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A novel real-time quantitative PCR method using attached universal template probe

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ABSTRACT

A novel real-time quantitative polymerase chain reaction (PCR) method using an attached universal template (UT) probe is described. The UT is an approximately 20 base attachment to the 5′ end of a PCR primer, and it can hybridize with a complementary TaqMan probe. One of the advantages of this method is that different target DNA sequences can be detected employing the same UT probe, which substantially reduces the cost of real-time PCR set-up. In addition, this method could be used for simultaneous detection using a 6-carboxy-fluorescein-labeled UT probe for the target gene and a 5-hexachloro-fluorescein-labeled UT probe for the reference gene in a multiplex reaction. Moreover, the requirement of target DNA length for UT–PCR analysis is relatively flexible, and it could be as short as 56 bp in this report, suggesting the possibility of detecting target DNA from partially degraded samples. The UT–PCR system with degenerate primers could also be designed to screen homologous genes. Taken together, our results suggest that the UT–PCR technique is efficient, reliable, inexpensive and less labor-intensive for quantitative PCR analysis.

INTRODUCTION

Nucleic acid analysis has become increasingly important in a variety of applications, such as the genotyping of individuals, the diagnosis of hereditary diseases, the detection of infectious diseases, tissue typing for histocompatability, identifying individuals in forensic diagnosis, paternity testing, and monitoring the genetic make-up of plants and animals in agricultural breeding programs. Techniques based on polymerase chain reaction (PCR) provide a powerful tool for the amplification of minute amounts of initial target sequences (1). The method has evolved from a low-throughput format with gel-based analysis to the use of fluorescence techniques (2). The amount of the DNA amplified by a PCR correlates with an increase in the fluorescent signal that results from an interaction between a fluorescent reporter and the amplified DNA sequences. The concentration of target DNA is then estimated by analyzing the fluorescent signal at each cycle of PCR (real-time). The real-time fluorescent PCR method for routine applications offers advantages including ease-of-use and high-throughput because it does not require post-PCR manipulations (‘closed tube’ format) (2) and avoids cross-contamination of PCR amplicons.

Many formats of real-time fluorescent PCR techniques have been developed in the past years. DNA-binding dyes, such as SYBR Green™, generate signal through unspecific binding to double-stranded DNA-amplicon during PCRs (3,4). The FRET probe method relies on the transfer of energy from one fluorescent dye to another (5). During the amplification cycle, once both probes hybridize correctly to the target sequence, energy from the fluorophore of the donor probe can be transferred to the acceptor fluorophore on the adjacent probe, then a fluorescent signal with a specific wavelength is emitted. Other methods incorporate the use of an oligonucleotide labeled with a fluorophore (the reporter) and a quencher moiety. Due to the spatial closeness of the quencher and the reporter, the fluorescence is suppressed. During PCR, the reporter and quencher are separated, resulting in an increase in fluorescent intensity. The separation occurs either by cleavage of the oligonucleotide (2,6,7), or by a change in the secondary structure of the oligonucleotide probe when it anneals to target DNA, as occurs with molecular beacons (8). An alternative approach employs reporter and quencher moieties attached directly to the PCR primers instead of the hybridization probe (9,10). In addition, Nazarenko *et al*. described a new method for nucleic acids detection and quantification using self-quenched fluorogenic primers labeled with only a single fluorescent dye (11). However, for all current probes or fluorescent dye labeled primers, a new specific primer or probe labeled with fluorescent dye is required for each target

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Table 1. Primer pairs used in SYBR Green I reaction in this study[a]

Gene target	Orientation	Sequence	Amplified target DNA (bp)	Reference
Invertase 1	Sense	5′-CGGCAGCCTCCAAACT-3′	97	This work
	Antisense	5′-AAGCACAGGGACCAAGC-3′		
Invertase 1-UT	Sense	5′-CGGCAGCCTCCAAACT-3′	97	This work
	Antisense	5′-taggaacaggcggcgacga*ATACAA*AAGCACAGGGACCAAGC-3′		
CryIA(b)	Sense	5′-CCCCCCTCAGAACAACAA-3′	56	This work
	Antisense	5′-GGCTCAGACGGTGGCT-3′		
CryIA(b)-UT	Sense	5′-taggaacaggcggcgacga*ATACAA*CCCCCCTCAGAACAACAA-3′	56	This work
	Antisense	5′-GGCTCAGACGGTGGCT-3′		
Lectin	Sense	5′-CTCTACTCCACCCCCATCC-3′	115	(20)
	Antisense	5′-GCCCATCTGCAAGCCTTTTTGTG-3′		
Lectin-UT	Sense	5′-taggaacaggcggcgacga*ATACAA* CTCTACTCCACCCCCATCC-3′	115	This work
	Antisense	5′-GCCCATCTGCAAGCCTTTTTGTG-3′		
35S-EPSPS	Sense	5′-GGAACGTCTTCTTTTTCCACG-3′	199	(20)
	Antisense	5′-CTTATTGCATTTCATTCAAAATAAG-3′		
35S-EPSPS-UT	Sense	5′-taggaacaggcggcgacga*ATACAA*GGAACGTCTTCTTTTTCCACG-3′	199	This work
	Antisense	5′-CTTATTGCATTTCATTCAAAATAAG-3′		

[a]UT sequence: taggaacaggcggcgacga, italicized nucleotide sequences are the linker region of the UT–PCR primer between the UT sequence and target DNA primer.

amplicon, which is relatively expensive and troublesome in practical use.

In this paper, we describe a novel attached universal template (UT) real-time quantitative PCR (12) method using universal nucleic acid probe. In this design, the UT sequence, which is ~20 bp in size and can hybridize to the UT probe, is attached to the 5′ end of a regular PCR primer that is specific to the target sequence. The UT probe is labeled with the fluorescent reporter on the 5′ end and the fluorescent quencher on the 3′ end. During the annealing phase, the UT probe specifically anneals to the 5′ end of the UT–PCR primer and the 3′ end of the UT–PCR primer specifically anneals to the target sequence and is extended. Due to the 5′ exonuclease activity of DNA polymerase, the hybridized UT probe is hydrolyzed, leading to the separation of the reporter moiety from the quencher moiety and the generation of a fluorescent signal. In this case, the same set of UT sequence and UT probe can be used for different target-specific primers. Multiplex quantitative fluorogenic PCRs with two UTs and UT probes labeled with different fluorescent dyes were also performed in this study. Moreover, the length of the target sequence to be amplified can vary from ~50 bp to hundreds of base pairs. This feature enables UT–PCR to effectively detect fragmented nucleic acid sequences. This is very useful for some highly processed samples with degraded DNA.

MATERIALS AND METHODS

Materials and DNA extraction

Three transgenic maize events, Bt11 and Event 176, developed by Novartis Seeds Inc. (Greensboro, NC, USA), and MON810, developed by Monsanto Company (St Louis, MO, USA), were used. Non-transgenic maize, hybrid 1412, dried seeds sold by Dairy-land Seed Co. (West Bend, WI, USA), was used. The transgenic Roundup Ready soybean and transgenic canola GT73 developed by Monsanto Company (St Louis, MO) and non-transgenic soybean and canola (obtained from the local market in Shanghai) were used. Plant genomic DNA was extracted and purified using the DNA extraction kit developed by Shanghai Ruifeng Agro-tech Co. Ltd (Shanghai, China). The quantity of DNA samples was calculated using absorbance measurements at 260 nm wavelength, and its copy number was estimated using the DNA quantity decided by the average size of maize genomic DNA (13).

Design and mechanism of the UT–PCR system

The target-specific portion of a UT primer of the UT–PCR was designed by modifying the analysis results of Primer 5.0 and Oligo 6, then the 5′ end of a regular PCR primer was attached to a UT DNA sequence with a linker region. To optimize the GC content and the melting temperature (T_m) of the UT–PCR primer, a linker region with a length of ~5–6 bp was added between the UT and target DNA-specific primer region. This linker region could serve as a flexible region of the UT–PCR primer during the PCR. Several UT sequences were first randomly created, and then blasted against nucleotide sequences available in public databases including EMBL, GenBank and DDBJ, to avoid significant sequence similarity between the UT sequence and any known genomic sequences especially those of maize, canola and soybean, as well as the sequence of the relevant transgenes. The UT probe sequence was complementary to the UT sequence and labeled with the fluorescent reporter on the 5′ end and the fluorescent quencher on the 3′ end. The T_m of the UT probe was approximately 5–10°C higher than that of the target-specific portion of the UT primer. When one designs the UT portion and UT primer, the same degree of caution has to be taken as for the regular TaqMan probe and primers to ensure high specificity. The UT primers and probe should be designed to minimize primer dimers, and other types of secondary structures between the UT probe and primers. Oligo 6 software can be used for analysis of secondary structure and T_m values. The major advantage of UT–PCR is that the UT and the UT probe can be used for detection of many target sequences. Users can choose to use our published UT portions available in this paper, or design their own.

To test whether the amplification efficiency of the PCR system was affected with the attachment of the UT in this

Table 2. Primer pairs and fluorogenic probes used in the UT–PCR and TaqMan PCR assay systems in this study

	Gene target	Orientation	Sequence	Amplified target DNA (bp)	Reference
1	Invertase 1	Sense	5′-CGGCAGCCTCCAAACT-3′		
		Antisense	5′-taggaacaggcggcgacga*ATACAA*AAGCACAGGGACCAAGC-3′[a]	97	This work
2	CryIA(b) (Event 176)	Sense	5′-taggaacaggcggcgacga*ATACAA*CCCCCCTCAGAACAACAA-3′	56	This work
		Antisense	5′-GGCTCAGACGGTGGCT-3′		
3	CryIA(b) (Event 176, Bt11, MON810)	Sense	5′-taggaacaggcggcgacga*CAACCAT*CCCCA/CCCA/TCAGAACAACAA-3′	72	This work
		Antisense	5′-CGGAACATGCT/GACACG-3′		
4	BnACCg8	Sense	5′-taggaacaggcggcgacga*AAAAGA*GGGTCTCAACGACTGCG-3′	69	This work
		Antisense	5′-CGTCCCTTTGGAAACTCA-3′		
5	EPSPS	Sense	5′-CGTCTTGAAGGTCGTGGTA-3′		
		Antisense	5′-taggaacaggcggcgacga*AAAGTT*ATGGGAAAGCAGTAGAGGAT-3′	76	This work
6	GOX	Sense	5′-taggaacaggcggcgacga*AAACAA*TGCTGGAAGACCAAACAAG-3′	56	This work
		Antisense	5′-TGATGAGGTTACGGAGTGC-3′		
7	UT probe		5′-*FAM* TCGTCGCCGCCTGTTCCTA *TAMRA*-3′		This work
8	CryIA(b) (Event 176)	Sense	5′-GTGGACAGCCTGGACGAGAT-3′		
		Antisense	5′-TGCTGAAGCCACTGCGGAAC-3′	106	(13)
		Probe	5′-FAM AACAACAACGTGCCACCTCGACAGG *TAMRA*-3′		

[a]UT sequence: taggaacaggcggcgacga, italicized nucleotide sequences are the linker region of the UT–PCR primer between the UT sequence and target DNA primer.

Table 3. Primer pairs and fluorogenic probes used in multiplex PCR in this study

Gene target	Orientation	Sequence	Amplified target DNA (bp)	Reference
Invertase 1	Sense	5′-CGGCAGCCTCCAAACT-3′		
	Antisense	5′-tgaggagcacgagacggaagt*ATACAA*TCAAGCACAGGGACCAA-3′[a]	99	This work
UT probe		5′-*HEX* ACTTCCGTCTCGTGCTCCTCA *TAMRA*-3′		This work
CryIA(b) (Event 176)	Sense	5′-taggaacaggcggcgacga*ATACAA*CCCCCCTCAGAACAACAA-3′	56	This work
	Antisense	5′-GGCTCAGACGGTGGCT-3′		
UT probe		5′-*FAM* TCGTCGCCGCCTGTTCCTA *TAMRA*-3′		This work

[a]UT sequence: taggaacaggcggcgacga, italicized nucleotide sequences are the linker region of the UT–PCR primer between the UT sequence and target DNA primer.

design, a group of several sets of target DNA-specific primers, with or without UT, were designed (see Table 1), to compare the amplification efficiency using SYBR Green I (Molecular Probes Inc.). Towards the development of UT–PCR quantitative analysis systems, two PCR systems were first chosen (see Table 2), one for the total quantification detection of endogenous maize (endogenous PCR system; primer set 1, Table 2), and the other for the quantification of Event 176 (transgenic maize; primer set 2, Table 2). The endogenous PCR system amplified a fragment of maize Invertase 1 gene (14), and the PCR system for transgenic maize amplified a fragment of the synthetic CryIA(b) gene (15). To further show the utility of this method, multiplex primers were also designed to detect the CryIA(b) gene and Invertase 1 gene in Event 176 with two UTs and UT probes (see Table 3). The UT probe to detect the variable target gene was labeled with the fluorescent reporter dye with 6-carboxy-fluorescein (FAM) on the 5′ end, and the UT probe to detect the endogenous reference gene was labeled with 5-hexachloro-fluorescein (HEX) on the 5′ end. The fluorescent quencher dye, 6-carboxytetramethylrhodamine (TAMRA) was located on the 3′ end of the probes. Other primers of the BnACCg8 (16), EPSPS (17) and GOX (18) genes of transgenic canola GT 73 were also designed to demonstrate the utility of this method using the BnACCg8 gene as an endogenous reference gene for canola (primer sets 4, 5 and 6, Table 2). The 5′ terminal fragments of different synthetic CryIA(b) genes with the varied nucleotide sequences in the three lines of insect-resistant maizes (Bt11, MON810, Event 176) share the same amino acids sequence. To screen the synthetic CryIA(b) genes in the unidentified maize-derived samples, one pair of degenerate UT–PCR primers for the synthetic CryIA(b) genes were also designed (primer set 3, Table 2).

Fluorescent PCR

Real-time PCR assays were carried out in a fluorometric thermal cycler (Rotor-Gene 2000; Corbett Research, Australia) with a final volume of 25 µl. Fluorescence was monitored during every PCR cycle at the annealing step. The UT–PCR contained the following mixture: 1× PCR buffer, 100 nM primers and probes, 400 µM each of dATP, dGTP, dCTP, 800 µM dUTP, 1.5 units of *Taq* DNA polymerase, 0.2 units of Amperase uracil *N*-glycosylase (UNG), 3 mM $MgCl_2$, 20 mM KCl. The TaqMan PCR contained the following mixture: 1× PCR buffer, 100 nM primers, 200 nM probes, 400 µM each of dATP, dGTP, dCTP, 800 µM dUTP, 1.5 units of *Taq* DNA polymerase, 0.2 units of Amperase UNG, 6.5 mM $MgCl_2$. The fluorogenic dye, SYBR Green I

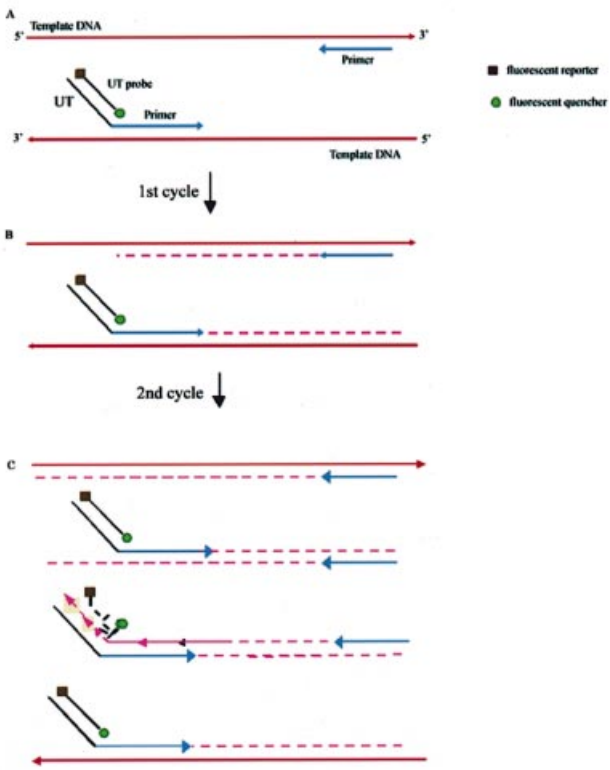

Figure 1. Schematic drawings of signal generation of the UT–PCR amplification. (**A**) The UT–PCR primer is composed of the 5′ end-attached UT sequence hybridized with the UT probe, and the 3′ end specifically hybridizes to the target sequence. (**B**) During the first cycle of the PCR amplification, the 3′ end of the UT–PCR primer is extended, generating a chimeric DNA fragment with the UT sequence on the 5′ end and newly synthetic target DNA on the 3′ end. (**C**) During the second cycle of the PCR amplification, the 3′ end of a free UT–PCR primer and the other primer anneal to available chimeric target DNA. The UT probe specifically anneals to the UT in the chimeric DNA fragments. Then, the 5′ exonuclease activity of DNA polymerase begins to hydrolyze the hybridized UT probe, and sets the reporter moiety free, thus generating a fluorescent signal. This amplification generates more chimeric DNA fragments.

was at 20 000 times dilution in the reaction. Real-time PCRs were run with the following program: 6 min at 94°C, 50 cycles of 20 s at 94°C, 40 s at 60°C and 30 s at 72°C. All the primers and fluorescent probes were synthesized and purified by Shanghai Shenyou Co. Ltd (Shanghai, China) and the other PCR reactants were purchased from Roche Molecular Biochemicals (Shanghai, China).

RESULTS

Signal generation and establishment of the UT–PCR assay

To illustrate the mechanism of signaling, a schematic of the UT–PCR amplification is presented in Figure 1. In this novel design, a UT–PCR primer is composed of UT sequence attached to the 5′ end of the primer that is specific to target DNA (Fig. 1A). During the first cycle of the PCR amplification, the 3′ end of the UT–PCR primer is extended, generating a chimeric DNA fragment with the UT sequence on the 5′ end and the newly synthetic target DNA on the 3′ end (Fig. 1B).

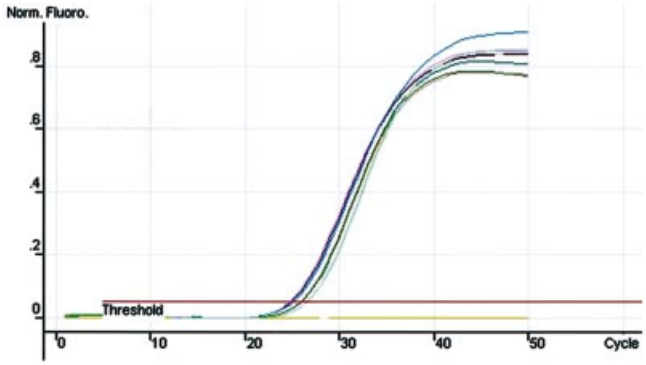

Figure 2. Comparison of the amplification efficiency between the target DNA-specific primers and the same primer pairs with one attached with UT using SYBR Green I fluorogenic dye (Lectin gene, four replicates per reaction).

During the second cycle of the PCR amplification, the 3′ end of a free UT–PCR primer and the other reverse primer anneal to available chimeric target DNA. Meanwhile, the UT probe specifically anneals to the UT in the chimeric DNA fragments. After that, the 5′ exonuclease activity of DNA polymerase begins to hydrolyze the hybridized UT probe, and sets the reporter moiety free, thus generating a fluorescent signal by proper excitation light source (Fig. 1C). This amplification generates more DNA fragments (Fig. 1C), and these are employed for amplified templates in the following cycle. Unlike the TaqMan PCR system (19), which can emit the fluorescent signal at the first cycle of the PCR amplification, the UT–PCR method generates fluorescent signal in the second cycle of the amplification.

To demonstrate that the amplification efficiency of the PCR system was not affected when one of the primer pairs was attached with the UT in this design, a comparison of the PCR efficiency was carried out using SYBR Green I as the fluorogenic dye for quantitative analysis (20). First, we compared the Ct and ΔCt measurements of the PCR amplification employing two previously published primer pairs (20) with that of the same primer pairs with one attached with the UT (see Table 1). The results of this analysis indicated that the UT being attached to one of the reported primers does not affect the amplification reactions of the PCR system (Fig. 2). Moreover, several other primer pairs (see Table 1) for comparison using this method were also tested and similar results were obtained. These results show that the UT being attached to one of the target DNA-specific primers does not obviously affect the PCR amplification efficiency.

Reproducibility of the UT–PCR assay

After optimization of the primers and the KCl and $MgCl_2$ concentrations in the UT–PCR system (see above), the reproducibility of the UT–PCR system was tested using a series of diluted DNA samples. The reproducibility of the Ct measurement was checked using 100 ng of total DNA varying in Event 176 DNA content from 0 to 5% (w/w) per sample in quadruple repeats (Table 4). For the endogenous UT–PCR system, the Ct values ranging from 24.46 to 25.46, and CV values of 0.59–1.35% were observed (Table 4). As expected, due to the fixed amount of maize DNA, Ct values did not vary with the transgenic material percentage. In the case of the

Table 4. Reproducibility of the Ct measurements of replicate standards from 0 to 5% of the transgenic maize content using the UT–PCR assay system

Transgenic content (%)	Ct value for reaction				Mean	CV[a] (%)
	1	2	3	4		
Invertase 1 gene						
0	25.33	25.29	25.26	24.90	25.20	0.67
0.1	25.13	24.56	24.46	25.19	24.84	1.33
0.5	25.06	25.28	25.46	25.34	25.28	0.59
5	25.11	24.61	25.36	25.50	25.14	1.35
CryIA(b) gene						
0	–	–	–	–	–	–
0.1	37.20	37.11	36.35	36.65	36.83	0.95
0.5	34.23	34.61	34.71	34.52	34.52	0.52
5	29.81	29.72	30.15	29.52	29.80	0.77

[a]Coefficient of variation.

transgenic PCR system, Ct values were correlated with the amount of transgenic material percentage, varying from 29.52 to 37.2, with a CV of 0.52–0.95% (Table 4). The results indicated that the CV derived from these methods was relatively small; the Ct measurements were highly reproducible.

Quantitative real-time PCR

In order to perform relative quantitative analysis of UT–PCR, both standard curves of the endogenous PCR system and the transgenic PCR system were constructed by serial dilution of the target DNA. Samples with templates comprising of serial dilutions of transgenic maize Event 176 ranging from 0.01 to 100 ng were discriminated by real-time PCR (Fig. 3 and primer set 2, Table 2). A linear relationship (R^2 = 0.994) was detected between the Ct and starting DNA concentrations. Comparable results were also obtained using primers of the maize endogenous Invertase 1 gene (primer set 1, Table 2) with a correlation coefficient of 0.995. Based on the standard curves of the endogenous PCR system and the transgenic PCR system, the transgenic content of unknown samples can be easily quantified. The transgenic amounts (%) were calculated based on the ratio of transgenic to total DNA quantities (13).

Other UT–PCRs were performed for amplicons of various target genes of transgenic canola GT 73 (primer sets 4, 5 and 6, Table 2). Comparable results were also obtained for BnACCg8 (16), EPSPS (17) and GOX (18) genes with correlation coefficients of 0.996, 0.992 and 0.997, respectively. Based on these results, we considered that this method was sufficiently precise and could be used for practical quantitative analysis of target DNA.

Multiplex quantitative PCR

Quantitative, real-time, multiplex fluorogenic PCR with two UTs and UT probes labeled with different fluorescent dyes were also performed. The multiplex system is useful because one primer set may be used to detect the amount of a transgene that is variable and another to detect an endogenous gene that is relatively constant and used as a reference. In one test, multiplex primers were designed to detect the CryIA(b) gene and Invertase 1 gene of Event 176 in one reaction tube (see Table 3). We performed a multiplex PCR with serial dilution

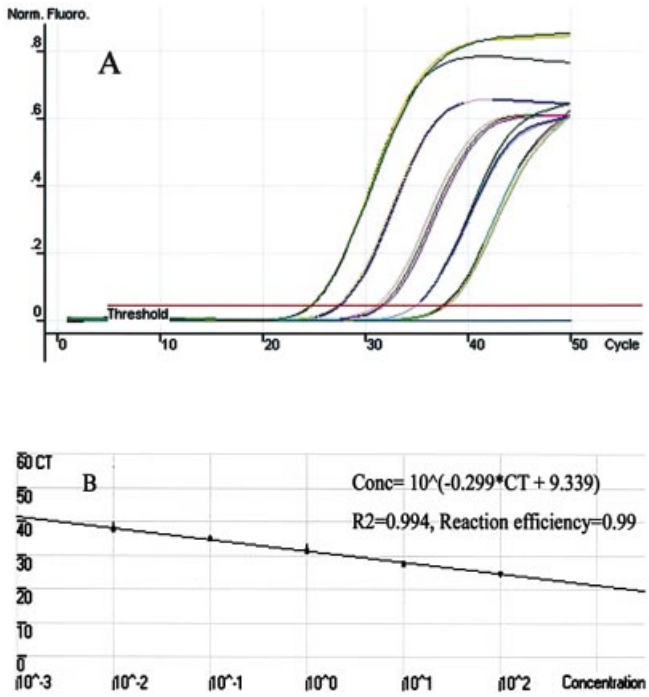

Figure 3. Sensitivity, precision and dynamic range of fluorogenic real-time PCR. Serial dilutions (10-fold) of transgenic maize Event 176 ranging from 0.01 to 100 ng were detected using a FAM-labeled fluorogenic primer (primer set 2, Table 2). (**A**) Amplification plot. (**B**) Initial DNA concentration versus Ct standard curve (R^2 = 0.994, reaction efficiency = 0.99, three replicates per dilution).

of transgenic maize Event 176 ranging from 0.01 to 100 ng, with each dilution containing 100 ng of total maize DNA. The correlation coefficient of Ct versus initial DNA concentrations was 0.993 (Fig. 4).

In multiplex PCR, the results of relative quantification can be achieved with the comparative Ct or ΔCt method (21). This method has the advantage of not requiring the construction of standard curves for both genes of endogenous reference gene and transgene. Instead, it requires a validation experiment to demonstrate that reaction efficiencies for the transgene and the endogenous reference gene are identical or very close (22). A sensitive method for assessing if two amplicons have the same efficiency is to examine how ΔCt varies ($Ct_{target} - Ct_{reference}$) with template dilutions (21). Since the reaction efficiencies of the target and reference gene in our tests were similar, the ΔCt calculation for the relative quantification of the target gene can be used. Therefore, the transgenic amount of the target gene could be calculated based on the difference between the Ct values of the target and an endogenous reference gene (Fig. 4D).

In multiplex PCR using primer pairs of Invertase 1 and CryIA(b) gene, we were able to detect 10 pg of initial genomic maize DNA (Fig. 4C). Referring to the genome sizes of maize (2.6 billion bases) (13), seven to eight copies of target DNA fragments could be detected in all amplification reactions with a CV of 1.02%. The reported TaqMan PCR detection limit was approximately 20 copies with the template of maize to amplify

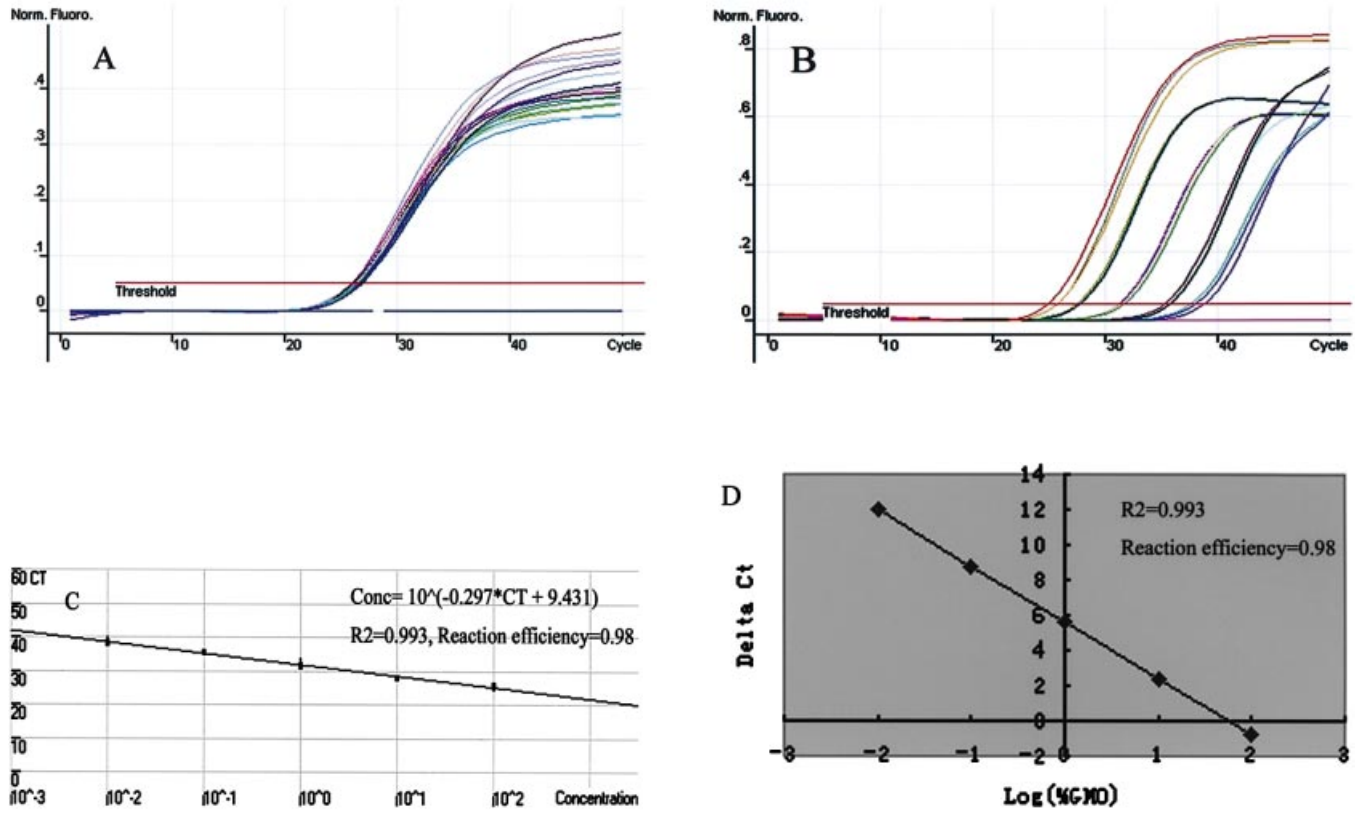

Figure 4. Multiplex fluorogenic PCR to detect the Invertase 1 and CryIA(b) gene using serial dilutions (10-fold) of transgenic maize Event 176. (**A**) Amplification plot of endogenous Invertase 1 gene. Each dilution contains 100 ng of total maize DNA. (**B**) Amplification plot of the transgenic CryIA(b) gene, serial dilutions of transgenic maize Event 176 ranging from 0.01 to 100 ng. (**C**) Initial Event 176 DNA concentration versus Ct standard curve. (**D**) Standard curve, plotting log (GMO amount) versus ΔCt (R^2 = 0.993, reaction efficiency = 0.98, three replicates per dilution).

the CryIA(b) gene (23). Then, we performed similar sets of other UT–PCRs with variable EPSPS (17) and GOX (18) genes of transgenic canola GT 73 using the BnACCg8 (16) gene as the endogenous reference gene. Standard curves yielded R^2 values of 0.992 and 0.997 for EPSPS and GOX genes, respectively. In our tests, for the EPSPS gene, we could detect the initial DNA template at the level of 50 pg in all amplifications. On the basis of the genome sizes of canola (1.2 billion bases) (24), the detectable copy number was approximately 40. For the GOX gene, however, the minimal detection of the initial DNA template was 20 pg with a correlation coefficient of 0.997.

Qualitative analysis of processed samples

Another feature of the UT–PCR design is that shorter length target DNA fragments could be amplified. This is useful for the detection of some processed samples with degraded target DNA. To this end, transgenic maize was subjected to heating at 121°C for 10, 30, 60 and 120 min by autoclaving. 100 mg of each treated sample was used for DNA purification and 1 µl of DNA of each sample was assayed in UT–PCR. The results showed that the Ct values increased with the prolonged autoclaved time (Table 5). The CryIA(b) gene could even be detected in the sample treated with autoclaving for 120 min. However, we failed to detect the target DNA fragment in the transgenic maize treated with autoclaving for more than 30 min using the TaqMan PCR system (Table 5).

Table 5. Ct measurements of the autoclaved samples with the same initial quantity using the UT–PCR and TaqMan PCR assay systems

Treatment time (min)	Ct value for reaction			Mean	CV[a] (%)
	1	2	3		
UT–PCR					
0	27.94	28.06	27.97	27.99	0.18
10	30.24	30.38	30.22	30.28	0.23
30	33.59	33.55	33.38	33.51	0.27
60	34.95	35.83	35.47	35.35	1.02
120	35.99	36.71	36.86	36.36	1.04
TaqMan PCR					
0	30.26	30.21	30.21	30.23	0.07
10	32.29	32.29	32.18	32.25	0.16
30	39.98	39.22	40.27	39.82	1.10
60	–	–	–	–	–
120	–	–	–	–	–

[a]Coefficient of variation.

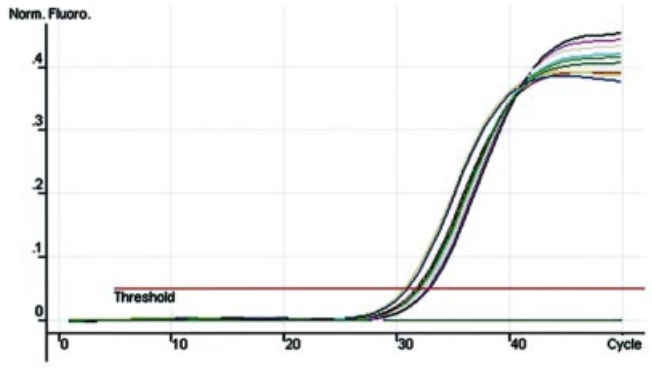

Figure 5. Screening of the CryIA(b) fragment in three lines of insect-resistant maizes (Bt11, Event 176, MON810) using degenerate UT–PCR primers (three replicates per sample).

Screening of different homologous DNA sequences

Three commercial lines of insect-resistant maize (Bt11, Event 176, MON810) all contain the synthetic CryIA(b) gene. Analysis of the CryIA(b) DNA sequence of Bt11 (sequence 22 of US Patent 5880275) (25), Event 176 (sequence 3 of US Patent 5625136) (15) and MON810 (sequence 1 of US Patent 6180774) (26), showed that the 5′ terminal region of the three synthetic CryIA(b) genes have the same amino acid sequence with different nucleotide sequences. As a result, it is difficult to get an optimal TaqMan PCR analysis system to screen these three different CryIA(b) genes in samples. In an attempt at using the UT–PCR technique to detect homologous synthetic CryIA(b) genes, a pair of degenerated primers was designed (primer set 3, Table 2). The results of UT–PCR analysis showed that the fluorescent signal was detected in all three transgenic maizes and no signal increase was observed in the absence of the DNA template (Fig. 5).

DISCUSSION

In this paper, we have successfully tested a novel UT real-time quantitative PCR method using attached universal nucleic acid probes. The unique design of this method is the attached UT and the UT probe. The UT probe is not specific, in comparison with sequence-specific probes such as the TaqMan probe (19) or molecular beacons (8), because it will detect any product of the primer that contains the UT. The UT system may generate non-specific signal if not designed with high competency. However, in the amplification, the UT probe is only attached to the UT tag of one primer. So, if the target primers generate non-specific products, the UT probe may not be able to bind. Therefore, no signal can be generated. Nevertheless, the UT system has some imperfections, one of which is that it may not be able to design a UT primer as easily as a regular PCR. This is because it requires the absence of significant sequence similarity between the UT and the sequence in the genome. Even so, it might not be more difficult than the design of the TaqMan probe for the TaqMan PCR assay, which is frequently complicated if the target sequence contains variations, e.g. HIV and HBV sequences. Compared with the TaqMan probe, it is easier to design a UT system using a single UT probe and multiplex regular degenerate primers to cover more subtypes or mutations.

While using the TaqMan probe (19) and molecular beacon (8), the probe hybridizes to the sequence inside the amplified sequence, in the UT–PCR the probe hybridizes to the external attachment, the UT. This enables the design of short UT–PCR systems that can detect even degraded DNA. This is in contrast to some conventional real-time PCR methods that require a longer target sequence. Furthermore, the universal attachment of the UT to any target-specific primers allows the use of different primers to distinguish several closely related target sequences by searching for the unique regions among them. In addition, we have proved that the attachment of optimized UT to the specific primer does not obviously affect the PCR amplification efficiency, suggesting no additional cost was caused by the attachment.

The relative quantification of UT–PCR is comparable in sensitivity and dynamic range with other published methods of quantification (23). With the primers designed in our study, it was possible to perform multiplex PCR for simultaneous detection of the target gene and the endogenous reference gene. With this system, there appeared to be no loss in sensitivity, with multiplex PCR giving a standard curve comparable with that obtained using the separate tube reaction. Moreover, the use of the multiplex system will allow different targets to be detected simultaneously and the random difference affected by separate tubes could be minimized. In multiplex PCR, the relative quantification can be estimated with the comparative Ct or ΔCt method (21). This method requires a validation experiment to demonstrate that reaction efficiencies for the transgene and the endogenous reference gene are identical or very close (22). Here we use FAM- and HEX-labeled probes for multiplex PCR, but other dyes, including 6-carboxy-4,7,2′,7′-tetrachloro-fluorescein (TET), 6-carboxy-4′,5′-dichloro-dimethoxyfluorescein (JOE), etc., may be used. The method may be highly specific as demonstrated by the lack of signal increase when no template is added to the UT–PCR in our study.

Based on the novel design of primers and probe, degenerate primers could also be designed to simultaneously detect some homologous DNA sequences. Although degenerate primers could also be designed for regular TaqMan PCR, it may be difficult for certain sequences of target DNA that contain variations. Since the restriction of the signal probe is relocated from the target sequence to an artificial UT sequence, UT–PCR is more capable of tackling difficult sequences with more subtypes or mutations.

Many kinds of foodstuffs contain ingredients that have been subjected to various processing which may lead to significant degradation and partial removal of DNA. Although the conventional PCR technique may be able to detect low copy numbers of target DNA molecules, it is still a prerequisite for successful PCR amplification that the target DNA sequences are intact. With the prolonged time of treatment, the average size of the DNA fragments in samples may be decreased. Consequently, the shorter the target DNA sequence of a PCR is, the more likely it is that intact fragments of the necessary size will be present in the foodstuff. For the UT–PCR assay, the target nucleic acid sequence could be as short as 56 bp in this study. The CryIA(b) gene could even be detected in the sample treated after autoclaving for 120 min using the

UT–PCR system. However, the target DNA fragment in the transgenic maize treated with autoclaving for more than 30 min was not detected using the TaqMan PCR system. Hence, we propose that the UT–PCR might be very useful for detection of degraded DNA in highly processed food products.

The major advantage of UT–PCR is that the universal probe can be used for many target sequences, and it will be less expensive compared with other sequence-specific fluorescent PCR techniques. In our study, six pairs of UT primers (see Table 2) shared the same UT probe. For other fluorescent PCR techniques that use FRET probes (5), the TaqMan probe (19), molecular beacons (8) and self-quenched methods (11), a specific probe or primer to each amplicon has to be labeled with fluorescent dye, which could be expensive and troublesome in practical assays. This is the greatest bottleneck for the wide application of real-time PCR. Since UT–PCR uses the same UT probe for detection of different target DNA sequences, it can be very inexpensive and convenient for regular users of nucleic acid tests. In addition, the multiplex system using two UTs will allow different targets to be detected simultaneously. We anticipate that the multiplex reactions with more UTs and UT probes will be feasible when more fluorescent reporters become available. Now we are currently working on the detection of other genetically modified organisms and on a mathematical model of the multiplex PCR, which will allow a more accurate quantification of the transgenic content.

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