Eur Food Res Technol (2003) 216:259–263
DOI 10.1007/s00217-002-0652-5

ORIGINAL PAPER

**Pieter Windels · Sophie Bertrand · Ann Depicker
William Moens · Erik Bockstaele · Marc Loose**

Qualitative and event-specific PCR real-time detection methods for StarLink maize

Received: 28 August 2002 / Revised: 5 November 2002 / Published online: 29 January 2003

Abstract In this paper we present qualitative detection and identification methods for StarLink maize (event CBH-351). The methodology proposed envisages detection of an internal target site in the *cry9c* coding region, as well as two event-specific target sites at the junction between the CBH-351 insert DNA and the genomic plant DNA. The *cry9c*-specific primer pair, generating a 180 bp amplicon, has been tested and optimised for conventional end-point PCR amplification. The event-specific primer pairs, generating amplicons of 138 bp and 100 bp respectively, give good performance in a conventional end-point PCR and in a real-time PCR assay. Our results clearly demonstrate that the primer pairs proposed can be used in an unambiguous and specific PCR identification assay for StarLink maize.

Keywords StarLink maize · Junction region · GMO · Identification · PCR

Introduction

Maize line CBH-351 (trade name StarLink) is a corn line that was registered in 1998 by the US Environmental Protection Agency (EPA) for use as animal feed and for industrial applications. In Europe, this genetically modified (GM) maize line is not authorized. As such, a qualitative assay for detection and identification of the presence of StarLink maize is sufficient for food analysis. StarLink maize contains a modified *cry9c* gene from *Bacillus thuringiensis* subsp. *tolworthi* and the *bar* gene from *Streptomyces hygroscopicus*. For both introduced genes, expression is driven by the 35S promotor, and termination of transcription for the *cry9c* and the *bar* gene is regulated by the nos adenylation signal and the 35S terminator respectively [1]. The *cry9c* gene product confers resistance to feeding damage of lepidopteran insects, such as the european corn borer (ECB). The bar gene is a source of resistance against the herbicide phosphinotricin.

In 1998, 1999 and 2000, respectively, 10 000 acres, 250 000 acres and 350 000 acres of this genetically modified maize line were grown in the United States. Although the use of StarLink corn was not approved for human consumption, in the summer of 2000, traces of the *cry9c* were detected in taco shells [2, 3] and in maize seeds of a non-StarLink variety [4]. This indicates that the GM corn had mingled the human food chain either by contamination of non-StarLink seed stocks during storage or transport or by means of inter-varietal crossing.

At the time of this study, there was a necessity to monitor the Belgian market for the potential, adventitious presence of CBH-351 maize DNA in the food chain. Therefore, it was of high importance to possess a method to unambiguously identify the presence of StarLink corn in raw materials and food samples. Since maize line CBH-351 was not documented at the regulatory level in the European Union, a strategy was applied to alleviate the lack of accurate knowledge of CBH-351 molecular characteristics. Therefore, two event-specific junction regions, amplified by anchor priming at the inserted 3' nos sequence and the *cry9c* coding sequence have been characterised and used for the design of CBH-351-specific primer pairs.

P. Windels · E. Bockstaele · M. Loose (✉)
Department for Plant Genetics and Breeding,
Centre for Agricultural Research,
Caritasstraat 21, 9090 Melle, Belgium
e-mail: m.deloose@clo.fgov.be
Tel.: +32 09 272 2876, Fax: +32 09 272 29 01

S. Bertrand · W. Moens
Section of Biosafety and Biotechnology,
Institute of Public Health,
Wytsmanstraat 14, 1050 Brussels, Belgium

A. Depicker
Department of Plant Systems Biology,
Flanders Interuniversity Institute for Biotechnology (VIB),
Ghent University,
K.L. Ledeganckstraat 35, 9000 Gent, Belgium

E. Bockstaele
Department for Plant Production, Ghent University,
Coupure Links 653, 9000 Gent, Belgium

Table 1 Primers used in this study and expected amplicon length

Primer name	Sequence (5'-3')
nos1NEST primer	GCG CGG TGT CAT CTA TGT TA
SL1 primer	TTG GCC CTT TTG AGA CAA TTA
SL2 primer	ACA ACG GGG AGT TGT ATG CT
9CA primer	TAC TGG AGC GGT CAC ACC TG
9CB primer	GTT CAC GCC GTA GAT GCC GAT
Primer pair	Amplicon length (StarLink maize)
Cry9c coding sequence	
9CA/9CB	180 bp
Junction A	
nos1NEST/SL1	138 bp
Junction B	
nos1NEST/SL2	100 bp

In this article, we present 3 primer pairs that can be used to screen and identify for the presence of CBH-351 maize in food and feed matrices.

Materials and methods

DNA isolation

In order to characterise Starlink junction regions and to optimise the designed event-specific primer pairs, DNA was extracted from lyophilised 100% CBH-351 leaf material. DNA was prepared using the DNeasy plant mini kit (Qiagen), starting from 20 mg of dried leaf material. DNA concentration of the extracted samples was estimated on agarose gel using a phage λ DNA dilution series.

DNA extraction for development of the *cry9c*-specific primers was done from 200 mg of CBH-351 maize kernels using the CTAB (cetyltrimethylammonium bromide) procedure as described elsewhere [5]. DNA quality and extraction yield was assessed by spectrophotometry.

Amplification and sequencing of 3' nos junctions

Amplification, isolation and sequencing of the 3' nos junctions was done as described previously [6]. Anchored-PCR reactions [7] were performed using a 3' nos specific anchor primer, the nos1 NEST primer (see Table 1), on StarLink DNA digested with MseI, BfaI and HindIII.

StarLink PCR identification system

According to the amplicon targeted, different PCR methodologies were used. First, for conventional end-point PCR amplification using the *cry9c*-specific primer pairs, PCR conditions were as follows. The PCR reaction mixture contained 1× amplification buffer (Perkin Elmer), $MgCl_2$ to a final concentration of 1 mM, 250 µM of dNTP mix (Pharmacia), 1 µM of each primer (see Table 1) and 1 unit of AmpliTaq Gold polymerase (Perkin Elmer). Autoclaved water was added to a final volume of 25 µL. PCR reactions were performed on 200 ng of template DNA. The PCR cycle profile used was as follows. After an initial denaturation at 95 °C for 12 min, the samples were submitted to 45 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, extension at 72 °C for 30 s and a final extension of 6 min at 72 °C. The obtained PCR products were analysed on a 2.5% agarose gel (Gibco BRL) and stained with ethidium bromide. A standard 123 bp ladder (Gibco BRL) was used as molecular weight marker.

Second, conventional end-point PCR amplification, using event-specific primer pairs, was carried out in a total volume of 25 µL under following conditions: 1× PCR-buffer (Perkin Elmer), 200 µM dNTP's (Pharmacia Biotech), 0.5 µM nosNEST1 primer, 0.5 µM plant-specific-primer (see Table 1), 1 unit Taq polymerase and 5 µL of the DNA sample containing approximately 25 ng of genomic DNA. The PCR reactions were performed using the following cycle profile. Step 1 : initial denaturation at 94 °C for 3 min; step 2 (35 cycles): 20 s at 94 °C, 30 s at 57 °C and 60 s at 72 °C; step 3 : 6 min at 72 °C. After the amplification, 10 µL of the reaction mix was separated on a 2% agarose gel and stained with ethidium bromide and visualised using a UV transilluminator. A GeneRuler 50 bp DNA ladder (MBI Fermentas) was used to estimate the length of the visualised PCR amplicons.

Event-specific primer pairs were also tested in a real-time PCR assay. Reaction conditions for this latter experiment were as follows: 1× SybrGreen PCR Master Mix (Applied Biosystems), 0.3 µM nos1Nest primer, 0.3 µM plant specific primer (see Table 1) and 5 µL template DNA. Water was added to a final volume of 50 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 reaction was followed using an ABI Prism 7700 sequence detection system. For event-specific real-time PCR, amplicon specificity was proven by means of melting curve analysis.

Determination of the absolute detection limit of event-specific primers

In order to determine the absolute limit of detection for two event-specific primer pairs, a dilution series was established containing 1, 5, 10, 20, 30, 50, 100, 250, 500, 1000 and 2500 CBH-351 genomic copies in a volume of 5 µL. The number of genomic copies was determined, taking into account an estimated haploid genome size for maize of 2671 Mbp and a molecular weight of approximately 2.7 pg.

Results and discussion

Screening for Starlink maize using *cry9c*-specific primer pairs

At the start of the present study, the precise genomic sequences used to construct the different CBH-351 expression cassettes was not known. In order to design primers specific for the *cry9c* gene we used the cry9ca1 sequence present in the EMBL genome database and described previously [8]. Several amplimers were designed to target the active site of the cry9ca1 protein but all were inoperative (data not shown). Therefore, we searched the EMBL sequence database for the translated cry9ca1 protein sequence. As a result a 1878 bp sequence was retrieved (A73544). Subsequently, a set of amplimers was designed, targeting the region of A73544 encoding the active site of the insecticidal protein and displaying a low sequence conservation with other cry coding sequences as found in Bt11, Bt176 or MON810. The position of the primers was also selected to target a short amplicon, making the proposed primer pair suitable for detection of Starlink maize in processed food or feed matrices.

The cry9ca1-specific amplimers (see Table 1) were tested for their ability to discriminate between different cry-containing maize lines such as Bt11, Bt176, MON 810 and CBH-351. This experiment was performed 3 times. A PCR product of the predicted size was only am-

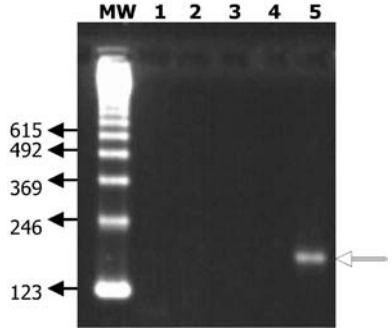

Fig. 1 PCR amplification of a CBH-351, *cry9c*-specific amplicon (180 bp). *Lane 1* non-GMO maize, *lane 2* Bt11 maize, *lane 3* Bt176 maize, *lane 4* MON810 maize and *lane 5* CBH-351 maize. *MW* molecular weight marker (length of the indicated fragments is given in bp)

plified from the CBH-351 DNA (Fig. 1) when using the 9CA/9CB primer pair. The PCR product was sequenced and elicited a 100% similarity with the corresponding amplicon theoretically selected in the A73544 sequence. There is no cross reaction with the other cry sequences from the other transgenic maize lines. Consequently, there is a very high probability that the DNA sequence A73544 or a close derivative was exploited to produce maize line CBH-351.

Amplification of the 3' nos junctions

Anchored PCR was performed on StarLink DNA using a 3' nos specific anchor primer and three different DNA digests (MseI, BfaI and HindIII). The anchor primer that was used, the nos1 NEST primer (see Table 1), is situated close to the end of the 3' nos terminator sequence. One fragment was amplified using the MseI StarLink DNA as template, 4 different 3' nos specific fragments were amplified using the BfaI digested StarLink DNA and no fragments were amplified using the HindIII digested DNA. Since the StarLink maize line contains at least 4 copies of the bar cassette [1], which contains the 3' nos terminator region, it is not unexpected to amplify different 3' nos specific junctions. After isolation of the amplified junctions, we were able to sequence and characterise unambiguously two out of the five amplified fragments situated at the 3' nos terminator border. The other amplified junctions were too short to give enough sequence information in order to develop valuable event-specific primer pairs.

The first junction, referred to as junction A (EMBL accession no AJ506040) (Fig. 2), is 239 bp long and contains 59 bp of insert DNA and 180 bp plant DNA. The insert DNA is composed of part of the 3' nos termination signal and part of a multiple cloning site. A BLAST search against the GenBank database was performed using the 180 bp plant DNA segment. This database search indicates that the plant DNA segment adjacent to the 3' nos signal of junction A is homologous with a *Zea mays*

Fig. 2a, b Sequence of two amplified 3' nos junctions. **a** Junction A, **b** junction B of StarLink maize. Plant DNA sequences are shown in *bold*. Insert DNA corresponding to the 3' nos terminator sequence is shown in *regular upper case*. The primers used within this study to amplify the junction regions are depicted as *underlined sequences*

chloroplast sequence, coding for a tRNA-Ile. The second junction, junction B (EMBL accession nr.: AJ506041) (Fig. 2), is 220 bp long and contains 39 bp of insert DNA. Similarly to junction A, the insert DNA contains part of the 3' nos sequence and part of a multiple cloning site. The remaining 181 bp of junction B are homologous with a *Zea mays* chloroplast genome locus that codes for a RNA polymerase alpha subunit.

Design of event-specific primer pairs

Based on the sequence of two StarLink plant DNA junctions, we developed two different event-specific primer pairs. For junction A, the SL1 plant-specific primer was developed, while for junction B the SL2 plant-specific primer was developed. Both plant-specific primers were used in combination with a 3' nos specific primer, the nos1 NEST primer (see Table 1). The expected amplicon length for the two different primer combinations is given in Table 1. Both primer pairs have been tested using conventional end-point PCR and real-time PCR. When real-time PCR was performed, a melting curve analysis of the amplicon was done. For the nos1NEST/SL1 and the nos1NEST/SL2 amplicons, experimental melting temperatures of 79.8 °C and 80.0 °C, respectively were determined (Fig. 3).

Sensitivity of the event-specific primer pairs

To test sensitivity of the event-specific primer pairs, we determined the absolute limit of detection for both prim-

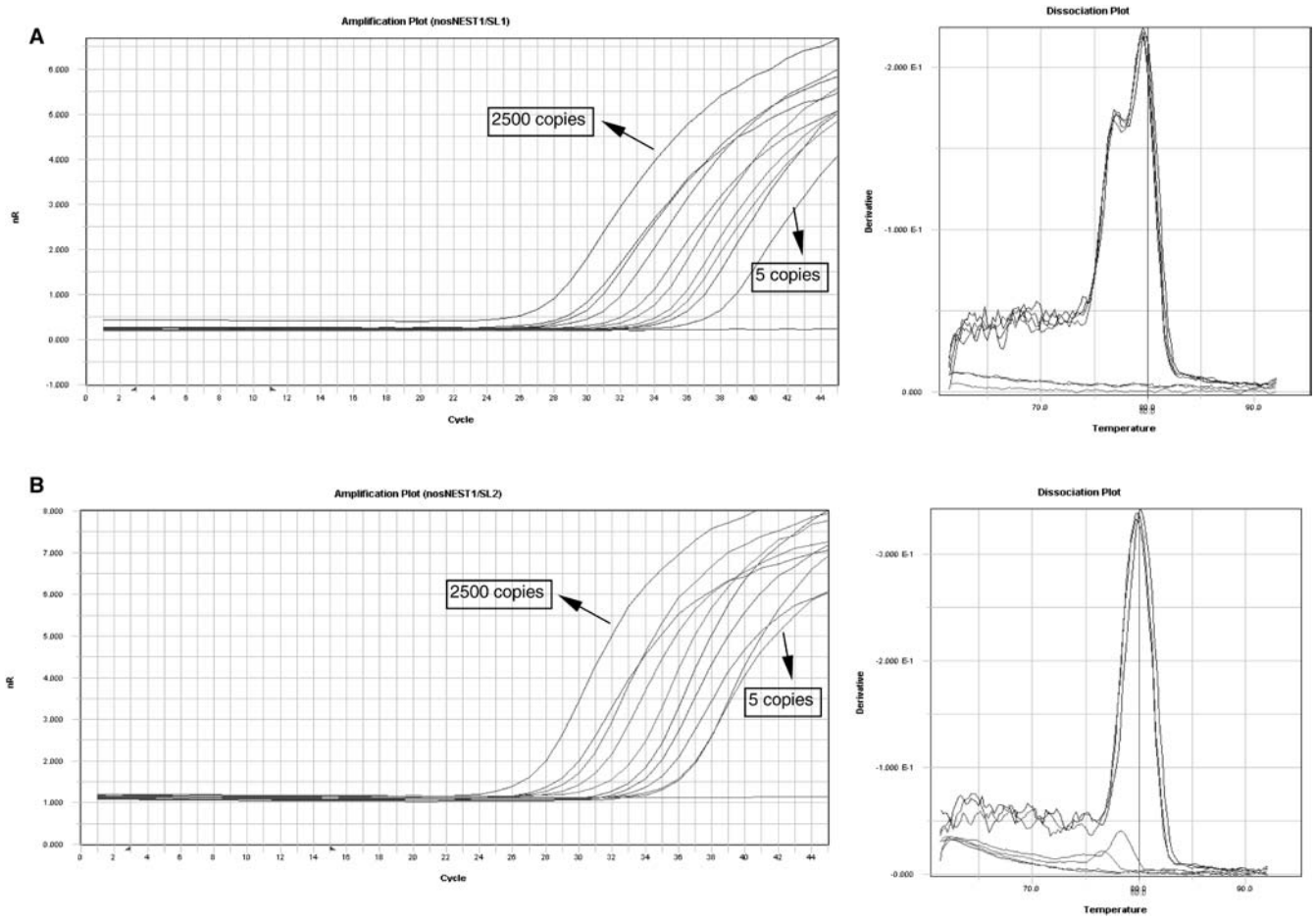

Fig. 3A, B Determination of the absolute detection limit of two event-specific primer pairs for CBH-351 in a real-time PCR assay and their respective melting curves. **A** nos1NEST/SL1 primer pair and **B** nos1NEST/SL2 primer pair. Amplification curves for 11 dilutions containing 2500, 1000, 500, 250, 100, 50, 30, 20, 10, 5 and 1 copy of the CBH-351 genome

er pairs. When a conventional end-point PCR amplification is performed, both event-specific primer pairs detect the CBH-351 genome down to approximately 10 copies. On the other hand, when the event-specific primer pairs are used in a real-time PCR assay, we can detect down to 5 copies of the CBH-351 genome (see Fig. 3).

Specificity of the event-specific primer pairs

Specificity of the developed primer pairs was tested in two different ways. First of all we analysed whether the developed event-specific primer pairs enabled us to distinguish between non-transformed maize and StarLink maize. PCR analysis, using the two different primer combinations on non-transformed maize DNA and StarLink DNA, revealed that both primer combinations give the expected amplicon when StarLink maize is used as template and no fragment is detected when the non-transformed maize DNA is used as template (Fig. 4). For

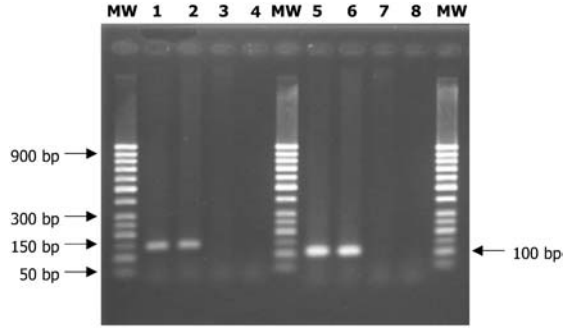

Fig. 4 Qualitative PCR analysis of two event-specific primer pairs for CBH-351 maize. *Lane 1–4*: event-specific primer pair nos1 NEST/SL1 (amplicon size 138 bp) tested on CBH-351 maize (analysis performed in duplo, lane 1 and 2), non-GMO maize (lane 3) and negative water control (lane 4). *Lane 5–8* event-specific primer pair nos1NEST/SL2 (amplicon size: 100 bp) tested on the same DNA samples as described for the nos1NEST/SL1 primer pair. *MW* GeneRuler 50 bp DNA ladder

the conventional end-point PCR, amplified amplicons were validated by direct sequencing. A 100% sequence similarity was observed between the amplified product and the theoretical sequence of the StarLink junction regions.

Secondly, we tested whether the event-specific primer combinations are specific for StarLink maize. Since the

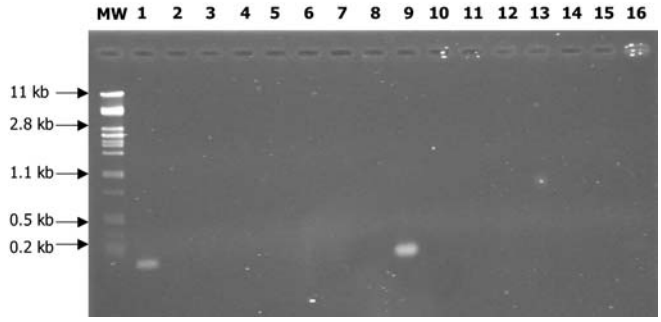

Fig. 5 Specificity PCR analysis of two event-specific primer pairs for CBH-351 maize. *Lane 1–8* event-specific primer pair nos1 NEST/SL2 tested on CBH-351 maize, non GMO maize, BtXtra maize, T25 maize, MON810 maize, GA21 maize, Bt11 maize and Bt176 maize. *Lane 9–16* event-specific primer pair nos1 NEST/SL1 tested on the same DNA samples as described for the nos1NEST/SL2 primer pair

event-specific primer pairs developed for StarLink maize consist of a primer specific for the nos terminator and a primer specific for a maize chloroplast genome sequence, it is not unthinkable that the primers give a background signal when other transgenic maize lines are present in the analytical sample. Therefore, the two primer combinations were tested on following maize samples: MON810 (Yieldgard) maize, T25 (Liberty-Link) maize, GA21 (Roundup Ready) maize, Bt11 maize, Bt176 (NaturGard, KnockOut) maize and BtXTra maize. The two event-specific primer combinations give the expected amplicon when StarLink maize is used as template, no amplification signal is seen for the other transgenic maize lines (Fig. 5). Taken together, these results indicate that the proposed event-specific primer pairs, based on two junction regions of CBH-351 maize, can be used to identify with high selectivity and high sensitivity the presence of CBH-351 maize.

Acknowledgements The authors thank Cindy Merckaert for helping with the sequencing of the junction fragments, Giliane Guillaume for the search of sequence A73544 and Jelle De Keukeleire for excellent technical assistance. The European Commission is acknowledged for financial support through the Fifth (EC) Framework Program 1998–2002, project QPCRGMO-FOOD, contract no QLRT-1999–01301. This work was supported by program 1510 of the Institute of Public Health.

References

1. Agricultural and Biotechnology Strategies (Canada) Inc. GMO database, CBH-351 info http://www.agbios.com/default.asp
2. EPA (2000) Assessment of scientific information concerning StarLink corn Cry9C Bt corn plant-pesticide. Federal Register 65, October 31 2000. Environmental Protection Agency, Arlington, pp 65246–65251 http://www.access.gpo.gov/su_docs/fedreg/frcont00.html
3. FDA (2000) FDA recommendations for sampling and testing yellow corn and dry-milled yellow corn shipments intended for human food use for Cry9C protein residues, Final guidance. Food and Drug Administration, Center for Food Safety Administration, Washington, DC http://www.cfsan.fda.gov/ ~dms/starguis.html
4. Orlandi PA, Lampel KA, South PK, Assar SK, Carter L, Levy DD (2002) J Food Prot 65:426–431
5. Hupfer C, Hotzel H, Sachse K, Engel K-H, (1998) Z. Lebensm Unters Forsch 206:203–207
6. Windels P, Taverniers I, Depicker A, Van Bockstaele E, De Loose M (2001) Eur Food Res Technol 213:107–112
7. Theuns I, Windels P, De Buck S, Depicker A, Van Bockstaele E, De Loose M (2002) Euphytica 123:75–84
8. Lambert B, Buysse L, Decock C, Jansens S, Piens C, Saey B, Seurinck J, Van Audenhove K, Van Rie J, van Vliet A, Peferoen M (1996) Appl Environ Microbiol 62:80–86