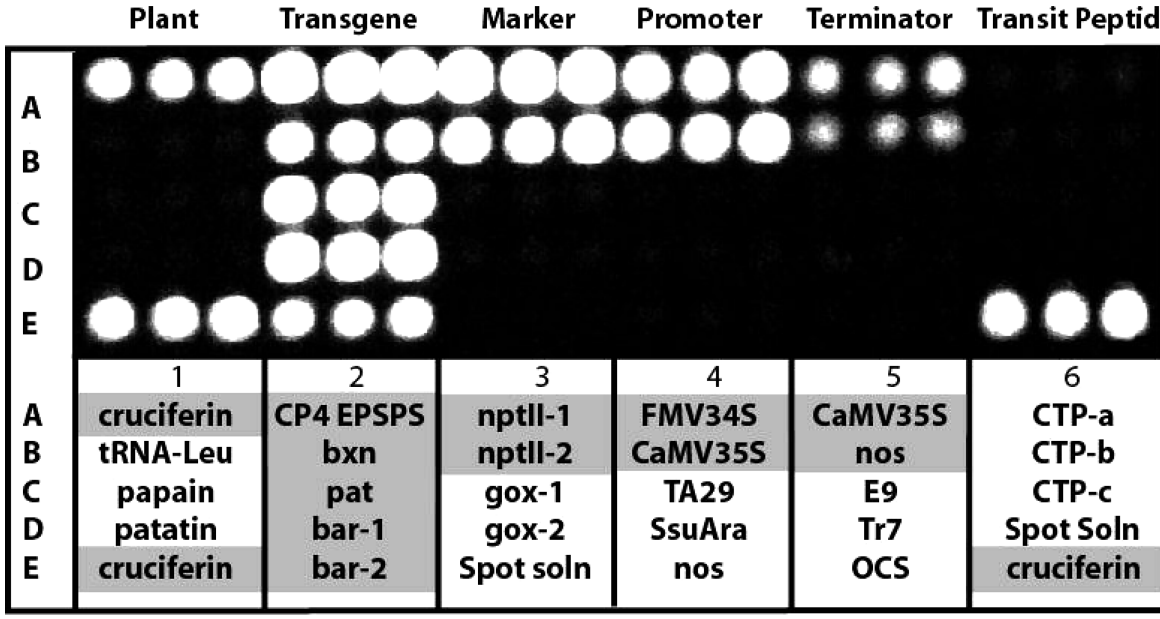

Figure 3. Oligonucleotide microarray format. Elements are printed in triplicate. A mixture of GM canola DNA which included all multiplex PCR targets was amplified and hybridized to the array (top). Highlighted text denotes expected elements targeted by the canola multiplex PCR (bottom).

specificity by hybridization of individual and multiplexed amplicons from appropriate GM canola lines corresponding to each probe sequence. Any probe showing cross-hybridization with nonspecific target was eliminated, and a new probe was subsequently designed and tested. The array elements were spotted in triplicate and, for increased confidence in target identification, some elements were designed to target different regions of the same PCR amplicon (**Figure 3**, bottom).

Assay Specificity. A great deal of effort was spent optimizing both the canola multiplex PCR and the microarray design and hybridization conditions. Final optimized conditions are outlined in the materials and methods.

The specificity of the elements spotted onto the array was assessed by the hybridization of purified and labeled PCR products amplified from various GM canola events and non-GM canola using the canola multiplex PCR primer pairs. A mixture of GM canola template which included all multiplex PCR targets was amplified and hybridized to the array; all expected elements were detected and no hybridization to nontargeted elements was observed (**Figure 3**, top). Analysis of the array with ImageQuant TL revealed that even the nos terminator, which gave the weakest hybridization signal of 1.7%, was still over 50 fold more intense than background which was measured at 0.03%. Typical array results for a non-GM canola ('AC Excel') are shown in **Figure 4A**, with hybridization to the endogenous canola-specific cruciferin elements only. Similar results were obtained for the non-GM varieties 'Westar', 'Hyola 401', and 'Sponsor' (data not shown). Hybridization specificity for the GM canola events Oxy235, MS1/RF1, MS1/RF2, HCN92 (**Table 3**; **Figure 4**) and GT73, T45, and MS8/RF3 (**Table 3**) was determined using a 5% mixture of GM-canola in non GM-canola DNA in the PCR. For each event, two sets of 12 arrays were analyzed and scored for the presence/absence of a positive signal to each element. Each set of 12 arrays was hybridized with labeled PCR product from a separate reaction. Arrays with excessively high background signals of 10% or greater were discarded from the analysis. Data from the remaining arrays are tabulated in **Table 3**, with positive signals indicated by a black bar. Of the 24 arrays from Oxy235 canola, six had excessively high backgrounds and were discarded; of the 18 remaining arrays, all gave positive hybridization signals to the cruciferin, p35S and bxn elements, and 15 gave a positive signal to the nos terminator element (**Table 3**; **Figure 4B**). All 24 arrays from MS1/RF1 canola were acceptable (**Table 3**; **Figure 4C**) and gave positive signals to the cruciferin, bar1, nptII 1 and nos terminator elements, while 22 were positive to the bar 2 element and only one positive to the nptII 2 element. Results from MS1/RF2 canola were similar (**Table 3**; **Figure 4D**), with positive signals on all 24 arrays to the cruciferin, bar 1, bar 2, and the nos terminator elements, 23 positive to the nptII 1 and 11 positive to the nptII 2 elements. Of the 23 good arrays from HCN92 (**Table 3**; **Figure 4E**), all gave positive signals to the cruciferin (with the exception of one of the three elements on one array), and pat elements. Twenty-three arrays were positive for the p35S, nptII 1, and 35S terminator elements and only 17 were positive to the nptII 2 element. All 24 arrays for GT73 canola (**Table 3**) hybridized to the cruciferin, pFMV34S, and CP4 EPSPS elements, with two false positives signals to the SsuAra promoter element and one false positive to the CTP-a element. Product from T45 canola (**Table 3**) hybridized to the cruciferin, p35S, and 35S terminator elements in all 24 arrays, and in 23 arrays for the pat element. One array gave a false positive to the EPSPS element, and there were four false positives to the CTP-b element. Product from MS8/RF3 canola (**Table 3**) hybridized to the cruciferin, bar, and nos terminator elements in all 24 arrays, with one array giving a false positive to the nos promoter element.

Overall, from 161 arrays containing 30 elements each, for a total of 4830 elements, there were only nine false positives and 48 false negatives among all the lines tested (**Table 3**). The majority of the false negatives (37) were for the nptII 2 element. Since another element, nptII 1, targeted the same gene sequence, the effects of the false negatives to nptII 2 did not prevent detection of nptII transgenic sequences when present in the canola lines.

Assay Sensitivity. Assay sensitivity was assessed for the three Canadian registered GM canola lines GT73, T45, and MS8/RF3. For each line, concentrations of 5, 1, 0.5, 0.1, and 0% GM canola target DNA in non-GM canola DNA was mixed and assayed. Eight sets of three arrays (24 arrays total) were analyzed for each canola line at each concentration and scored as positive or negative for each element. At least 2 of the 3

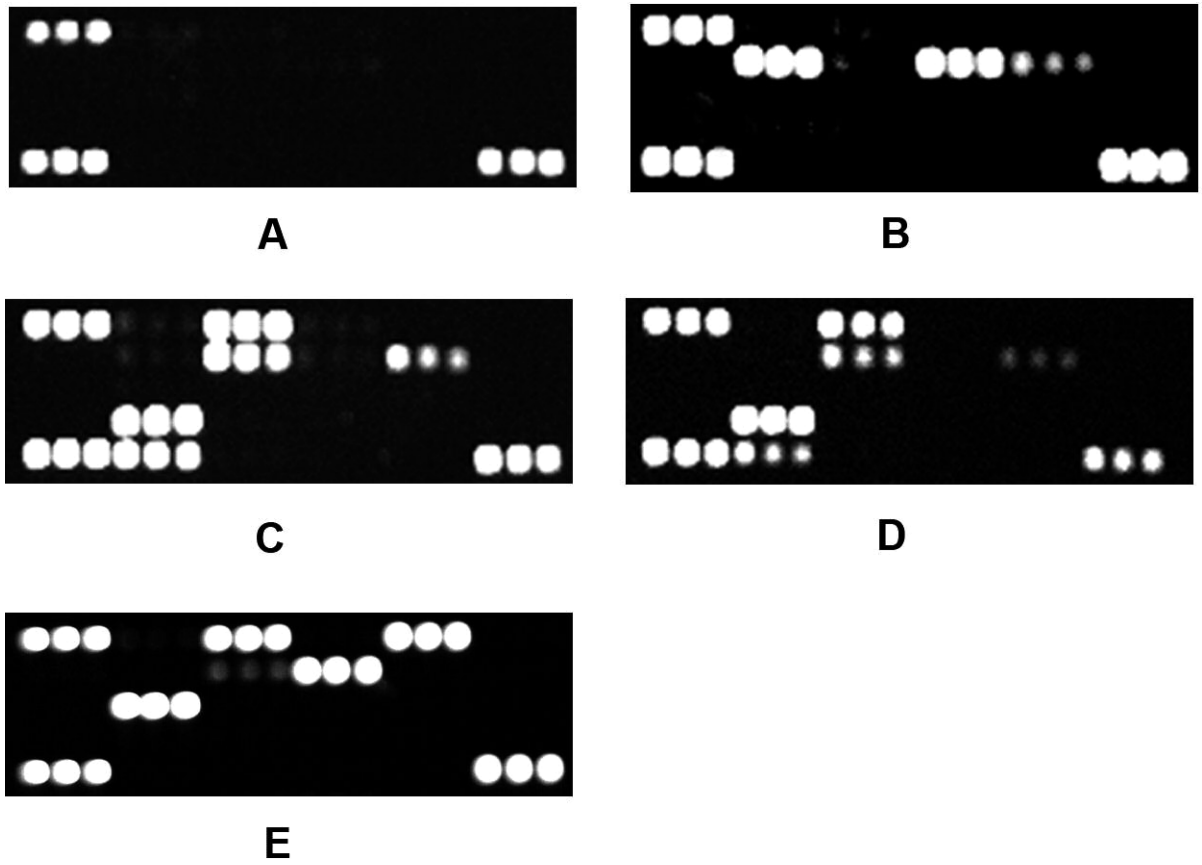

Figure 4. Specificity of the canola microarray. Detection of oligonucleotide elements after hybridization with Cy3-labeled multiplex PCR amplicons from *B. napus* 'AC Excel' non-GM canola (**A**); and the GM lines Oxy235 (**B**); MS1/RF1 (**C**); MS1/RF2 (**D**); and HCN92 (**E**).

arrays in a set had to score positive for an element to be considered positive. Intensity values for a representative set of three arrays for the lowest positive concentration for each line are shown in **Table 4**. For GT73, all eight arrays sets gave the expected positive and negative hybridization results at the lowest concentration of 0.1%, with no false positives/negatives in any of the 24 individual arrays. Identical results were obtained for both T45 and MS8/RF3 at 0.5%. At 0.1%, one of the array sets from the T45 line gave a weak signal slightly below background in two of the three arrays for the 35S terminator. Of the remaining seven sets of three arrays, a single array in three of the sets also gave a signal for the 35S terminator that was slightly below background, but this did not affect the overall score. All other expected elements in this line (cruciferin, p35S, and pat) gave strong signal intensities. The 0.1% MS8/RF3 sample gave positive signal for all expected targets except for the nos terminator, which was below background in seven of the eight array sets and was deemed below the limit of detection for MS8/RF3.

GM Canola Detection in Complex Matrices. Application of the canola multiplex-array assay for the practical detection of GM canola from material other than seed was determined using DNA extracted from two major groups of processed foods derived from canola: canola meal and animal feed. As shown in **Figure 5**, amplified product from canola meal, poultry and gamebird grower mash, hog pellets, and horse feed all hybridized to the cruciferin elements, indicating presence of canola in the samples, as well as to various GM elements on the array: product from canola meal hybridized to the cruciferin, pFMV34S, CP4 EPSPS, p35S, npt II, bar, and nos terminator elements; product from poultry and gamebird grower mash hybridized to the cruciferin, pFMV34S, CP4 EPSPS, p35S, bar, and nos terminator elements; product from hog pellets hybridized to the cruciferin, pFMV34S, CP4 EPSPS, p35S, and nos terminator (weakly) elements; and product from horse feed hybridized to the cruciferin, pFMV34S, CP4 EPSPS, p35S, nos terminator, pat and bar elements. Hybridization of an additional eight sets of three arrays (24 arrays total) for the canola meal and poultry and gamebird grower mash products was performed to check for variability of detection (**Table 3**). For the canola meal, all eight sets were positive for cruciferin, pFMV34S, CP4 EPSPS, p35S, npt II, bar and nos terminator elements, three sets were positive for the CaMV-35S terminator element, and one set was positive for the pat element. Scored individually, however, 11 of the 24 arrays were positive for the CaMV-35S terminator and nine were positive for pat. Product from the poultry and gamebird grower mash scored positive in all eight array sets for cruciferin, pFMV34S, CP4 EPSPS, p35S, bar and nos terminator elements.

For all processed food samples (meal, mash and feed), signals to the pFMV34S and CP4 EPSPS elements were very strong, indicating the presence of GT73 canola. To confirm the presence of GT73, real-time PCR was performed using a method specific for the detection and quantification of GT73 canola (*17*). The canola meal, gamebird mash, hog pellets, and horse feed contained 15.5%, 2.1%, 2.6%, and 1.0% GT73 canola, respectively (data not shown). Hybridization to GM elements other than pFMV34S and CP4 EPSPS indicates the presence of additional GM events in the processed samples, either from canola or other species.

DISCUSSION

We have developed a method utilizing a single multiplex PCR coupled with an oligonucleotide DNA array capable of simul-

Table 3. Specificity of the Canola Microarray

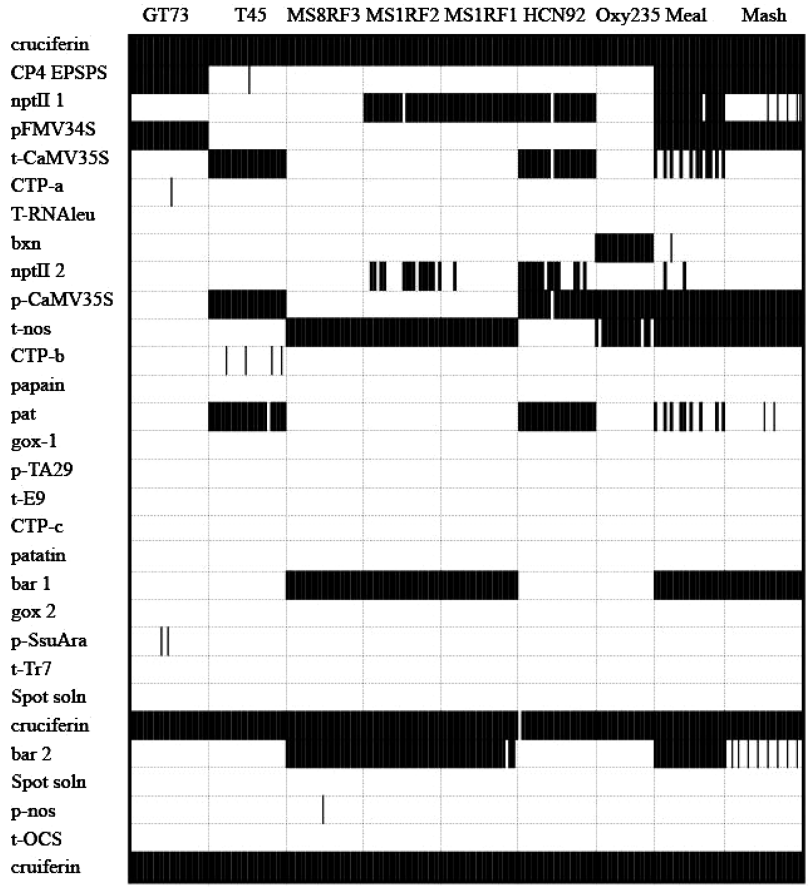

Table 4. Relative Array Signal Intensities

element	0.1% GT73-1	0.1% GT73-2	0.1% GT73-3	0.1%T45-1	0.1%T45-2	0.1%T45-3	0.5% MS8/RF3-1	0.5% MS8/RF3-2	0.5% MS8/RF3-3
cruciferin	**83.2**	**80.6**	**92.4**	**83.3**	**80.5**	**99.3**	**99.0**	**93.7**	**85.3**
CP4 EPSPS	**60.6**	**66.6**	**50.0**	0.1	0.2	0.2	1.0	0.7	0.4
nptII 1	0.2	0.0	0.6	0.1	0.2	0.0	0.2	0.1	0.1
p-FMV34S	**42.5**	**34.8**	**23.3**	0.3	0.2	0.2	0.4	0.2	0.3
t-CaMV35S	0.1	0.0	0.3	**9.7**	**23.0**	1.7	0.0	0.0	0.1
CTP-a	0.2	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.1
T-RNA leu	0.0	0.0	0.2	0.0	0.1	0.0	0.0	0.0	0.0
bxn	0.4	0.7	0.9	0.3	0.2	0.3	0.1	0.0	0.0
nptII 2	0.2	0.3	0.4	0.1	0.1	0.1	0.1	0.1	0.1
p-CaMV35S	1.1	1.2	1.1	**41.5**	**78.0**	**6.6**	0.3	0.3	0.2
t-nos	0.1	0.1	0.2	0.0	0.1	0.0	**2.9**	**4.1**	1.6
CTP-b	0.2	0.0	0.0	0.3	0.3	0.1	0.1	0.0	0.0
papain	0.1	0.1	0.3	0.1	0.1	0.2	0.1	0.0	0.0
pat	0.1	0.2	0.1	**4.3**	**5.8**	**3.8**	0.1	0.0	0.2
gox 1	0.2	0.2	0.1	0.0	0.0	0.1	0.1	0.0	0.1
p-TA29	0.1	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0
t-E9	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0
CTP-c	0.2	0.0	0.0	0.1	0.0	0.1	0.1	0.1	0.0
patatin	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0
bar 1	0.3	0.3	1.2	0.2	0.4	0.1	**19.4**	**19.3**	**8.7**
gox 2	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
p-SsuAra	0.4	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.1
t-Tr7	0.1	0.0	0.1	0.1	0.0	0.1	0.1	0.0	0.1
Spot soln	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.1	0.0
cruciferin	**100.0**	**100.0**	**100.0**	**85.7**	**88.4**	**100.0**	**100.0**	**100.0**	**90.7**
bar 2	0.2	0.2	0.3	0.1	0.1	0.1	**2.1**	**2.3**	0.9
spot soln	0.1	0.2	0.2	0.1	0.0	0.2	0.0	0.1	0.0
p-nos	0.1	0.1	0.2	0.3	0.9	0.1	0.0	0.0	0.1
t-OCS	0.0	0.1	0.1	0.0	0.1	0.1	0.0	0.0	0.0
cruciferin	**91.1**	**94.8**	**93.8**	**100.0**	**100.0**	**97.7**	**91.0**	**91.2**	**100.0**
background (spot soln)	0.03	0.13	0.13	0.05	0.08	0.11	0.09	0.09	0.04
background 10×	0.3 (2.0)	1.3 (2.0)	1.3 (2.0)	0.5 (2.0)	0.8 (2.0)	1.1 (2.0)	0.9 (2.0)	0.9 (2.0)	0.4 (2.0)

taneously detecting at least two elements from each of the 12 approved GM canola lines in Canada. This is a useful qualitative method for the rapid screening of plants and plant products for the presence of GM canola events. For regulatory purposes, GM canola detected and identified using this method can be further analyzed using real-time PCR for quantitative analysis. The

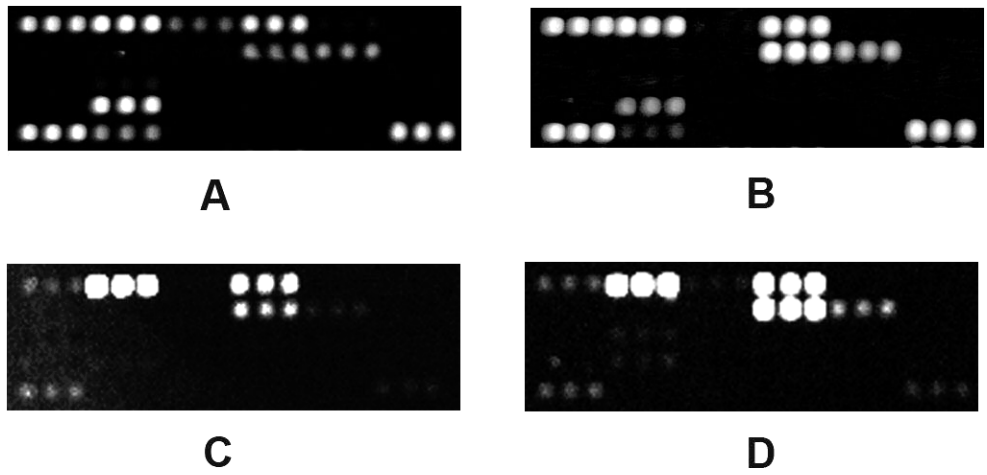

Figure 5. Detection of oligonucleotide elements after hybridization with Cy3-labeled multiplex PCR amplicons from processed foods: canola meal (**A**); poultry and gamebird grower mash (**B**); hog pellets (**C**); and horse feed (**D**).

assay includes construct-specific elements for identification of approved lines, common elements (e.g., CaMV 35S promoter, *Agrobacterium tumefaciens* nos terminator, or *npt*II gene) for screening of unidentified or unapproved lines, a canola-specific endogenous control, and endogenous genes from heterologous crops to serve as additional controls. This method has several advantages over other nucleic acid amplification techniques that target multiple sequences. Used on its own, end-point multiplex PCR is a widely applied method of detecting multiple GM targets in a single assay. For example, James et al. (*18*) developed a multiplex comprised of six primer pairs targeting four GM canola lines as well as an endogenous canola gene and a plant-specific gene. However, used alone, end-point multiplex PCR becomes problematic when expanding the number of targets in the reaction due to increased nonspecific amplification products and poor resolution of amplification products on a gel. The coupling of multiplex PCR with microarray hybridization is more specific, because the sequence of a substantial portion of the amplified PCR product must match the array oligonucleotide element sequence, rather than relying on a gross size estimate for identification. This also permits the use of less stringent PCR conditions, allowing for the incorporation of more primers, yet reliably amplifying the desired products. The disadvantage of real-time multiplex PCR is the limitation of targets that can be detected in a single reaction, due to the number of fluorophores that can be simultaneously detected in probe-based reactions, or the T_m restraints in SYBR Green-based reactions. Both probe- and SYBR Green-based real-time multiplex PCR assays have been developed for the detection of GMOs (*19, 20*), but simultaneously detect only two or three different targets, respectively.

Elements on our canola array were validated for functionality and specificity by hybridization with labeled amplified products of specific transgene sequences from appropriate GM canola lines. Each target sequence hybridized specifically to its corresponding array element. Furthermore, the hybridization pattern of elements on the microarray for amplified product from seven available GM canola lines was in concordance with the published data of the construct content of these lines. In most cases, background signals were very low and positive array signals were strong and easily identifiable by direct visual observation. This result was confirmed using the ImageQuant TL software to obtain relative quantitative intensity values for the array elements. To help normalize the data, the set of triplicate array elements with the most intense signal was assigned a mean value of 100% in ImageQuant TL. This was preferable to, for example, normalizing the data to the intensity of the control cruciferin elements, because while cruciferin often gave the most intense signal, this was not always the case, and is likely due to the different relative levels of target present in each sample and differences in amplification efficiency of the primer pairs in the multiplex PCR. Normalizing to the highest signal makes the assumption that overall amplification efficiency is relatively constant (at least for the purposes of this analysis) as compared to the specific amplification of cruciferin. Several methods were tried to determine spot background, and the spot edge average method was chosen because it gives good localized background intensity and is relatively tolerant of noise in the image (*21*). To set a threshold level for a positive result, we averaged the intensities of the six elements for the spotting solution control and set the threshold at 10-fold higher. In some cases, background as determined in this manner was exceptionally low, resulting in a very low threshold and, subsequently, a large number of false positives. To reduce these false positives, the threshold was determined to be 10-fold above background or a minimum of 2%, whichever was greater. It should be noted that the threshold levels as determined applies to the system of spotting/hybridization and array analysis used in this study. It is likely that the use of commercially spotted arrays or active mixing hybridization systems would result in re-evaluation of threshold levels.

Overall, very few false positives and negatives were observed among the lines tested. Each array contains 30 elements spotted in triplicate, with 161 arrays analyzed for GT73, T45, MS8/RF3, MS1/RF2, MS1/RF1, HCN92, and Oxy235 (**Table 3**), for a total of 4830 elements. Of these, only nine were false positives (0.2%) and were randomly distributed. A greater number of false negatives were observed (48), representing 1% of all elements analyzed. Most of the false negatives (37) were to the nptII 2 element, with only three false negatives to the nptII 1 element. Since both elements target the same gene, and considering the poor performance of the nptII 2 element, its presence is redundant and could be removed without affecting the ability to detect the nptII transgene in a sample. If we remove the nptII 2 data, the number of false negatives drops to 11, or 0.2%, similar to the number of false positives. As with the false positives, distribution of false negatives appears random. Some, such as the false negative for cruciferin in one array for HCN92 (**Table 3**), are due to failure of the SpotBot to print that particular triplet of spots. The detection of false positives/negatives does not appear to be related to the labeled PCR product, since hybridization of the same PCR product to multiple

arrays still results in random false positives/negatives on individual arrays. More likely, the randomness is due to either uneven spotting or hybridization. The facilities used to spot our arrays are not fully climate controlled and may have contributed to inconsistent spotting. A more controlled environment, or the use of commercially printed array slides, may result in higher consistency. Alternatively, the use of a hybridization system that allows active mixing of hybridization solution may also improve consistency and reproducibility of results. While the number of correctly scored elements was very high at 99.6% with only 0.4% false positives/negatives, if arrays are randomly analyzed in triplicate, to compensate for the slight variability between arrays, the number of false positives and negatives can be reduced further. As a result, for the analysis of assay sensitivity and detection of GM canola in meal and mash, eight sets of 3 arrays were analyzed both as a set (8), and as individual arrays (24).

The sensitivity of the assay was assessed using decreasing concentrations of GM canola target DNA diluted in non-GM canola DNA. Sensitivity was determined by analyzing three sets of arrays hybridized with the same labeled PCR product, with a total of eight array sets for each concentration tested. Sensitivity limits for the three Canadian registered GM canola lines GT73, T45, and MS8/RF3 were determined as the lowest concentration of GM canola that resulted in a positive signal for *every* element on the array expected to hybridize with labeled DNA amplified from a particular GM line. The GM lines GT73, T45, and MS8/RF3 gave the expected hybridization at concentrations of 0.1%, 0.1%, and 0.5%, respectively. This degree of sensitivity is more than sufficient to conform to the more strict European food and feed labeling threshold level of 0.9% for approved GM products. Levels of sensitivity observed in this study are similar with those from other reported GMO PCR-microarray methods. Using a two-step competitive multiplex PCR array-based method, Rudi et al. (*7*) reported detection limits of 0.1−2% for seven different GM maize events. Bordoni et al. used a ligation detection reaction-universal array (LDR-UA) approach, and reported a sensitivity of 0.1% for Bt176 GM maize (*8*) and reliably identified the presence of 0.5% transgenic events within complex mixtures of Roundup Ready soy and Bt11, MON810, GA21, and Bt176 maize (*9*). A similar LDR-UA platform utilizing primers targeting GM junction sequences developed by Peano et al. (*10*) could detect the five aforementioned GM events at 0.4%. Focusing on the same five GM events but using a peptide nucleic acid array platform, Germini et al. (*11*) reported high selectivity of these GM events at 5% GM content. More recently, and using multiplex PCR DNA array methods, Leimanis et al. (*14*) report detection limits of less than 0.3% for nine specific GM events, five plant species and three GMO screening elements, while Xu et al. (*15*) report detection limits of 0.5% for Roundup Ready soy and 1.0% for MON810 maize.

To test the practicality of our method for detection of GM canola from complex matrices, DNA extracted from processed canola meal and various animal feed was tested and detectable levels of GM material was observed from all samples. For example, GT73 canola could be positively identified in all of the samples, which was then verified and quantified using a real-time PCR assay specific for GT73. PCR quantification revealed levels as low as 1% GT73 in animal feed were clearly detectable on the microarray. This indicates that the method is effective for processed foods, even from complex matrices such as animal feed that may contain highly degraded DNA. DNA from both the canola meal and animal feeds showed significant degradation as determined from agarose gel electrophoresis (data not shown). The design of relatively short amplicons (<300 bp) in the multiplex PCR facilitates the efficient amplification of DNA from highly processed food samples.

GM elements other than those found in GT73 were also detected from the canola meal and animal feed samples, including the 35S promoter, nos terminator, and the *bar* transgene. The combination of the *bar* transgene and nos terminator indicate the possible presence of the GM canola line MS8/RF3. Alternatively, noncanola GM material could be present in the meal and feed samples. For instance, several GM maize constructs contain the 35S promoter, nos terminator, and/or *bar* transgene, with maize making up a greater proportion of the meal and feed samples than canola. Further development of the array to include additional elements targeting other endogenous plant genes would help identify the GM content in unknown samples that contain a mixture of different GM materials.

The coupling of multiplex PCR with oligonucleotide microarray hybridization offers a specific, sensitive, and high-throughput alternative to existing methods intended for multiple target detection. The current canola multiplex PCR amplifies at least two transgenic sequences found in each GM canola event approved in Canada. Unapproved events would likely contain one or more of these sequences and would also be detected. The array contains additional elements not yet targeted by the current multiplex PCR, and could be expanded easily to include several other elements. Development of other multiplex PCR assays targeting these elements is underway.

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