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Event-Specific Plasmid Standards and Real-Time PCR Methods for Transgenic Bt11, Bt176, and GA21 Maize and Transgenic GT73 Canola

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Since the 18th of April 2004, two new regulations, EC/1829/2003 on genetically modified food and feed products and EC/1830/2003 on traceability and labeling of GMOs, are in force in the EU. This new, comprehensive regulatory framework emphasizes the need of an adequate tracing system. Unique identifiers, such as the transgene genome junction region or a specific rearrangement within the transgene DNA, should form the basis of such a tracing system. In this study, we describe the development of event-specific tracing systems for transgenic maize lines Bt11, Bt176, and GA21 and for canola event GT73. Molecular characterization of the transgene loci enabled us to clone an event-specific sequence into a plasmid vector, to be used as a marker, and to develop line-specific primers. Primer specificity was tested through qualitative PCRs and dissociation curve analysis in SYBR Green I real-time PCRs. The primers were then combined with event-specific TaqMan probes in quantitative real-time PCRs. Calibration curves were set up both with genomic DNA samples and the newly synthesized plasmid DNA markers. It is shown that cloned plasmid GMO target sequences are perfectly suitable as unique identifiers and quantitative calibrators. Together with an event-specific primer pair and a highly specific TaqMan probe, the plasmid markers form crucial components of a unique and straighforward tracing system for Bt11, Bt176, and GA21 maize and GT73 canola events.

KEYWORDS: GMO; event-specific marker; PCR identification and quantification; GA21 maize; Bt176 maize; Bt11 maize; GT73 canola

INTRODUCTION

From April 18th, 2004 on, a very comprehensive labeling and traceability system for GM crops and derived food and feed products has come into effect within the Member States of the European Union. First, "horizontal" Directive 2001/18/EC regulates the experimental release and the placing on the market of genetically modified crops. This directive aims to protect human health and the environment with regard to the deliberate release of transgenic crops. Central ideas are the requirement for performing a risk evaluation prior to release and the need to propose a post-release monitoring plan (*1*). Second, when GM crops are intended for food or feed use, "vertical" regulations 1829/2003 and 1830/2003 have to be applied. Regulation (EC) number 1829/2003 on GM food and feed regulates the placing on the market of food and feed products containing or consisting of GMOs and provides for the labeling of such products (*2*). Labeling of food and feed products is mandatory in cases where more than 0.9% of the food ingredients, considered individually, is of GM origin. Regulation (EC) number 1830/2003 on traceability and labeling of GMOs introduces a harmonized system to trace and label GMOs and food and feed products produced from GMOs (*3*). Within the context of the present EU legislative framework, an applicant seeking authorization for a certain GM event also needs to provide a prevalidated event-specific identification method and appropriate reference samples (*2*). The Community Reference Laboratory (CRL) is in charge of receipt, preparation, storage, and distribution of the samples and of the conductance of a full-scale interlaboratory validation study of the proposed method (http://gmo-crl.jrc.it). The new regulatory framework

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in the EU more than once emphasizes the need of an adequate tracing system that might ensure greater consumer acceptance. It is in this framework that the present study was conducted.

PCR-based techniques, targeting well-chosen transgene-specific DNA segments, have been proven to be among the most adequate and efficient methods for the qualification and quantification of transgenic crops. This is primarily due to the stable nature of the DNA molecule as well as the extreme sensitivity of the PCR technology (*4*). Whereas conventional, qualitative PCRs detect the amplified DNA after the PCR has completely finished (end-point measurements), real-time PCR enables quantification by measuring the DNA in the linear-logarithmic phase. The fluorescent TaqMan chemistry is one of the most powerful tools for DNA quantification. According to the region that is targeted during PCR amplification, several levels of PCR specificity are distinguished (*5*). From a low to high level of specificity, we can differentiate screening for widely used regulatory elements (e.g., 35S promoter, nos terminator, ...), amplification of transgene specific regions (e.g., *bar* gene, *pat* gene, *cry* genes, ...), targeting cross-border construct-specific regions and finally event-specific targets. Event-specific methods target DNA regions that are unique for a specific transgene event. This can be the border junction between the inserted DNA and the flanking plant DNA or a rearranged DNA sequence that arose during DNA repair processes that drive the integration of the exogenous DNA. Qualitative and quantitative PCR methods have been published, targeting event-specific DNA sequences of Roundup Ready soybean (*6*−*9*), Mon810 maize (*10*−*11*), CBH-351 or StarLink maize (*12*), Bt11 maize (*13*−*14*), NK603 maize (*15*), and GA21 maize (*16*).

Quantitative real-time PCR methods involve the establishment of calibration curves based on the analysis of a set of calibrators with precisely known contents of the measured target. Because PCR measures DNA bioanalytes, pure DNA calibrators are preferable. Pure DNA reference materials (RMs) for PCR analysis of GMOs are favored in the framework of the modular approaches for GMO analysis and validation of GMO methods (*17*). In addition, DNA standards allow us to measure copy numbers of DNA targets and are in line with the new "haploid genome copy number unit" for measurement and expression of GMO contents (*18*). DNA calibrators are solutions containing either genomic or plasmid DNA sequences. Plasmid DNA markers containing cloned transgenic sequences have been used since 2001 (*7*) and are increasingly promoted as the standards of choice for GMO trace analysis (*10*, *15*, *19*−*23*). Thus far, event-specific plasmid DNA markers have been described only for Roundup Ready soybean (*7*), Mon810 maize (*10*), and NK603 maize (*15*).

In this paper, we describe four new event-specific marker systems, consisting of a set of primers, a TaqMan probe, and a single target plasmid (STP) standard. The marker systems allow event-specific identification and quantification of Bt176 maize, Bt11 maize, GA21 maize, and GT73 canola. Maize crop Bt176 (Ciba−Geigy) was introduced on the European market in 1997 under the then current Directive 90/220/EC (*24*). Bt11 maize (Novartis) received a 258/97/EC (*25*) authorization in 1998. In 2004, a marketing authorization under regulation (EC) 1829/2003 has been granted for Bt11 sweet maize (Syngenta Seeds) (http://europa.eu.int/comm/food/food/biotechnology/authorisation/comm_register_en. htm). Bt11 and Bt176 events express a herbicide tolerance trait, because of the presence of the *pat* gene and the *bar* gene, respectively, in combination with an insect resistant trait based on expression of a *Bacillus thuringiensis cry* gene. Event GA21 (Monsanto) harbors a modified maize *EPSPS* gene, which imparts tolerance to glyphosate herbicide (Roundup). Finally, GT73 canola (Monsanto) is a glyphosate-tolerant canola event that harbors both the *CP4 EPSPS* gene as well as the *goxv247* gene. At present (March 2005), GA21 and GT73 are not yet authorized; however, approvals under 1829/2003/EC for both events are underway (http://gmo-crl.jrc.it).

First, the transgene insertion loci of the events were characterized and sequenced. From the obtained sequence, a fragment was cloned into a plasmid vector, and subsequently, primers were designed, giving rise to a PCR amplicon of about 100 base pairs. The specificity of the primers was tested in conventional PCRs as well as in real-time PCRs using the fluorescent SYBR Green I dye. The primers were finally used in combination with a fluorescently labeled TaqMan probe in real-time PCR assays. For absolute quantification of the transgene targets, calibration curves were set up, both with plasmid calibrators in the range from 10 to 10^7 copies and with genomic DNA calibrators in the range from 10 to 10^4 copies (haploid genome equivalents) of the target sequence.

MATERIALS AND METHODS

Plant Materials and Genomic DNA Isolation. For each of the commercial transgene events investigated in this study, DNA was prepared from lyophilised leaf material that was harvested from a single plant grown in the greenhouse. In this way, 100% GM DNA was obtained for each transgenic event. DNA was prepared using the Qiagen DNeasy plant mini kit (Westburg, The Netherlands), starting from 20 mg of dried leaf material. The quality and concentration of the extracted DNA samples were assessed with a GeneQuant spectrophotometer (Amersham Pharmacia Biotech, The Netherlands).

The copy numbers of the transgene target sequences in the solutions were calculated as follows. First, the number of maize respectively canola haploid genome equivalents were calculated. On the basis of the molecular weight of double-stranded DNA (965 Mb weigh 1 pg, *10*) and the haploid genome length of maize (2694 Mbp, *26*), we calculated that one haploid maize genome equivalent can be detected in 2.73 pg of DNA. In the same way, from the haploid genome length of canola (5428 Mbp, *26*), we know that one equivalent of the canola haploid genome is present in 5.5 pg of DNA. In this way, measured DNA concentrations (ng/μL) were translated to absolute copy numbers of the haploid, maize respectively canola genome and thus of endogenous target sequence per PCR (5 μL). Second, because we work with haploid genome equivalents (*18*) and assuming that the GM material used in this study is 100% transgenic, these calculated copy numbers also represent the numbers of copies of the transgene targets. Serial dilutions (10-fold) of the transgenic genomic DNA samples were made (10^4−10 copies) for the quantitative assays.

Molecular Characterization of Transgene Loci. Aiming at sequence data that could be used for the development of event-specific primer pairs, we determined the molecular structure of the transgene locus for the commercial events under investigation. Therefore, we focused on either the transgene borders between the inserted DNA and the flanking plant DNA or we targeted DNA rearrangements unique for the transgene event.

To obtain an event-specific amplicon, for each event, we used several primers, specific for either a gene from the insert (Bt176 maize and GA21 maize) or a part of the plasmid used for transformation (GT73 canola). For the amplification of transgene border junctions, we used the anchored-PCR protocol as described previously (*27*, *28*). For the amplification of transgene rearrangements (GA21 maize), a regular PCR amplification protocol was used. The PCR reaction mixture contained 1× amplification buffer (Applied Biosystems, Roche Molecular Systems, Branchburg, NJ), $MgCl_2$ to a final concentration of 1 mM, 200 μM of dNTP mix (Amersham Pharmacia Biotech, Inc.), 0.5 μM of each PCR primer and 1 unit of AmpliTaq DNA polymerase (Applied Biosystems, Roche Molecular Systems, Branchburg, NJ). Autoclaved

Table 1. Oligonucleotides Used in This Study

PCR system	name	orientation	sequence	amplicon (bp)
		Qualitative Assays for Characterization of Bt176 Maize		
maize plant genome	Bt176pl1	forward	5′-TCTCTCCCACTCCCATCCTATAA-3′	160
	Bt176pl2	reverse	5′-AGCTCCGTTGAACGACAGAC-3′	
maize plant genome	Bt176pl3	forward	5′-GCTCCCTCTTCTCGTGTTCTC-3′	260
	Bt176pl4	reverse	5′-TCTACCTTGGGTTAATCACTTGG-3′	
		Qualitative Assays for Characterization of GT73 Canola		
canola plant genome	GTjunc1	forward	5′-ATCATCCTCCTTCCTTTCCTTG-3′	151
	GTjunc4	reverse	5′-CTAGCCGTCGATTTCCACAT-3′	
canola plant genome	GTjunc1	forward	5′-ATCATCCTCCTTCCTTTCCTTG-3′	134
	GTjunc3	reverse	5′-CATGTGGAATGTTCAATACCTTG-3′	
canola plant genome	GTjunc2	forward	5′-CTTGCCTTCGTATAAGCTTGTGT-3′	114
	GTjunc4	reverse	5′-CTAGCCGTCGATTTCCACAT-3′	
		Qualitative Assays for Cloning of Event-Specific Fragments		
Bt11 maize	3nos1	forward	5′-AATCCTGTTGCCGGTCTTG-3′	505
	Bt11junc2	reverse	5′-GAGCCTCTGGTCGATGATAAATG-3′	
Bt176 maize	bar3	reverse	5′-AAGCACGGTCAACTTCCGTAC-3′	570
	Bt176plant	forward	5′-TCGACTTTATAGGAAGGGAGAGG-3′	
GA21 maize	GA21a	forward	5′-AGAGCTGTAGTTGTTGGCTGTG-3′	528
	act11	reverse	5′-ACGGGGGAAAGCTATTTTAATCT-3′	
GT73 canola	GT73junc-F1	forward	5′-TGCATTTTATGACTTGCCAAT-3′	512
	GT73junc-R1	reverse	5′-CACTAGCCGTCGATTTCCAC-3′	
		Qualitative and Quantitative Event-Specific Assays		
Bt11 maize	Bt113JFor (*14*)	forward	5′-GCGGAACCCCTATTTGTTTA-3′	93
	Bt11-b	reverse	5′-CAAGAAATGGTCTCCACCAAA-3′	
	TQ-Bt11	forward	5′-FAM-TATCCGCTCATGGAGGGATTCTTGGA-TAMRA-3′	
Bt176 maize	Bt176-a	forward	5′-GACTTCAGCCTGCCGGTACT-3′	77
	Bt176-b	reverse	5′-GTGCATCAATGGAGGAGAGAAC-3′	
	TQ-Bt176	forward	5′-FAM-TCTCGGTGACGGGCAGGACC-TAMRA-3′	
GA21 maize	GA21-a	forward	5′-AGAGCTGTAGTTGTTGGCTGTG-3′	88
	GA21-b	reverse	5′-GCTGGGGGATCCACTAGTTCT-3′	
	TQ-GA21	forward	5′-FAM-TGGAAAGTTCCCAGTTGAGGATGCT-TAMRA-3′	
GT73 canola	GT73-a	reverse	5′-TCAGCAAGATTCTCTGTCAACAA-3′	106
	GT73-b	forward	5′-TAGATTTCCCGGACATGAAGAT-3′	
	TQ-GT73	forward	5′-FAM-TCCTTTTCTTGCCTTCGTATAAGCTTGTG-TAMRA-3′	

water was added to a PCR mix of 20 μL, to which 5 μL of DNA was added. PCR reactions were performed on 25 ng of template DNA. The PCR cycle profile used was as follows. After an initial denaturation at 95 °C for 3 min, the samples were submitted to 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 40 s, and a final extension of 10 min at 72 °C.

Amplified fragments obtained after anchored-PCR analysis were subject to gel isolation and PCR reamplification as described by Windels et al. (*27*). Subsequently, fragments of interest were cloned in a pCR2.1-TOPO plasmid vector using the TOPO cloning kit (Invitrogen, The Netherlands), and plasmid DNA was prepared using the GFX plasmid DNA preparation kit. For sequence determination of the cloned PCR fragments, we used the Big Dye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, U.K.). The junctions were sequenced in both directions. An ABI Prism 377 DNA Sequencer and matching software were used to separate the sequencing reactions and to analyze the sequence information. All kits were used according to the guidelines provided by the respective manufacturers.

Construction of Plasmid DNA Markers. For each of the events, a 500−600-bp fragment of the event-specific sequence (junction region or rearranged insert sequence) was amplified with the primers listed in **Table 1**. Plasmid DNA markers, containing a cloned PCR fragment, were constructed as described earlier (*7*). Obtained plasmid DNA concentrations (μg/mL) were measured with GeneQuant and calculated in copy numbers of the event-specific target, taking into account the size of the plasmid and the molecular weight of ds DNA (see above). A 10-fold dilution series was made from 10^7 to 10 copies per reaction (5 μL), to be used as a calibrator set in the real-time PCR reactions. In cooperation with the European Network of GMO Laboratories (ENGL), an official database collection, named "pENGL", is currently under construction, which contains plasmid standards with GM event-specific and endogenous, species-specific sequences. This initiative was taken by different partners of a Belgian OSTC project (2002−2004, "Tracing and authentifying GMOs and derived products in the agro-food sector", PODO II) and is coordinated by the Section of Biosafety and Biotechnology from the Scientific Institute of Public Health (Brussels, Belgium). The four plasmids described in this paper were constructed with the aim of depositing them into the pENGL database.

Qualitative PCR Analyses. Throughout this study, different PCR methodologies were used. First, the quality of the plasmid DNA solutions was verified by means of a conventional PCR with the primers used for cloning (**Table 1**). Reactions were performed in a total volume of 50 μL, containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 nM of dNTPs, 0.4 μM of each primer, 2 units of AmpliTaq DNA polymerase, and 5 μL of plasmid DNA (approximately 600 ng). For Bt176, GT73, and GA21 event-specific PCRs, an initial denaturation step at 95 °C for 3 min was followed by 35 cycles of denaturation (95 °C for 20 s), primer annealing (56 °C for 40 s), primer extension (72 °C for 90 s), and a final extension step of 72 °C for 6 min. For the Bt11 event, the same program was applied however with an annealing temperature of 60 °C.

Second, conventional end-point PCR amplifications were performed as described above (see the molecular characterization of transgene loci), with the exceptions that, for the Bt176, Bt11, and GA21 assays, primer concentrations were 0.3 μM and annealing temperatures were 60 °C.

All qualitative PCR products were analyzed on a 2% agarose gel and stained with ethidium bromide. As molecular weight markers, a ready-to-use GeneRuler 100-bp DNA ladder (MBI Fermentas, Vilnius, Lithuania) or phage Lambda DNA, digested with *Pst*I, were used.

Quantitative PCR Analyses. Event-specific primer pairs were tested in real-time PCR assays as well. Real-time PCR reactions were performed with a ABI Prism 7000 High Throughput Sequence Detection System (Applera Belgium, Lennik, Belgium).

In a first stage, SYBR Green I reactions were performed to test the sets of plasmid DNA calibrators and the primer specificity. Real-time

PCR reaction mixtures contained 1× SYBR Green PCR Master Mix (Applera Belgium, Lennik, Belgium), 0.3 μM of each primer (**Table 1**), and 5 μL of template plasmid DNA. Water was added to a final volume of 25 μL. Samples were subjected to the following thermal profile: 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCR program was followed by a dissociation step, during which the temperature was gradually increased from 60 to 95 °C. This additional step allows us to check the melting temperature of the formed amplicons and thus the specificity of the primers and probes. Reactions were not replicated, because the objective was only to have a indication on primer specificity and on quality (purity) and concentration levels of the plasmid calibrators.

In a second stage, the same plasmid DNA standards were used to set up calibration curves for quantification by means of TaqMan PCRs. The sequences of the used TaqMan probes are shown in **Table 1**. Standard rules for probe design were followed: (1) melting temperatures of about 70 °C (about 10 °C higher than melting temperatures of primers), (2) no G at 5′ (to avoid quenching of the reporter dye), (3) more C's than G's (because of the quenching activity of G bases), (4) no more than two C's or G's in the last five bases at the 3′ end, and (5) if possible, no more than three equal, sequential bases in the whole probe. The four probes were fluorescently labeled with the reporter dye FAM at the 5′ end and with the quencher dye TAMRA at the 3′ end. The TaqMan probes were 5′-labeled with FAM (reporter) and 3′-labeled with TAMRA (quencher). Plasmidic and genomic DNA dilution series were analyzed simultaneously to be able to compare the two types of pure DNA calibrators. TaqMan reactions were performed in duplicate, in a 25 μL volume mixture containing 1× GMO MasterMix (Diagenode, Luik, Belgium), 0.3 μM of each primer, 0.2 μM of TaqMan probe, and 5 μL of template DNA. Cycling conditions were the same as those for SYBR Green I reactions, without the melting step.

After the real-time PCR reactions were finished, the results were evaluated and interpreted with the ABI Prism 7000 SDS software. Amplification plots were visualized in the logarithmic graph for manual setting of the fluorescent threshold value. This threshold was chosen in the middle of the linear phase of the PCR plots. The same plots were then evaluated in the linear graph, for manual setting of the baseline. The latter was generally set between cycles 3 and 15 but adjusted to a smaller cycle number where necessary. After manual adjustment of the fluorescence threshold value and the baseline, a linear standard curve was obtained. Where necessary, outlier points were excluded keeping in mind the following general rules for establishing real-time PCR calibration curves: (1) a correlation coefficient of at least 0.98 must be obtained; (2) the slope should match the ideal value of −3.32 (corresponding to a maximum PCR efficiency of 2, according to $E = 10^{(-1/\text{slope})}$) as much as possible; and (3) the *y* intercept should match the ideal value of 40 (corresponding to the theoretical number of cycles needed to amplify a single copy) as much as possible (*29*).

RESULTS

Sequence Characterization of a Border Region for Bt176 Maize (EMBL Accession Number AJ878607). For Bt176 maize, we focused on the characterization of the *bar* gene cassette that contains the *bar* gene linked to the 35S promoter and the 35S terminator sequence (see **Figure 1A**). Because the 35S promoter sequence and the 35S terminator sequence are also used to drive expression in the *cry* gene cassette, we developed primers specific for the *bar* coding sequence (Genbank accession number X05822). Subsequently, the Bt176 genomic DNA was digested with the *Mse*I restriction enzyme, and anchor primers specific for the *bar* coding sequence were used. In this way, we succeeded in the amplification of a 821-bp fragment. Upon sequencing of the fragment, we could demonstrate that the fragment spans the 3′ end of one particular *bar* expression cassette (see **Figure 1A**). The sequence of this fragment and the origin of the observed sequence motifs is shown in **Figure 2A**. For this fragment, the 3′ end of the *bar* coding sequence is truncated for 124 bp and this truncated *bar*

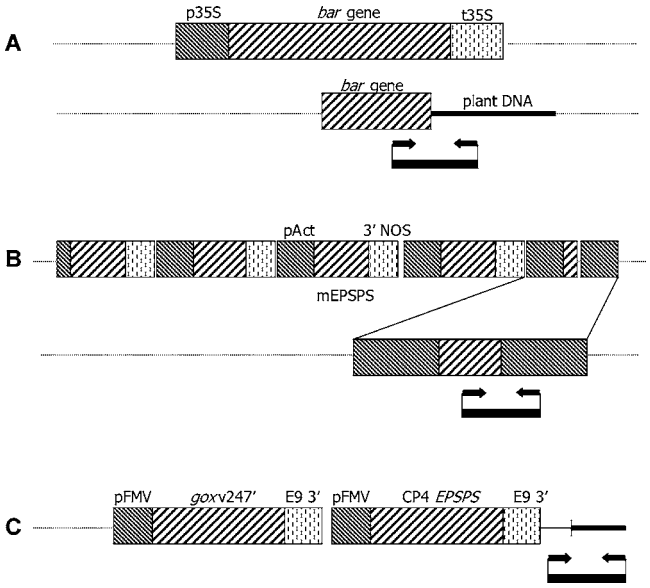

Figure 1. Schematic representation of the transgenic maps of (A) Bt176 maize, (B) GA21 maize, and (C) GT73 canola, with the location of the primers (indicated as arrows).

cassette is followed by flanking DNA for which we could not show significant sequence similarity. A BLAST homology search with the sequence revealed no significant sequence homology with known sequences. Therefore, we performed a PCR analysis to determine whether this flanking sequence is maize nuclear DNA. Two primer pairs, i.e., Bt176pl1/Bt176pl3 and Bt176pl2/Bt176pl4 (see **Table 1**), have been designed that cover the flanking sequence. These primer pairs should give the expected amplicon when the flanking sequence is plant DNA; otherwise, no amplification will be observed. Indeed, both primer pairs gave the expected amplicon fragment when used on Bt176 maize as well as nontransgenic maize genomic DNA (see **Figure 3A**). In this way, we demonstrate that the Bt176 fragment that we have amplified consists of a truncated *bar* coding sequence followed by a flanking maize genomic DNA sequence (**Figures 1A** and **2A**).

Sequence Characterization of an Internal Rearrangement of GA21 Maize (EMBL Accession Number AJ878608). To amplify and characterize an event-specific region of the GA21 maize line, we initially focused on a border region between the insert DNA and the plant DNA. Earlier studies as done by the company and presented in their dossier indicate that transgenic maize harbors three complete and three incomplete copies of the GA21 expression cassette. Each cassette harbors a rice *actin* promoter sequence, an optimized transit peptide derived from sunflower and maize *rbcS* sequences, a modified version of the EPSPS sequence, and the *nos* terminator. Several approaches that we tested to amplify this region, either at the 5′ or 3′ end of the insert DNA, were unsuccessful. We put forward that this is due to the complex structure and repetitive nature of the GA21 insert DNA. Therefore, we changed our strategy and aimed at an internal DNA segment that is unique for the GA21 event. Because the two incomplete GA21 expression cassettes at the 3′ end of the GA21 insert are between two truncated GA21 expression cassettes, we hypothesized that this was a good region for the development of a unique GA21 primer pair. This region was amplified by PCR analysis. We used a mEPSPS-specific primer in combination with a rice *actin* promoter primer. Using this primer combination, we estimated to amplify two fragments: one longer fragment that was derived from two adjacent complete copies and a short one that was due to a

A

AAGCACGGTCAACTTCCGTACCGAGCCGCAGGAACCGCAGGAGTGGACGGACGACCTCGT

CCGTCTGCGGGAGCGCTATCCCTGGCTCGTCGCCGAGGTGGACGGCGAGGTCGCCGGCAT

CGCCTACGCGGGCCCCTGGAAGGCACGCAACGCCTACGACTGGACGGCCGAGTCGACCGT

GTACGTCTCCCCCCGCCACCAGCGGACGGGACTGGGCTCCACGCTCTACACCCACCTGCT

GAAGTCCCTGGAGGCACAGGGCTTCAAGAGCGTGGTCGCTGTCATCGGGCTGCCCAACGA

CCCGAGCGTGCGCATGCACGAGGCGCTCGGATATGCCCCCCGCGGCATGCTGCGGGCGGC

CGGCTTCAAGCACGGGAACTGGCATGACGTGGGTTTCTGGCAGCTGGACTTCAGCCTGCC

GGTACTGCCCCGTCCGGTCCTGCCCGTCACCGAGATCTGA*tgttctctcctccattgatg*

cacgccatcaatggccttgaagccttggccgaccgtttctcccttcccccctgggctccct

ctctctccctctcccttcctataaagtcgataccacgcccacggagttctccctcccaca

tccgagctcgctccctcttctcgtgttctcgctccctcgccgttcgccgccgtgaagctc

gtcgccggaccgtctgcgcccgtctgtcgttcaacggagctccgcccgggcctacgcgtt

gtccgtcgttcgccgtccgcccttccggtcaagtcccctgtcccgttccctactcgaccg

accactcccctcgccgagctccttccctgtgcgtgattaa

B

TGTCGAAGCGGACAAAGCTGCCAAAAGAGCTGTAGTTGTTGGCTGTGGTGGAAAGTTCCC

AGTTGAGGATGCTAAAGAGGAAGTGCA*GCTCT***AGAACTAGTGGATCCCCCAGCTTGCATG**

CCTGCAGGTCGAGGTCATTCATATGCTTGAGAAGAGAGTCGGGATAGTCCAAAATAAAAC

AAAGGTAAGATTACCTGGTCAAAAGTGAAAACATCAGTTAAAAGGTGGTATAAAGTAAAA

TATCGGTAATAAAAGGTGGCCCAAAGTGAAATTTACTCTTTTCTACTATTATAAAAA

C

TGAGTAACGACTAGGTACATCTAAAGGGCCTGTACTTC*tagtaggaggaaggaaaggaac*

ggaaaggaaggaaaagaacggaagcatattcgaacacagttaacaactgtctcttagaac

gacttcttaatgagtctttgtctcatgaagttccataacttgtaaggtgtacacctttag

ctgccgatc

Figure 2. Characterized sequences used for development of event-specific primer pairs. (A) 821-bp Bt176 junction region (EMBL AJ878607) between a truncated *bar* coding sequence (capitals) and the adjacent maize genomic DNA (small italic letters). (B) 297-bp GA21 rearranged junction sequence (EMBL AJ878608) between a truncated mEPSPS coding sequence (capitals) and a truncated *actin* promotor sequence (bold capitals), linked to each other through a 5-bp microhomology region (italic capitals). (C) 189-bp GT73 canola junction fragment (EMBL AJ878609) between part of the pTi15955 plasmid used for transformation (bold capitals) and plant genomic DNA (small italic letters).

rearrangement. After adapting our PCR program, including a very short elongation step at 72 °C, we could inhibit the amplification of the longer fragment. Indeed, we clearly obtained a short fragment of 297 bp. Upon sequence analysis, we could demonstrate that the fragment consists of part of the truncated mEPSPS coding sequence and part of the rice *actin* promoter sequence. Both fragments were ligated by taking advantage of a 5-bp microhomology region (**Figures 1B** and **2B**).

Sequence Characterization of a Border Region for GT73 Canola (EMBL Accession Number AJ878609). According to the Summary Notification Information Format as presented by the company (*30*), transgenic GT73 oilseed rape was transformed using a binary vector that harbors the goxv247 coding sequence as well as the CP4 EPSPS coding sequence (**Figure 1C**). Because GT73 canola was transformed using *Agrobacterium*-mediated transformation, we targeted the border regions of the insert to start our walking experiments for cloning of the junction regions. First, we developed primer pairs specific for the left and right border regions of both the octopine and nopaline Ti plasmids, and we used these primer pairs to perform PCR analysis on the GT73 canola genomic DNA. A positive PCR signal indicates that a specific border has been used for the development of the GT73 binary vector. In this way, we could demonstrate that the GT73 canola contains a border sequence that is derived from the *Agrobacterium tumefaciens* Ti plasmid pTi15955. Subsequently, we digested the GT73 canola genomic DNA with the *Mbo*I restriction enzyme, and we used Ti plasmid pTi15955-specific anchor primers to walk across the GT73 border region. Indeed, we succeeded in amplification of a 189-bp fragment. Sequence analysis of this fragment shows that the amplified border sequence consists of part of the pTi15955 plasmid border sequence followed by a unknown flanking sequence (see **Figure 2C**). For this flanking DNA segment, we found sequence homology with an *Arabidopsis thaliana* genomic sequence. The *Arabidopsis thaliana* sequence that shows sequence identity is derived from *Arabidopsis* chromosome 3, clone MOA2. Using PCR, we verified that the observed sequence is indeed plant DNA. Therefore,

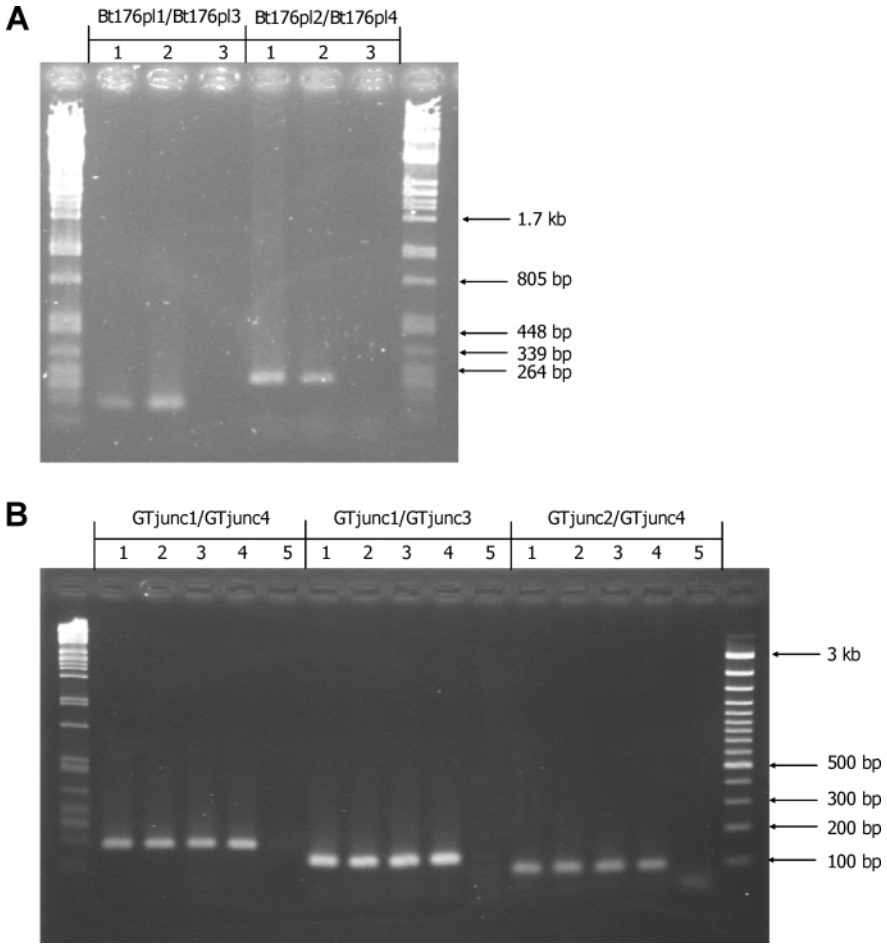

Figure 3. Results on 1.5% agarose gel of conventional PCRs performed for molecular characterization of Bt176 maize and GT73 canola. (A) 160-bp amplicon of primer pair Bt176pl1/Bt176pl3 (left) and 260-bp amplicon of primer pair Bt176pl2/Bt176pl4 (right), performed on wild-type maize (lane 1), 100% transgenic Bt176 maize (lane 2), and a no template control (NTC, lane 3). Lambda Pst DNA was used as a marker. The results show that the sequence adjacent to the *bar* gene, as part of the amplified 821-bp junction fragment shown in **Figure 1A** and **Figure 2A**, is indeed plant genomic DNA. (B) 151-bp amplicon of primer pair GTjunc1/GTjunc4 (left), 134-bp amplicon of primer pair GTjunc1/GTjunc3 (middle), and 114-bp amplicon of primer pair GTjunc2/GTjunc4 (right), performed on wild-type canola (lanes 1 and 2), 100% transgenic GT73 canola (lanes 3 and 4), and a NTC (lanes 5). Both Lambda Pst (first lane) and a 100-bp ladder (last lane) were used as length markers. All three primer pairs are specific for the 151-bp plant DNA segment as part of the amplified 189-bp junction shown in **Figure 1B** and **Figure 2B**. The results thus confirm that this segment is indeed plant DNA.

we used three primer pairs, i.e., GTjunc1/GTjunc4, GTjunc1/GTjunc3, and GTjunc2/GTjunc4 (**Table 1**), that cover the 151-bp plant DNA segment. When using these primer pairs on GT canola, amplicons with the expected length are observed; in addition, identical PCR amplicons are obtained on nontransformed canola DNA as the template (see **Figure 3B**). This indicates that the 151-bp DNA segment that flanks the GT insert is indeed canola plant DNA. For the three transgene events, the regions characterized as described above are schematically presented in **Figure 1** and the sequences are shown in **Figure 2**.

Construction of Plasmid DNA Markers. The characterized event-specific sequences formed the basis for the generation of a larger fragment to be cloned. The Bt176 junction sequence of 821 bp (**Figure 2A**) could be used directly to design primers for cloning. For the other events, only small sequences were obtained after transgene characterization. For Bt11, the 70-bp 3′ integration junction sequence as revealed by Ronning et al. (*14*) was used. In the present study, a 297-bp rearranged sequence of GA21 (**Figure 2B**) and a 189-bp junction sequence of GT73 canola (**Figure 2C**) were obtained. After a BLAST homology search was performed with both ends of the event-specific sequences, larger sequences of the respective elements were found: maize plant DNA for Bt11, *actin* promotor DNA for GA21, and canola plant DNA for GT73. Those sequences could be ligated to the respective ends of the yet available sequences. In this way, we could construct larger fragments of at least 500 bp for Bt11, GA21, and GT73 events. The four event-specific amplicons listed in **Table 1** were successfully generated, ligated into a plasmid vector, and transformed into *Escherichia coli*, following the cloning procedure as described previously (*7*). Plasmid DNA, finally obtained from transformed bacterial cultures, was subjected to the same PCRs targeting the cloned junction sequences. The results for one plasmid DNA sample per event are shown in **Figure 4**. The good results of this PCR test provide the evidence for the presence of the junction sequence in the plasmid vector, as well as the good quality of the plasmid DNA solutions. The same plasmid DNA preparations were measured spectrophotometrically. On the basis of the known length of the plasmid, including the inserted fragment, and the molecular weight of single-stranded DNA, the concentration in ng/μL was calculated back to the number of target copies per PCR reaction (5 μL of DNA template). For each plasmid DNA marker, 10-fold dilution series were then made from 10^7 to 10 copies.

Design of Event-Specific Primer Pairs. On the basis of the sequences of the amplified event-specific DNA regions of Bt176 maize, GA21 maize, and GT73 canola (**Figures 1** and **2**), we

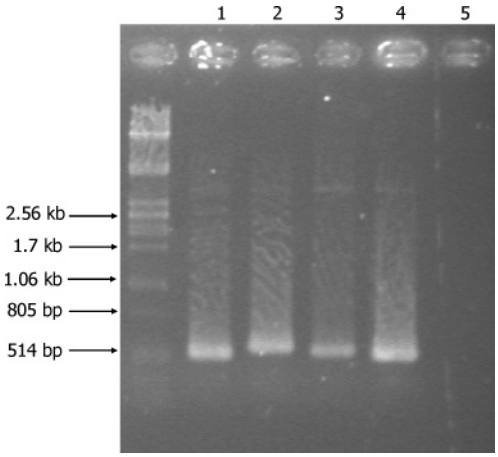

Figure 4. Agarose gel (1.5%) with large PCR products to be used as targets for cloning into pCR2.1-TOPO vectors. The length of the amplicons is determined based on a Lambda Pst marker. Lane 1, 505-bp amplicon of primer pair 3nos1/Bt11junc2 for Bt11 maize; lane 2, 570-bp amplicon of primer pair Bt176plant/bar3 for Bt176 maize; lane 3, 528-bp amplicon of primer pair GA21a/act11 for GA21 maize; lane 4, 512-bp amplicon of primer pair GT73junc-F1/GT73junc-R1 for GT73 canola; lane 5, no template (negative PCR) control.

developed event-specific primer pairs. Primer 3 software was used for the design, aiming at melting and annealing temperatures of about 60 °C and amplicon lengths between 70 and 120 bp. For Bt11 maize, the forward primer Bt113JFor as published by Ronning et al. (*14*) was used. A new reverse primer and TaqMan probe were designed (**Table 1**), because these sequences were more matching with the proposed standard rules for probe design (see above), which we put forward as minimum requirements. The selection of those two new oligos is linked to the preferences of the used probe design software.

Ronning et al. (*14*) reported that the Bt11 construct is integrated into a maize-specific, 180-bp tandemly repeated DNA sequence motif (EMBL AF030935). Because of priming in this highly repetitive sequence, the authors observed multiple PCR products when using a primer entirely located in the repeat sequence. The 93-bp Bt11 amplicon targeted in our study (**Table 1**) contains a 61-bp transgene sequence, which is 100% similar to pUC18 (EMBL L08752, *14*), followed by 32 bp of maize plant DNA. Because of this risk for formation of multiple amplification products and thus for low specificity, we designed a reverse primer that is located entirely in the maize plant DNA and a TaqMan probe that spans the junction. This is the ideal configuration of an event-specific PCR assay (*14*). Despite the above-mentioned particularities of the Bt11 junction, we did not expect problems with low specificity in the TaqMan assays. This is because successful amplicon detection in real-time PCR requires three oligonucleotides to anneal to their specific target, instead of two for a conventional PCR.

Specificity Testing of Primers. Primer specificity was first tested by means of qualitative PCR analysis. Each of the event-specific primer pairs was tested on genomic DNA isolated from the transgenic line under study, the wild-type, nontransformed plant, as well as a set of other transgenic soybean, maize, and canola events. The amplicon obtained for the transgene sample was verified each time by direct sequencing. For all four amplicons, a 100% sequence similarity was observed between the amplified product and the theoretical sequence of the characterized event-specific domains. No amplification was observed for the other genomic DNA samples, which confirms that the developed, event-specific PCR primer sets are unique for the transgene event for which they were designed (results not shown).

The sensitivity of the primers and the specificity for their target were also tested by using the plasmid DNA calibrators. Per transgene event, a dilution series from 10^7 to 10 copies of plasmid DNA was subject to a real-time PCR with SYBR Green I, including a dissociation step. Results are shown in **Figure 5**. For each of the plasmid marker sets, amplification plots are shown together with the calculated calibration curve as well as the melting peak of the amplicon. Amplification plots are generated for all samples (10^7-10 cp) in the SGI assays for Bt11 (A) and GA21 (C) maize and GT73 canola (D). For those three events, dissociation curve analysis resulted in one clear peak, without formation of aspecific or primer−dimer products and with no observed signal for the no template control (NTC, flat curves). Mean melting temperatures with their standard deviations (SD) are (73.9 ± 0.3) °C for Bt11 maize (A), (79.8 ± 0.1) °C for GA21 maize (C), and (77.3 ± 0.3) °C for GT73 canola (D) (**Figure 5**). The Bt176 event-specific assay seems to be a little less sensitive and specific. This is reflected in amplification plots generated down to only 100 copies and in a small primer−dimer peak for the 10 cp sample (NTC is fine). Specificity is however acceptable, because a single melting peak (mean $T_m = 83 \pm 0.04$ °C) is obtained for the desired event-specific PCR products and because no primer−dimer or aspecific products occur in the plasmid DNA samples used for constructing the calibration curve ($100-10^7$ cp, **Figure 5B**).

For Bt11 maize and GT73 canola, we observe an effect of the template concentration on the melting temperature. More in particular, higher initial amounts of the target give lower T_m values (parts **A** and **D** of **Figure 5**). T_m values over the different target concentrations are however within a SD of 0.3 °C and can therefore be ascribed to the normal dispersion around the mean experimental T_m values (*31*, *32*).

These first experiments on the plasmid calibrators also allow us to establish calibration curves. Linear regression curves can be seen for each plasmid series on the graph of amplification plots. With correlation coefficients (R^2) of at least 0.99 and slopes between −3.1 and −3.4 (PCR amplification efficiencies between 1.102 and 0.968, respectively), it can be said that the curves are good (**Figure 5**).

Absolute Quantification Methods and Commutability of Genomic and Plasmid DNA Calibrators. Reactions were performed on plasmidic DNA series (the same series as used in the SGI assays, $10-10^7$ copies) as well as genomic DNA series ($10-10^4$ copies). The objective of these experiments was to check whether good calibration curves can be established, usable for specific absolute quantification purposes, and whether plasmidic DNA calibrators are comparable to genomic DNA calibrators. Because plasmid DNA is used for quantification of genomic DNA extracted from "real-life" samples, comparability or "commutability" forms an important issue. Comparable behavior between plasmidic and genomic DNA in the PCR is reflected in the characteristics of the obtained standard curves (**Figure 6**). The larger the deviation between the two slopes and the deviation between the two intercepts of plasmidic and genomic DNA standard curves, the less comparable they are to each other. For Bt11 and Bt176, the genomic and plasmidic standard curves show nearly equal slopes and intercepts. This results in two standard curves that are more or less overlapping (parts **A** and **B** of **Figure 6**). This is the ideal case. The standard curves for GT73 canola result in more or less equal slopes but

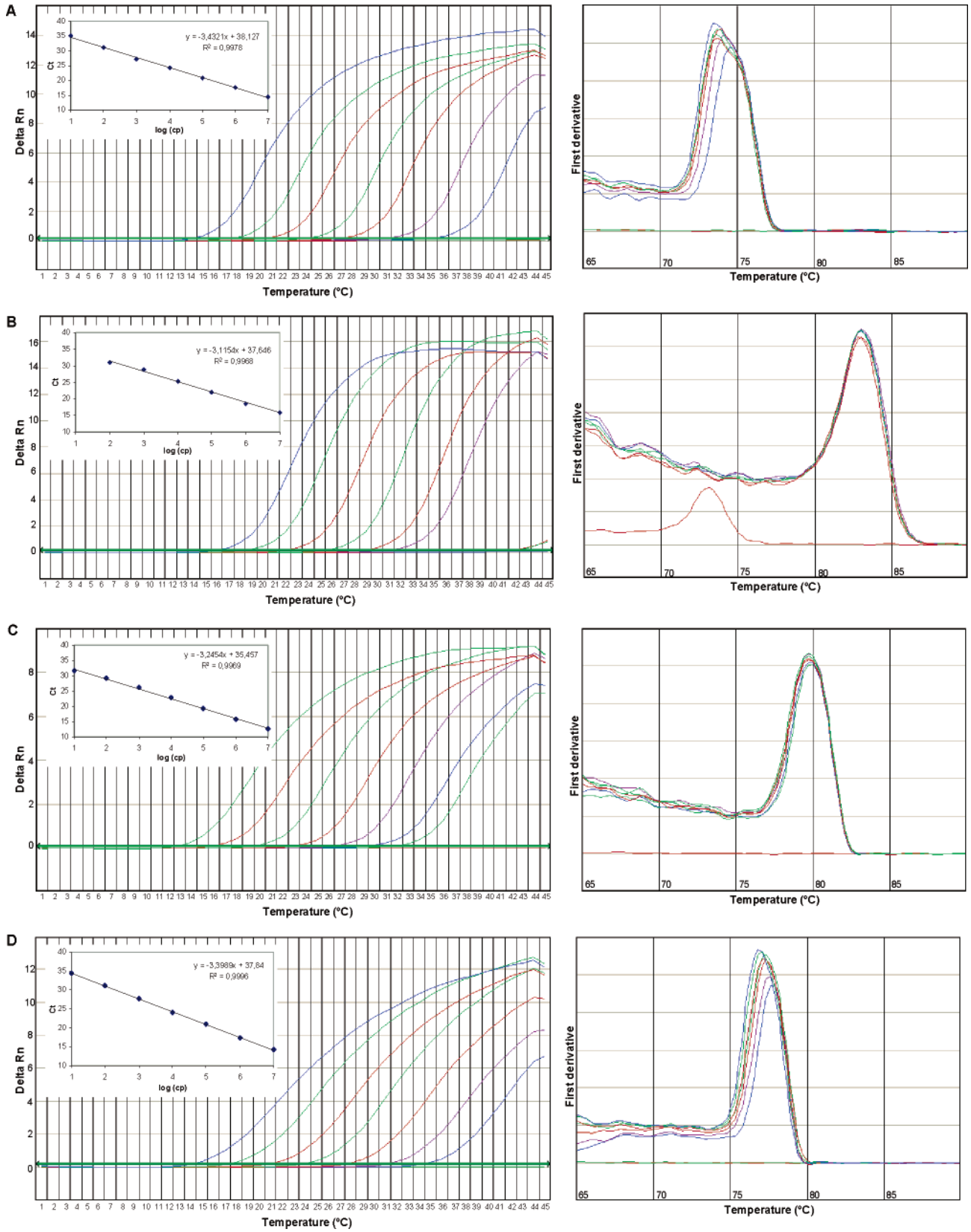

Figure 5. Amplification plots, corresponding calibration curves, and melting peaks resulting from real-time PCR analysis with SYBR Green I, on plasmid DNA standards specific for transgene events Bt11 maize (A, 10–1E7 copies), Bt176 maize (B, 1E2–1E7 copies), GA21 maize (C, 10–1E7 copies), and GT73 maize (D, 10–1E7 copies). Fluorescence threshold values were set manually at 0.2, and baselines were set manually at 3–13. Standard curves were obtained with all points (10–10^7 cp) for Bt11, GA21, and GT73 and without one outlier (10 cp) for Bt176.

show a difference in the intercept. In the graph, this is visible as a "shift" between both curves (**Figure 6D**). For GA21 maize finally, both the slopes and the intercepts differ from each other (**Figure 6C**). This may indicate that the GA21 event-specific plasmid DNA markers are less commutable for quantification of genomic DNA samples.

Figure 6 allows us to visually examine the slopes, intercepts, and R^2 values for genomic and plasmid DNA calibrators. Mutual

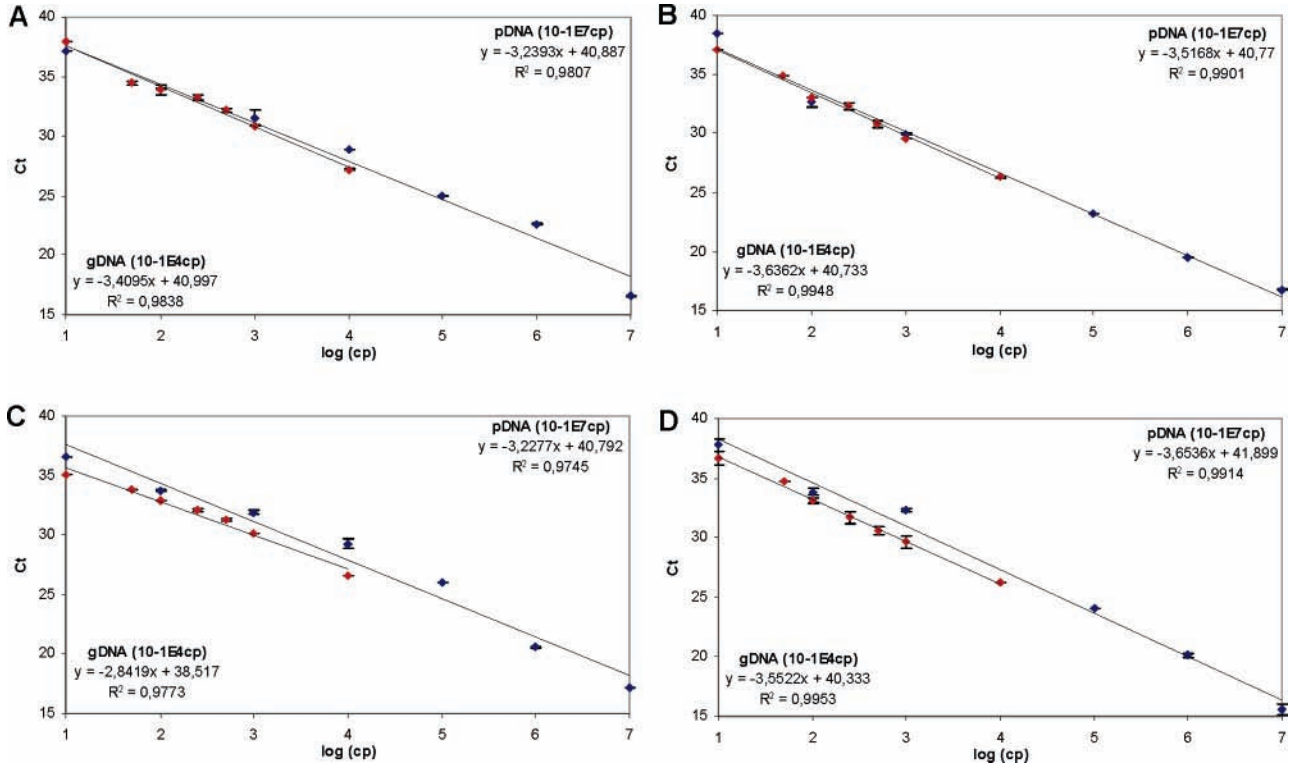

Figure 6. Calibration curves resulting from real-time PCR analysis with TaqMan chemistry, for 10–1E4 cp gDNA (lower curves, calibrator points indicated in red) and 10–1E7 cp pDNA (upper curves, calibrator points indicated in blue) for (A) Bt11 maize, with the following calibration curve settings: manual threshold of 0.237, manual baseline of 3–13, and outliers 1 cp (two replicates) and 10^4 cp (one replicate); (B) Bt176 maize, with a manual threshold of 0.279, a manual baseline of 3–15, and no outliers; (C) GA21 maize, with a manual threshold of 0.2, an automatically set baseline, and outliers 1 cp (two replicates); and (D) GT73 canola, with a manual threshold of 0.214, an automatically set baseline, and outliers 1 cp (two replicates) and 10^4 cp (two replicates).

Table 2. Statistical Analysis of the Results of Calibration Curves Set Up with Plasmid DNA (pDNA) and Genomic DNA (gDNA) Calibrators[a]

criterium	type of calibrator	A	B	C	D	mean of data set	variance of data set	*n*	estimated difference between means	*T* statistical data	$P(T \leq t)$ two-sided	critical *t* (*a*; *n* – 1)
slope	pDNA	3.2393	3.5168	3.2277	3.6536	**3.409 35**	0.044 372 4	4	0	0.389 946	0.722 613	3.182 449
	gDNA	3.4095	3.6362	2.8419	3.5522	**3.359 95**	0.128 035	4				
intercept	pDNA	40.887	40.77	40.792	41.899	**41.087**	0.295 619 3	4	0	1.613 147	0.205 112	3.182 449
	gDNA	40.997	40.733	38.517	40.333	**40.145**	1.252 458 7	4				
R^2	pDNA	0.9807	0.9901	0.9745	0.9914	**0.984 175**	6.433×10^{-5}	4	0	–8.490 33	0.003 431	3.182 449
	gDNA	0.9838	0.9948	0.9773	0.9953	**0.9878**	7.717×10^{-5}	4				

[a] The mean values for slope, intercept, and correlation coefficient for both series were tested for significant differences in a two-sided *t* test for paired data sets. Calculated *T* values are based on a value of 0.05 for α.

differences between the mean values for both types of calibrators were additionally checked by performing a statistical *t* test for two paired data sets. The results are shown in **Table 2**. It was assumed that the data are normally distributed. For each criterium, the *T* value calculated in the statistical analysis is compared to the critical value for $t_{\alpha,n-1}$ with *n* being the number of data values and α set at 0.05. If the absolute value of *T* is greater than $t_{\alpha,n-1}$, the null hypothesis that the means would be equal to each other is not valid. It is clear from **Table 2** that the slopes and intercepts are not significantly different for both types of DNA, whereas the correlation coefficients seem to differ significantly from each other. The meaning of different R^2 values is less relevant in the context of commutability. The correlation coefficient only shows the relationship between the independent variable, the logarithm of the copy number in our case (*x* axis) and the dependent variable, which is the C_T value (*y* axis). The value of R^2 does not necessarily indicate a linear relationship between the dependent variable (C_T) and the independent variable (cycle number) but says something about the dilution effect between the different samples of the calibrator series.

From the statistical analysis, we can conclude that slopes and intercepts are not significantly different for genomic and plasmidic DNA. This means that both types of calibrators have the same amplification efficiency in the PCR and thus behave equally.

DISCUSSION

Because Windels et al. (*27*) reported on a rearrangement at the insertion locus of transgene Roundup Ready soybeans, the requirement for characterizing the exact end points of inserted DNA and the sequences following those end points has been widely recognized and stressed. The new regulation (EC) 1829/2003 requires detailed sequence information of the transgene

plant DNA junctions as well as the plant target locus, to be included in the technical dossier submitted to the competent authorities. Together with the scientific dossier, the company must submit a prevalidated, event-specific detection procedure and deliver positive and negative control samples (*2*).

Despite the more severe and comprehensive regulatory framework, molecular tools for characterization of transgene loci and identification of GMOs in the lab remain and will even become more necessary, to check the accuracy of technical dossiers and to complete already existing information. Two main issues in this respect are (1) the molecular characterization of transgenes at the site(s) of integration and (2) the production of reliable and commutable reference materials. It is self-evident that tracing transgenic crops in a unique way needs event-specific marker systems, consisting not only of primer pairs and DNA assays but also of reference materials and calibrants enabling the accurate quantification of the event. In this work, we developed such transformation event-specific marker systems for three transgenic maize events and one transgenic canola line (**Figure 1**). After the characterization of a unique, event-specific locus for each event, part of this sequence was cloned in a plasmid vector, to be used further as pure DNA calibrators in real-time PCR.

For two of the four events under study, Bt11 and GA21 maize, event-specific sequences have already been characterized and reported in the literature (*14*, *16*). For Bt11 maize, we targeted the 3′-integration junction between the transgene construct and the host plant DNA, determined by means of a modified inverse PCR protocol by Ronning et al. (*14*). For GA21 maize, a specific internal sequence, spanning parts of a 3′ terminator *nos* and a 5′ promotor *actin*, was amplified, sequenced, and considered as line-specific by Hernandez et al. (*16*). Because of the complex structure of the inserted GA21 expression cassettes, we concentrated on another region in the inserted DNA. We focused on a rearrangement sequence containing part of a truncated mEPSPS coding sequence at the 3′ end of one incomplete mEPSPS cassette and part of a truncated *actin* promotor sequence belonging to another incomplete GA21 inserted cassette. Both sequences were ligated through a 5-bp microhomology region (**Figures 1B** and **2B**). Thus far, no efforts had been made toward molecular characterization of Bt176 maize and GT73 canola. Event Bt176 was characterized at the locus of insertion by anchored PCR. We were able to amplify a junction region of 821 bp between the inserted truncated *bar* coding sequence and the adjacent maize DNA (**Figures 1A** and **2A**). For GT73 canola finally, we succeeded in amplifying a 189-bp junction sequence between the pTi15955 plasmid border sequence and flanking genomic DNA (**Figures 1C** and **2C**).

The quantitative assays developed in this study consist of an event-specific primer pair, a TaqMan probe, and a set of plasmid DNA markers usable as calibrators to establish absolute calibration curves. The specificity and sensitivity of the primers have first been tested qualitatively. Analysis of genomic DNA isolated from the nontransgenic, conventional variety as well as other GM events and phylogenetically related plant species revealed that the four primer sets are highly specific. These high specificities were also confirmed in SYBR Green I real-time PCR experiments with plasmidic DNA samples (10^7-10 copies). Dissociation curve analysis revealed that a unique, single amplicon was present in each of the PCRs (**Figure 5**). Our results on dissociation curves and T_m values for Bt11, Bt176, and GA21 maize and GT73 canola events confirm the identification possibilities of this technology.

Dissociation curve analysis provides for a simple, cheap, rapid, and reliable alternative to conventional PCR methods for GMO identification. Amplicons are identified and distinguished by their melting temperatures, which are determined by the product's GC content, length, and sequence and the concentrations of dye, salt, and specific template in the tube (*32*, *33*, *34*). SYBR Green I-based melting curve analysis allows differentiation and thus multiplexing of different amplicons, provided that the different amplicons melt at significantly distinguishable temperatures. Ririe et al. (*32*) reported that products differing in T_m by less than 2 °C could be distinguished. Hernandez et al. (*31*) were able to identify amplicon pairs differing at least 1.5 °C in T_m, in a duplex assay, while products differing in T_m by only 1.2 °C were not distinguishable anymore. We found slight differences in T_m (SD of ±0.3 °C) for the same amplicon, however, for different initial template concentrations for Bt11 and GT73 targets (parts **A** and **D** of **Figure 5**). However, given that the resolution of melting curve analysis and amplicon determination is 1.2 °C, the observed dispersions may be ascribed to normal deviations around the mean experimental T_m values and therefore do not present a problem. In the context of differentation of PCR products, it is worthwile to mention that optical capillary electrophoresis (CE), also called LabChip technology, is an alternative to quantitative real-time PCR. Several authors reported on successful separation of PCR products based on UV absorption or laser-induced fluorescence and using automatic gel images or electropherograms as results (*9*, *35*−*37*).

After the primers had been tested in nonspecific real-time PCR assays based on SYBR Green I, specific TaqMan PCRs were performed on both genomic (10^4-10 copies) and plasmidic (10^7-10 copies) DNA samples. For GMO analysis, a discussion is ongoing for what concerns the type of reference material and calibrator to use for this purpose. Both genomic DNA (gDNA) and plasmidic DNA (pDNA) pure analyte RMs have been developed and used, diluted either in water or in other genomic DNA (*7*, *10*, *15*, *19*−*23*). Arguments against plasmid molecules (compared to genomic DNA RMs) are the potential risk for contamination when working with plasmids and the idea that these molecules would not be applicable to real-life, genomic DNA samples. The first argument, the risk for contamination, does not make sense if plasmid DNA solutions are used in the same molar concentrations as genomic DNA; in this situation, there is no difference. The second argument, the commutability issue, deserves more attention. As a consequence of food and feed processing and of DNA extraction procedures, gDNA quality can be extremely poor; e.g., DNA may be degraded, and PCR inhibitors may be present. To the contrary, because plasmid DNA solutions contain purified templates without the risk for competition with "bulk" genomic DNA sequences, these are very likely to be favored in PCR reactions. Since the introduction of pDNA calibrators (*7*), the fear has been growing among GMO analysts that gDNA and pDNA would show different PCR amplification efficiencies and would therefore not be commutable. Per definition, commutability is the similarity between the analytical response of a certain material and the response of routine samples (*38*). In a real-time PCR reaction, the direct response is the amplification plot (fluorescent signal), while C_T values form indirect responses. For a certain number of copies, it was thus important to obtain the same C_T value regardless of the type of DNA target (plasmid versus genomic). Commutability is reflected in the behavior of the standards, relative to that of the unknown samples. Comparable behavior in the PCR is on its turn reflected in the slope of the

calibration curve, which is linked to the PCR amplification efficiency. In this study, the objective was to demonstrate the suitability of plasmid DNA calibrators for quantification of genomic DNA. We set up real-time PCR standard curves with pDNA and gDNA calibrators and compared the responses. **Figure 6** and **Table 2** demonstrate that, for each of the sequences targeted here, slopes of the resulting standard curves, obtained with gDNA and pDNA, respectively, do not differ significantly from each other.

This study demonstrates that plasmid and genomic DNA behave in a similar way in the PCR and are thus commutable. An aspect of commutability, which is often forgotten, is commutability between different sequence targets within genomic or plasmid DNA. As much as commutability is focused on the type of DNA (genomic versus plasmid), it should stress also and even more on the background and origin of the DNA. Two pools of genomic DNA, isolated from two different matrixes, are not necessarily likely to behave similar in a PCR reaction. Here, we think about genomic DNA extracted from, e.g., seeds or grains, leaf material or a processed end product. In practice, this means that PCR amplification efficiencies can differ from DNA target to target. This commutability compound deserves careful consideration. We believe that mutual commutability between different sources of genomic DNA is more relevant than commutability of genomic versus plasmid DNA and that experimental work on comparison between calibrator and unknown sample DNA should be more directed toward this aspect.

The event-specific plasmid DNA markers, constructed in this study for Bt11, Bt176, and GA21 maize and for GT73 canola, allow us to set up calibration curves for absolute copy number determination of each target. For relative quantification of the GMO content (%) in routine samples, each event-specific TaqMan assay should be combined with a wild-type, endogenous PCR assay. This is another issue that forms the elongation of this work, however, falls outside of the aim of this paper. With this work, we principally aimed at molecular characterization of three transgene events and subsequent in-house development of plasmid DNA markers. Quantitative TaqMan assays, using this new and promising type of standards, did not yet exist for either of the four targeted events.

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