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Development of real-time PCR systems based on SYBR® Green I, Amplifluor™ and TaqMan® technologies for specific quantitative detection of the transgenic maize event GA21

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Abstract

Maize GA21 line has integrated several tandemly repeated copies of the *r-act* 5-enol-pyruvylshikimate-3-phosphate synthase construct used for plant transformation. We were able to amplify a nucleotide sequence corresponding to the polylinker plasmid vector flanked by the *r-act* promoter and nopaline synthase 3′-terminator. A method for specific detection and quantification of Roundup Ready® transgenic maize line GA21 DNA using conventional and real-time PCR and based on this transgenic sequence is described. GA21 specific primers and probe were designed targeting the vector–promoter junction region and amplifying a 72-bp DNA fragment. Quantification methods were optimized through three different real-time PCR chemistries, i.e. SYBR® Green I, Amplifluor™ and TaqMan®. All three methods proved to be specific, highly sensitive and reliable for both identification and quantification of GA21 DNA.

Plasmid *pGAivr* containing single copies of the GA21 and *invertase* amplicons was constructed for use as external standard in calibration curves. Using *pGAivr*, a TaqMan® based real-time PCR assay was optimized in duplex format targeting the maize species-specific *ivr1* gene and the GA21 junction region. The detection limit of the method was 0.01% GA21, which is far below the established threshold for accidental presence of genetically modified organisms (GMO), this method therefore being suitable for use in routine GMO analysis.

Keywords: Transgenic maize; GA21; Real-time PCR; Genetically modified organisms quantification

1. Introduction

Worldwide commercialisation of genetically modified organisms (GMO) has led to the approval of labelling regulations in several countries intended to protect the consumers' right to information. EEC (European Commission, 2000a,b) and Japanese Ministries of Health and Welfare (MHW) and Agricultural Food and Fisheries (MAFF) (Matsuoka et al., 2001) regulations have established the compulsory labelling of foods containing more than 1 or 5% GMO, respectively, which is considered to be the upper threshold for an accidental GMO contamination during field culture or seed transport. GM soybean and maize are the most extensively cultivated GMO, with the traits introduced in these lines being, basically, resistance to herbicides and increased tolerance to insects and pests. The *Zea mays* line GA21, for example, was developed by Monsanto in collaboration with DEKALB Genetics Corporation to exhibit tolerance to the Roundup® glyphosate herbicide. This line was obtained by direct transformation of a DNA construct containing the rice-*actin* (*r-act*) promoter with its leader intron sequence, a modified 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene from maize and the termination signal nopaline synthase (NOS) 3′ from *Agrobacterium tumefaciens*. During transformation, several copies of the construct were tandemly inserted in a head-to-tail conformation leading to a multicopy arrangement of the transgene.

Abbreviations: CRM, certified reference materials; CTAB, cetyl trimethylammonium bromide; EPSPS, 5-enol-pyruvylshikimate-3-phosphate; FAM, 6-carboxyfluorescence; GMO, genetically manipulated organisms; IRMM, Institute for Reference Materials amd Measurements; LOD, limit of detection; LOQ, limits of quantification; OPS, Optimized transit peptide; PCR, Polymerase chain reaction; TAMRA, 6-carboxy-tetramethylrhodamine; UNG, Uracil N-glycosylase.

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The more robust GMO detection methods currently in use are those based on DNA amplification, because of the high stability of the DNA molecule under the harsh conditions used in food processing (Hübner et al., 2001; Wiseman, 2002). Quantitative real-time PCR methods have been developed for several GMO events including the maize 176 (Vaïtilingom et al., 1999), MON810 (Holck et al., 2002; Hernández et al., 2003), Bt11 (Rønning et al., 2003), CBH-351 (Windels et al., 2003) events, the soybean GTS 40-3-2 (Berdal and Holst-Jensen, 2001; Terry and Harris, 2001; Vaïtilingom et al., 1999; Wurz et al., 1999) event, and also a durum transgenic wheat line, transformed to express a tobacco gene (*rab*1) (Terzi et al., 2003); but assays specific to the maize line GA21 are not yet available, probably due to the complexity of the insertion. All these methods are based on the parallel amplification of transgene specific sequences and an endogenous reference gene, which serves as internal control for the quality of the extracted DNA, and as estimation of the total amount of target plant DNA present in the sample. Nucleic acid quantification in real-time PCR assays is performed in a closed tube through fluorometric analyses, which reduces the risk of cross-contamination and allows easy automation of the method. Different fluorometric detection systems have been developed to the present. The TaqMan® chemistry (Applied Biosystems, Foster City, CA, USA) is one of the most commonly used for GMO detection and is based on the simultaneous addition of two primers and a specific probe that yields fluorescence emission upon DNA synthesis (Heid et al., 1996). The Amplifluor™ Universal Amplification and Detection System (Intergen Co., Purchase, NY, USA) is based on a universal hairpin primer (Uniprimer™). It anneals to the Z sequence appended to one of the gene-specific oligonucleotides and produces fluorescence upon incorporation into the amplification product (Nazarenko et al., 1997). The intercalating dye SYBR® Green I (Molecular Probes, OR, USA), which exhibits fluorescence enhancement upon binding to the double-stranded amplification product (Howell et al., 1999; Morrison et al., 1998), offers an inexpensive and sequence unspecific alternative. In all three types of reaction, the fluorescence signal produced directly correlates with the accumulation of PCR product at each cycle, thus providing a quantification of the amount of template DNA in the reaction.

Here we report on the development of SYBR® Green I, TaqMan® and Amplifluor™ based real-time PCR strategies for specific quantification of maize line GA21. This is the first time that an Amplifluor™-based real-time PCR assay is optimized for GMO quantification. Specific GA21 nucleotide sequences were obtained by PCR amplification and sequencing of a transgene region located between two inserted copies of the construct, which have fused in a head-to-tail arrangement. We have used the plasmid–promoter junction sequence, unique to this insertion event, as target for the PCR assays. We have compared the limits of detection (LOD) and quantification (LOQ) of the three real-time PCR methods and obtained similar values. Furthermore, a plasmid containing the GA21 target sequence and a region of the maize endogenous control *invertase A* gene (*ivr1*, U16123) was constructed for use as standard for quantification; and both GA21- and *ivr1*-specific real-time PCR reactions were optimized in duplex format.

2. Experimental

2.1. Plant materials

Transgenic maize seeds of the GA21, T25 and CBH-351 lines were kindly provided by the Laboratory of the Government Chemist (LGC, Teddington, Middlesex, UK) and Aventis CropScience (Gent, Belgium). Powdered certified reference materials (CRMs) for Roundup Ready® (GTS 40-3-2) soybean and maize Bt176, Bt11 and MON810 were from the Institute for Reference Materials and Measurements (IRMM) and commercialized by Fluka (Buchs, Switzerland). Certified powdered Bollgard® (event 531) and Roundup Ready® cotton was also used. Leaves of *Zea mays* cultivar W64A, *Zea diploperennis*, *Sorghum bicolor*, *Oryza sativa*, *Hordeum vulgare*, *Brassica napus*, *Brassica oleracea*, *Arabidopsis thaliana* ecotype *Columbia*, *Solanum tuberosum* var. *Désirée*, *Lycopersicum esculentum* var. *Ailsa Craig*, *Helianthus annuus* were from plants cultivated in the green-houses at the IBMB-CSIC. DNA samples of *Secale cereale*, *Triticum aestivum*, *Panicum miliaceum*, *Glycine max*, *Lens esculenta*, *Vicia faba*, *Phaseolus aureus* and *Lupinus albus* were provided within the framework of the 'Reliable, Standardised, Specific, Quantitative Detection of Genetically Modified Food' (Qpcrgmofood) European project.

2.2. DNA isolation and quantification

Large-scale genomic DNA was isolated from 30 g of leaves according to Dellaporta et al. (1983). Small-scale genomic DNA was isolated from 0.1 g of plant material using a CTAB-based protocol as described (Meyer and Jaccaud, 1997). DNA concentration was quantified using the spectrophotometer GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). Concentrations were further checked by agarose gel electrophoresis and ethidium bromide staining (Sambrook and Russel, 2001). UV-fluorescent emission was recorded and quantified with the Quantity One™ software (Bio-Rad Laboratories Inc., Hercules, CA).

2.3. PCR reactions

The Primer Express™ v2.0 software (Applied Biosystems division of Perkin–Elmer Corp., Foster City, CA) was used to design the oligonucleotides shown in Table 1. Primers and probe were purchased from MWG-Biotech AG

Table 1
Oligonucleotides

PCR system	Name	Type	Sequence (5′–3′)
Mapping integrated junction	*NTGAF1*	Forward primer	GGTTTTTATGATTAGAGTCCCGCAA
	APGAR1	Reverse primer	GACTATCCCGACTCTCTTCTCAAGC
	APGAR2	Reverse primer	CGAACTTAAAACCCGACTCAAATACAG
	NTGAF2	Forward primer	TTGCCGGTCTTGCGATGATTAT
Junction sequence cloned	*NTGAF1*	Forward primer	GGTTTTTATGATTAGAGTCCCGCAA
	APGAR1	Reverse primer	GACTATCCCGACTCTCTTCTCAAGC
SYBR® Green I	*GA141F*	Forward primer	GGATCCCCCAGCTTGCAT
GA21 r-t[a] PCR	*GA212R*	Reverse primer	TTTGGACTATCCCGACTCTCTTCT
TaqMan®	*GA141F*	Forward primer	GGATCCCCCAGCTTGCAT
r-t[a] GA21	*GA212R*	Reverse primer	TTTGGACTATCCCGACTCTCTTCT
PCR	*GA160P*	TaqMan® probe	FAM-CCTGCAGGTCGAGGTCATTCATATGCTT-TAMRA
Amplifluor™	*GA141ZF*	Amplifluor™ primer	actgaacctgaccgtacaGGATCCCCCAGCTTGCAT[b]
GA21 r-t[a] PCR	*GA212R*	Reverse primer	TTTGGACTATCCCGACTCTCTTCT
TaqMan®	*IVR7F*	Forward primer	GCGCTCTGTACAAGCGTGC
Invertase	*IVR8R*	Reverse primer	GCAAAGTGTTGTGCTTGGACC
r-t[a] PCR[c]	*IVR78P*	TaqMan® probe	VIC-CACGTGAGAATTTCCGTCTACTCGAGCC-TAMRA

[a] r-t: real-time.
[b] lower case: Z sequence of the Amplifluor™ primer.
[c] Hernández et al. (in preparation).

(Ebensburg, Germany). The TaqMan® probe *GA160P* was labelled at the 5′-end with the fluorescent 6-carboxyfluorescein (FAM) dye whereas the TaqMan® probe *IVR78P* was 5′-end labelled with the fluorescent VIC™ reporter dye. The quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) was attached to the 3′-end of both probes. For the Amplifluor™ Universal System a forward primer was designed containing the target-specific sequence in addition to the Z sequence at its 5′ end. All PCR reactions were performed in a 20 μl reaction volume. The TaqMan® PCR Core reagents were used for the TaqMan® and SYBR® Green I reactions (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, NJ).

SYBR® Green I assay. Primer *GA141F* matching the vector sequence was used in combination with primer *GA212R* annealing to the 5′ *r-act* sequence. PCR reactions contained 1 × PCR buffer II (100 mM Tris–HCl, pH 8.3, 500 mM KCl), 3 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP, dUTP, 300 nM primers, 1:10,000 dilution of SYBR® Green I (Sigma-Aldrich Co., St Louis, MO), 1 unit of Ampli*Taq* Gold™ DNA polymerase, 0.2 units of AmpErase® uracil *N*-glycosylase (UNG), and 2 μl of the DNA solution. Real-time PCR reactions were performed using the following program: 2 min 50 °C, 10 min at 95 °C and 50 cycles of 15 s at 95 °C and 1 min at 60 °C. In the absence of SYBR® Green I dye, the same conditions can be used for conventional PCR followed by agarose gel analysis.

TaqMan® assay. Primers *GA141F* and *GA212R* were used in combination with the *GA160P* probe. PCR conditions were as stated before except for the omission of SYBR® Green I dye, and that 1 × PCR TaqMan buffer A (including ROX as a passive reference) was used together with 6 mM $MgCl_2$ and 200 nM probe in the PCR mix.

Multiplex TaqMan® assay. Primers and probe oligonucleotides *GA141F/GA212R/GA160P* specific to the transgene were used in combination with the endogenous reference gene oligonucleotides *IVR7F/IVR8R/IVR78P*. PCR reactions were performed using the same conditions as in the TaqMan® uniplex reactions, using 300 nM of each primer and 200 nM of each probe.

Amplifluor™ assay. PCR reactions were performed according to the conditions described by the manufacturer and included 1 × Ex *Taq*™ Buffer (TaKaRa Bio Inc., Japan), 1.5 mM $MgCl_2$, 250 μM dNTPs, 50 nM *GA141ZF* primer, 500 nM *GA212R*, 500 nM UniPrimer™ (Intergen Co., Purchase, NY, USA), 1 unit of TaKaRa Ex *Taq*™ polymerase (TaKaRa Bio Inc., Japan) and 2 μl of the target DNA solution. Amplification reactions were run using the following conditions: 2 min at 95 °C and 50 cycles of 15 s at 95 °C, 20 s 55 °C and 40 s 72 °C. Fluorescence was measured only at the melting point of the amplification products.

All real-time PCR reactions were run on an ABI PRISM® 7700 Sequence Detection System device (Applied Biosystems division of Perkin–Elmer Corp., Foster City, CA) and the PCR products were analyzed using the sequence detection system v.1.7 software (Applied Biosystems). Quantification was performed by interpolation in a standard regression curve of C_T values generated from DNA samples of known concentrations.

2.4. Construction of standard curves

A 248 bp fragment encompassing nucleotides 2289–2536 from the *ivr1* gene (AN: U16123) from maize was cloned onto the pGEM®-T Easy vector to be utilized as endogenous reference gene. The primer pair *NTGAF1/APGAR1* was used to amplify a discrete PCR product of 208 bp from genomic *GA21* DNA, which was subsequently cloned onto the *Sma*I/*Sac*I restriction site of the pGEM-T*ivr* plasmid to yield plasmid *pGAivr*. Plasmid DNA was purified through a QIAGEN® Plasmid Midi Kit column (QIAGEN GmbH, Hilden, Germany) and quantified using the PicoGreen® kit (Molecular Probes Inc., Eugene, OR, USA) in a Luminescence Spectrometer LS50B (Perkin–Elmer Corp., Norwalk, CT, USA). The purified plasmid DNA was serially diluted in a solution containing 50 ng/μl BSA to yield 2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2, 20, 2, 1, 0.5 molecules of plasmid/μl. According to the plasmid size (3463 bp), 1 molecule corresponds to 3.82 ag of plasmid DNA.

3. Results and discussion

3.1. Plasmid–transgene junction sequences integrated in the maize event GA21

The maize event GA21 was obtained by particle acceleration-driven transformation of the plasmid vector *pDPG434* that includes the *r-act* promoter with the intron leader sequence, a modified EPSPS gene from maize fused to an optimised transit peptide sequence (OTP derived from the corn and sunflower ribulose-1,5-bisphosphate carboxylase oxygenase transit peptide sequences) and the termination signal NOS 3′ from *A. tumefaciens*. According to a Monsanto notification (Scientific Committee of Plants, 2000) a single insertion of 18.5 kb occurred, which includes three complete copies of the cassette, in addition to two partial copies, both of them lacking the NOS 3′ terminator.

Based on the reported sequences for the *r-act* promoter and the *A. tumefaciens* NOS 3′-termination sequences, we designed a battery of oligonucleotides aimed to amplify the sequences located between two consecutive cassettes which would allow us to identify specific sequences for the GA21 event (Table 1). Junction regions between the transgene and plant genomic sequences or transgene and plasmid vector sequences have been found very useful for GMO identification due to the lack of cross-reactivity with other GMO events or plant genomic sequences. Indeed, neither the *pDPG434* plasmid construct nor a different plasmid containing a similar polylinker *r-act* promoter/NOS 3′ terminator sequence arrangement have been used for production of any other plant GMO, these sequences thereby being specific for the GA21 insertion event.

Long-range PCR amplifications were performed using combinations of different oligonucleotides located within the NOS 3′ terminator and *r-act* promoter, respectively, with GA21 and non-GM maize genomic DNA as a template. For all combinations, a discrete product was obtained only with the GA21 template DNA (data not shown), with the lengths of the obtained fragments (208 bp for primers *NTGAF1* and *APGAR1*; 447 bp for *NTGAF1* and *APGAR2*; 302 bp for *NTGAF2* and *APGAR1*; and 541 bp for *NTGAF2* and *APGAR2*) being consistent with a unique junction sequence comprised by 54 bp of vector.

To further characterize this junction sequence, we cloned the *NTGAF1/APGAR1* amplification product into the pGEM®-T Easy vector, and fully sequenced this transgene fragment (GenBank accession number AY255709). The sequence of this cloned DNA region is shown in Fig. 1, in which the 3′ sequence of the NOS terminator and the 5′ sequence of the *r-act* promoter are indicated, together with the 54 bp nucleotide sequence located in between these two sequences. Comparison of this 54-bp sequence with DNA and patent databases showed that it would correspond to plasmid DNA, with nucleotides 91–136 matching to the polylinker restriction sites *Not*I, *Xba*I, *Sph*I and *Pst*I, present in the plasmid *pDPG434* used for transformation. Thus, these data indicate that multiple copy insertion of the construct occurred by multimerization (i.e. ligation of multiple molecules) of the vector through the *Not*I restriction site used for linearization. In fact, we assessed whether the two vector sequences located between the three complete inserted cassettes (the additional partial cassettes do not include the NOS 3′ terminator) were identical by sequencing several of the *NTGAF1/APGAR1* amplified fragments. We obtained identical nucleotide sequences for all clones indicating that both intergenic sequences are identical.

3.2. Use of the plasmid–transgene junction to design GA21-specific real-time PCR quantification methods

Based on the transgenic sequences reported above, we designed the oligonucleotides *GA141F/GA212R/GA160P* (Table 1) suitable to be used in real-time PCR assays for specific detection and quantification of the GA21 insertion event. The forward primer was located within the plasmid sequence and the reverse primer was complementary to the 5′ region of the *r-act* promoter, thus yielding a 72-bp amplicon product. A combination of primers *GA141F* and *GA212R* was used in SYBR® Green I based real-time PCR reactions; and the same transgene sequences were targeted in the Amplifluor™ based reactions (primers *GA141ZF/GA212R* were used in these reactions as indicated in Table 1, in combination with the UniPrimer™ oligonucleotide). For TaqMan® based reactions, the former nucleotides were used, together with a TaqMan® probe (*GA160P*) designed over the plasmid–transgene junction sequence, which contained annealing sequences complementary to both the plasmid and the *r-act* promoter, thus providing an extra component of specificity to the detected signal

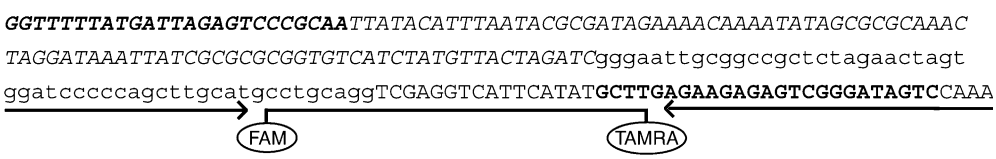

Fig. 1. *Genome organization of event GA21 in transgenic maize and design of a real-time PCR assay for event GA21 maize*. The nucleotide sequence of part of the GA21 transgene used to design specific primers for amplification is shown. Capital letters represent the 3′ sequence of the NOS terminator (italics) and the 5′ sequence of the *r-act*; and lowercase letters show the plasmid flanking sequence. Primers used for PCR amplifications detailed in Table 1 are highlighted in bold and underlined. Forward and reverse primers *GA141F* and *GA212R* amplify a 72 bp amplicon with annealing sequences for dual-labelled fluorescent probe *GA160P* placed in the transgene-plasmid border sequence.

(this vector/*r-act* promoter junction arrangement is exclusive of the GA21 event).

To compare these methodologies, we first optimised all three real-time PCR systems using 100 ng of GA21 purified DNA (protocol P/N 402823 Rev D: Applied Biosystems 2003), with optimal PCR conditions being those reported in Section 2. It must be noted that although optimal conditions for the TaqMan® and SYBR® Green I amplification reactions were very similar; the Amplifluor™ reactions had significant intrinsic differences with respect to the other two methods. First, elongation cannot be performed in the presence of dUTP, which in combination with the uracil-*N*-glycosylase (UNG) enzyme allows cleavage of contaminant amplicons. Second, in these reactions a DNA polymerase lacking 5′–3′ exonuclease activity needs to be used in order to avoid hydrolysis of the Uniprimer™ primer region. This is in clear contrast, with the TaqMan® based reactions, which specifically require the use of a DNA polymerase enzyme with this type of activity (as for example the Ampli*Taq* Gold™ DNA polymerase) which will result in cleavage of the TaqMan® probe and subsequent fluorescence emission.

To evaluate the specificity of the developed PCR assays, we run real-time PCR reactions on 100 ng of DNA extracted from 19 different plant species that phylogenetically were closely related to maize (i.e. *Zea diploperennis*) or more distantly related to this plant species. We also assayed the lack of cross-reactivity with other GMOs currently approved, particularly with GM maize and GMO tolerant to glyphosate-containing herbicides (Roundup Ready® soybean, YieldGard® maize and Roundup Ready® cotton). In these assays, detectable amplification was only observed with the GA21 event (Fig. 2).

3.3. Construction of an external standard plasmid for evaluation of the detection (LOD) and quantification (LOQ) limits of the GA21 detection chemistries

GMO PCR quantification is based on the parallel amplification of a region of the transgene and a species-specific endogenous gene, which gives an estimation of the total amount of amplifiable DNA from the species of interest present in the sample. CRM validated by the IRMM are usually used as standards for amplification, with genomic DNA obtained from these certified materials used to construct the quantification calibration curves. One major advantage of using CRM DNA as calibration standards is that the transgene and the species-specific target gene are in a constant relative amount, which minimizes errors in relative concentrations. However, calibration curves obtained with certified materials have the disadvantage that at high amounts of genomic DNA as required in the upper limits of the standard curve, signal inhibition occurs which narrows down the linear range of the obtained calibration curves (Berdal and Holst-Jensen, 2001). CRMs, on the other hand, have not been commercialised for every GMO, as for example GA21 maize, for which validated reference material is not currently available.

The absence of GA21 CRM prompted us to construct plasmid *pGAivr* to be used as external standard suitable to build the calibration curves required for quantification. This plasmid contains a single copy of the *NTGAF1/APGAR1* PCR product and a single copy of a 248 bp fragment of the maize *ivr1* gene, which was optimised for use as maize species-specific endogenous control (Hernández et al., in preparation).

We have determined the LOD and the LOQ of the three real-time PCR systems through reactions (nine replicated) performed with appropriate dilutions of *pGAivr* to yield an estimated number of target DNA molecules ranging from 4×10^6 to 1. As shown in Fig. 3 and Table 2, high reproducibility and accuracy could be obtained with all three methods. The slopes of the linear regression curve analyses indicated very efficient amplification rates in the TaqMan® (−3.449) and SYBR® Green I (−3.138) PCR systems, being the Amplifluor™ based reactions slightly less efficient (−3.862). Correlation coefficients were about 0.99 for all three systems, with down to 40 target molecules detected in each of the nine replicate reactions performed for each method. The three methods allowed detection of four target molecules in six (TaqMan® and SYBR® Green I) or five (Amplifluor™) out of nine replicates, and down to one target molecule in half of the replicates.

Theoretical LOQ was calculated for this experimental design, taking into consideration the error associated with the serial dilutions of the DNA. Table 2 shows the range of plasmid copies that with a 95% probability were present in each reaction. In the higher dilution ranges (below 40 copies of plasmid) confidence intervals overlap with each other and reliable quantification is not possible. Therefore, based on these statistical and experimental considerations, the LOQ was determined to be of 40 copies for all three methods.

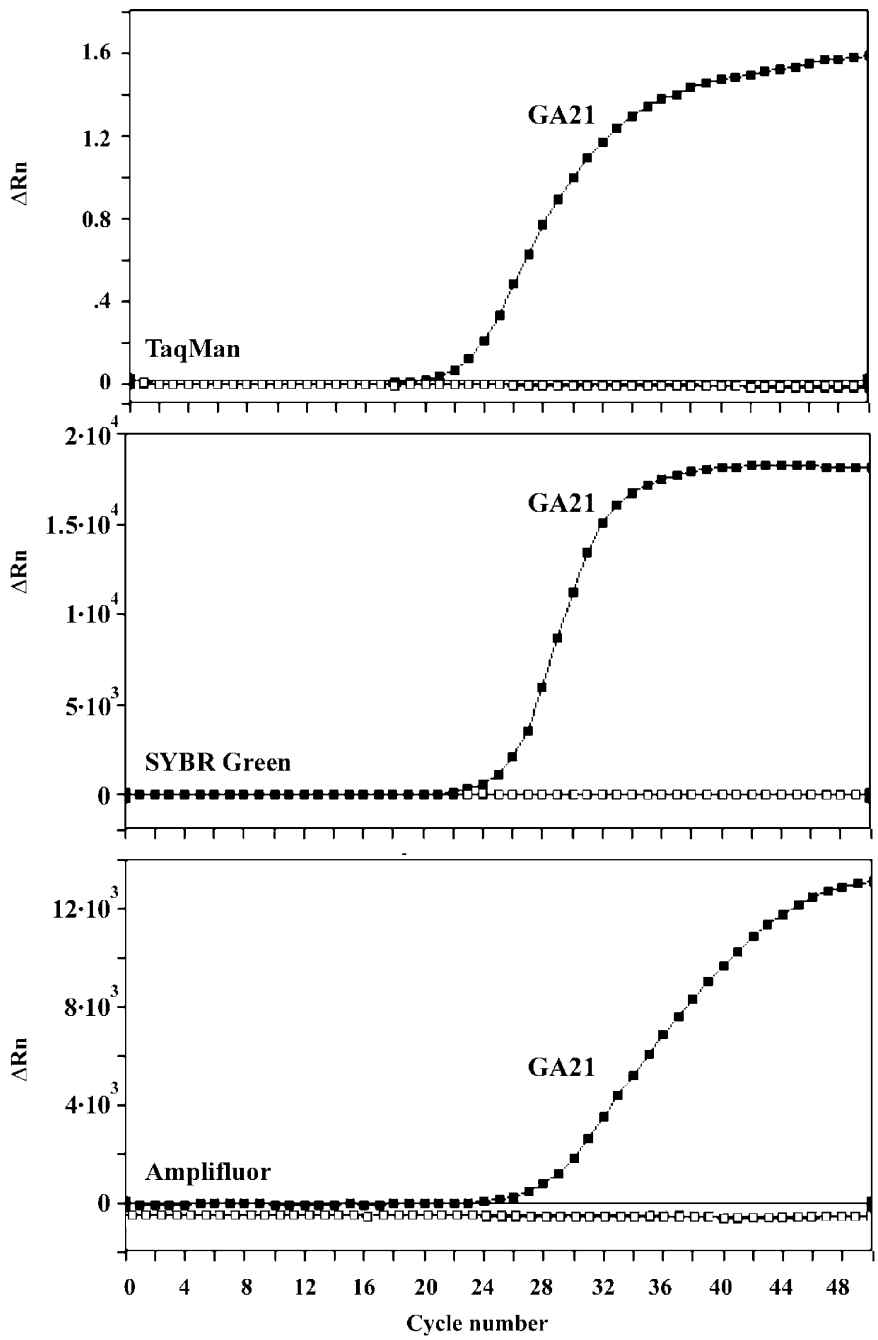

Fig. 2. *Specificity of the GA21 real-time PCR-based assays*. Amplification plots generated in TaqMan®, Amplifluor™ and SYBR® Green I real-time PCR reactions with 100 ng of DNA purified from (■) event GA21 maize; and (□, all samples beneath the threshold) Bt176, Bt11, MON810, T25 and CBH-351 transgenic maize lines; GTS 40-3-2 transgenic soybean line; Bollgard® (event 531) and Roundup Ready® transgenic cotton lines; and the wild-type plant species listed in Section 2.

3.4. Duplex detection and quantification of GA21 and the maize invertase gene using TaqMan® real-time PCR

To simplify the TaqMan® based real-time PCR experimental design, we performed the TaqMan® GA21 event-specific PCR reaction in a duplex format with the reaction targeting the maize species-specific *ivr1* gene, used as endogenous reference gene. Plasmid *pGAivr*, containing both target DNAs in the same number of copies, was used as DNA template for these reactions and three reaction replicates were set for each plasmid DNA concentration that ranged from 4×10^6 to 1 target molecule. Results obtained for the GA21 (data not shown) and *ivr1* (Table 2) reactions were the same as in uniplex format. The linear regression curve derived from the *ivr1* reactions showed both very efficient amplification rates (slope of − 3.152) and

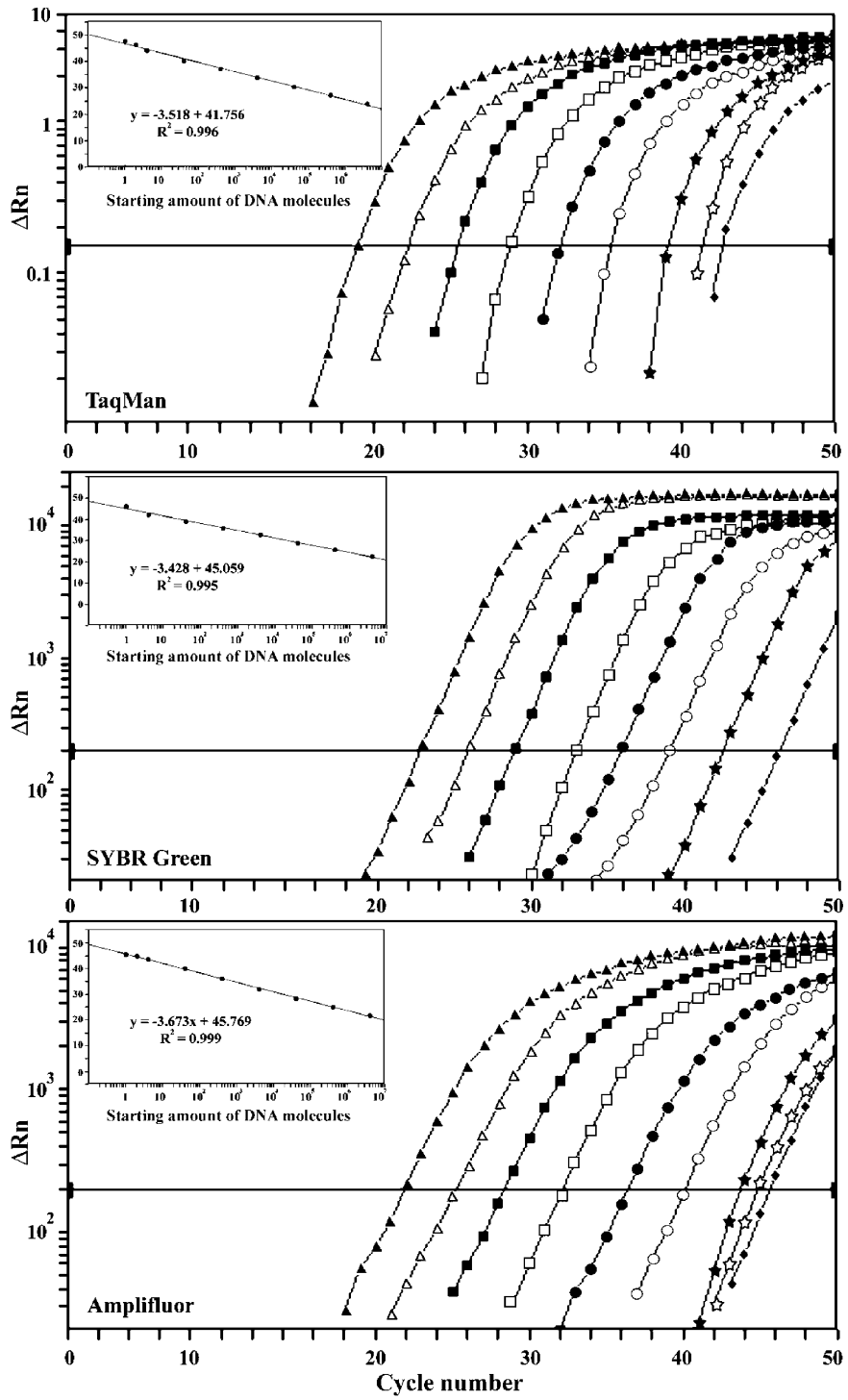

Fig. 3. *Detection and quantification of event GA21 using real-time PCR-based assays*. Typical amplification plots generated in real-time PCR reactions containing 4×10^6 (▲), 4×10^5 (△), 4×10^4 (■), 4×10^3 (□), 4×10^2 (●), 40 (○), 4 (★), 2 (☆), and 1 (◆) plasmid *pGAivr* molecules. Inset, representative standard curve generated from the amplification data of TaqMan®, Amplifluor™ and SYBR® Green I PCR systems, respectively.

a high correlation coefficient (R^2 of 0.989). This allowed to conclude that *pGAivr* is suitable to be used as external standard to elaborate both GA21 and *ivr1* standard curves in a duplex format, thus decreasing both the amounts of reagents and the number of PCR positions devoted to the controls in the quantification assay.

Furthermore, taking advantage of the fact that the plasmid *pGAivr* contains a single copy insert of each of the *ivr1* fragment and GA21 junction region, we determined the number of copies of the junction plasmid sequence located between the NOS terminator and the *r-act* promoter sequences in the tandemly arranged construct present in the GA21 maize

Table 2
Real-time PCR detection and quantification limits obtained with external standards

p*GAivr* DNA									
Template molecules approximate	4×10^6	4×10^5	4×10^4	4000	400	40	4	2	1
Upper limit confidence interval	4 003 914	401 238	40 392	4124	439	52	8	5	3
Lower limit confidence interval	3 996 086	398 762	39 608	3876	361	28	1	0	0
GA21 uniplex									
SYBR® Green I Signal ratio	9/9	9/9	9/9	9/9	9/9	9/9	6/9	4/9	5/9
SYBR® Green I Mean C_T values	22.12	25.21	27.51	32.20	34.09	37.81	44.37	46.38	45.46
SYBR® Green I SD C_T values	0.54	0.36	0.24	0.57	0.33	0.45	4.25	4.38	4.47
TaqMan® Signal ratio	9/9	9/9	9/9	9/9	9/9	9/9	6/9	5/9	5/9
TaqMan® Mean C_T values	18.33	21.63	25.00	28.44	31.91	35.38	45.6	45.11	45.95
TaqMan® SD C_T Values	0.17	0.08	0.03	0.10	0.33	0.56	5.31	5.66	5.85
Amplifluor™ Signal ratio	9/9	9/9	9/9	9/9	9/9	9/9	5/9	7/9	5/9
Amplifluor™ Mean C_T values	20.67	24.20	26.80	31.45	35.63	39.91	46.17	44.66	46.39
Amplifluor™ SD C_T values	0.32	0.60	0.30	0.34	0.33	0.61	3.65	3.08	3.45
ivr duplex									
TaqMan® Signal ratio	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2	1/2
TaqMan® Mean C_T values	19.90	23.78	25.44	29.24	32.27	31.11	38.07	43.57	44.03
TaqMan® SD C_T values	0.21	0.54	0.03	0.76	0.11	7.51	0.21	9.10	8.45

C_T refers to threshold cycle value, SD is the standard deviation. Signal ratio corresponds to positive/total reactions.

line. We used DNA extracted from leaves of GA21 plants that were shown to be heterozygous for the insertion event (glyphosate tolerance assays were performed with the F1 plants) and quantified both the *ivr1* and GA21 junction regions through duplex real-time PCR assays. In these reactions, a 100–309.5 (approximately 1:3) ratio of amplification was obtained for both products, indicating that six copies of the *r-act* promoter–plasmid junction sequence are present in the GA21 tandemly arranged insert (the single-copy *ivr1* gene is represented twice in the 2n maize genome, while the DNA template was extracted from lines that were heterozygous for the GA21 insertion event). These results, besides being consistent with the data provided by Monsanto (Scientific Committee of Plants, 2000), demonstrate that a correction factor should be applied to the quantification results, i.e. divide by 3 for heterozygous material or by 6 for homozygous material, to calculate the percentage of material accounting for GA21 maize in the sample analysed.

The suitability of this method to detect and quantify GA21 DNA in duplex format even for tiny amounts of GMO, was assessed by performing duplex reactions with 50 ng of maize genomic DNA that contained either 100, 10, 1, 0.1 or 0.01% of GA21 DNA (Table 3). All reactions yielded positive fluorescence signal corresponding to *ivr1* but also to GA21, thus indicating that the system has a detection limit of 0.01% GMO with 50 ng of starting maize template DNA. Moreover, using *pGAivr* in duplex format to build standard curves, these reactions resulted in quantification figures of 103.17, 8.49, 1.39 and 0.24% of GA21 for reactions containing 100, 10, 1 and 0.1% GMO, respectively, which corresponds to a high accuracy in quantification. GMO percentages corresponding to 0.01% GA21 could not be determined since they were below the LOQ.

In conclusion, we report three different real-time PCR strategies, i.e. SYBR® Green I, Amplifluor™ and TaqMan® for the reliable and accurate detection, identification and quantification of the transgenic maize line GA21. Its specificity relies on the amplification of a DNA region that is unique to the event GA21 since it includes plasmid vector together with *r-act* promoter sequences. The assays proved to be suitable for analytical purposes, with excellent limits of detection and quantification. In the absence of CRM, a plasmid containing the GA21 and a maize endogenous control PCR targets was constructed and was used to derive standard curves either in uniplex or duplex format.

Table 3
TaqMan® based real-time PCR systems targeting GA21 (FAM) and *ivr1* (VIC) in duplex format

% GA21	100	10	1	0.1	0.01
FAM signal ratio	2/2	2/2	2/2	2/2	2/2
FAM mean C_T values	25.19	28.79	31.46	33.37	33.06
FAM SD C_T values	0.04	0.09	0.21	0.07	0.91
VIC signal ratio	2/2	2/2	2/2	2/2	2/2
VIC mean C_T values	28.88	28.77	29.04	28.65	28.83
VIC SD C_T values	1.50	0.14	0.04	0.44	0.57

50 ng of genomic DNA containing different percentages of GA21 line were used as template. C_T refers to threshold cycle value, SD is the standard deviation. Signal ratio corresponds to positive/total reactions. Serial dilutions of *pGAivr* were run in parallel to build the standard curves.

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