Comparison Of Different Molecular Methods In Screening Genetically Modified Lentil

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Abstract-Currently transgenic plants are grown in more than 20 countries with maize, soybean, canola and cotton being the most predominant crops. Inexperience in the outcomes of the technology and growing public concern necessitates proper detection and regulation of genetically modified organisms (GMOs) from farmland to market. Due to their high specifity and sensitivity, polymerase chain reaction (PCR) based systems are currently the method of choice in detection of genetic modifications. This study compares the efficiency of three different PCR based methods; reverse-transcription PCR (RT-PCR), real-time PCR (qPCR) and conventional PCR in reference with the transgene copy numbers assessed by Southern blot hybridization, in detection of genetic modification. In the study, first generation transgenic lentil (*Lens culinaris* M.) plants carrying beta-glucuronidase (*gus*) gene in control of CaMV-35S promoter and *A.tumefaciens nos* terminator was used. Conventional PCR was used in detection of *gus* gene signal and RT PCR was performed in detection of gene's expression. qPCR was used to detect expression signals of both 35S promoter and *nos* terminator. All of the methods were successful in producing amplification signals for each target gene. Although qPCR signal strengths were in consistency with the band intensities obtained by RT-PCR to some extent, outcomes of both PCR-based methods appeared to be independent from copy number of genes detected in Southern blot

hybridization. Band intensities obtained by conventional PCR showed no particular correlation with any other PCR-based method. Inconsistency in copy number of gene and qPCR signal strength, even in pure DNA samples may have a contribution for the debates on the influence of various factors on qPCR and reliability of the method in genetic modification quantification.

Keywords- Real time PCR, reverse transcription PCR, conventional PCR, lentil, genetically modified organism

Genetiği Değiştirilmiş Mercimeğin Taranmasında Farklı Moleküler Yöntemlerin Karşılaştırılması

Özet-Transgenik bitkiler günümüzde, başlıca mısır, soya fasülyesi, kanola ve pamuk olmak üzere yirmiyi aşkın ülkede yetiştirilmektedir. Rekombinat DNA teknolojisinin ortaya çıkarabileceği sonuçlarla ilgili deneyimsizlik ve artmakta olan toplumsal kaygılar, genetiği değiştirilmiş organizmaları (GDOlar), tarladan markete kadar gerektiği anda tespit edebilmeyi ve gerekli düzenlemeler getirmeyi gerekli kılar. Yüksek spesifite ve duyarlılıkları nedeniyle polimeraz rincir reaksiyonuna (PZR) dayalı sistemler günümüzde genetik modifikasyonların tespitinde en çok tercih edilen yöntemlerdir. Bu çalışmada, PCR'a dayalı üç farklı yöntem olan ters ifadeli PZR (RT-PZR), gerçek zamanlı PZR (qPZR) ve geleneksel PZR, genetik modifikasyon tespitinde, Southern blot hibridizasyonu ile belirlenen transgen kopya sayıları referans alınarak karşılaştırılacaktır. Çalışmada, CaMV-35S promotoru ve *A.tumefaciens nos* terminatörü kontrolünde beta-glukoronidaz (*gus*) geni taşıyan birinci nesil transgenik mercimek bitkileri kullanılmıştır. Geleneksel PZR, *gus* gen sinyalinin belirlenmesinde, RT-PZR, gen ifadesinin belirlenmesinde kullanılmıştır. qPCR ise 35S promotor ve *nos* terminator ifade sinyallerinin belirlenmesinde kullanılmıştır. Kullanılan tüm yöntemler, hedef gen için çoğaltım sinyalleri üretmede başarılı olmuştur. qPCR sinyal yoğunlukları RT-PZR tarafından oluşturulan bant yoğunluklarıyla bir ölçüde tutarlılık göstermekle birlikte, PZR'ye dayalı bu iki yöntemle elde edilen sonuçlar Southern blot hibridizasyonuyla belirlenen gen kopya sayılarından bağımsız görünmektedir. Geleneksel PZR ile elde edilen bant yoğunlukları ise diğer PZR'ye dayalı yöntemlerle belirli bir ilişki göstermemiştir. Saf DNA örneklerinde ölçülmüş olan genin kopya sayısı ve qPZR sinyal yoğunluğu arasındaki tutarsızlığın, çok çeşitli faktörlerin qPZR üzerindeki etkileri ve yöntemin genetik modifikasyonun sayısallaştırılmasındaki güvenilirliği üzerine yapılan tartışmalara katkıda bulunabileceği düşünülmektedir.

Anahtar Kelimeler- Gerçek zamanlı PZR, ters ifadeli PZR, geleneksel PZR, mercimek, genetiği değiştirilmiş organizma

1.INTRODUCTION

Plants can be consumed directly or they can be processed into many types of foods. During the past decade, the development of biotechnology has revolutionized agriculture by the introduction of genetically modified organisms (GMO) [1]. GMOs can be defined as organisms in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating or natural recombination but by the use of recombinant DNA technology. The addition of foreign genes has often been used in plants to produce novel proteins that confer pest and disease tolerance and, more recently, to improve the chemical profile of the processed product such as vegetable oils [2]. In a decade of use of genetically modified organisms (GMOs), planting of GM crops has consistently increased and areas sown with GM crops exceeded 100 million hectares in 21 countries during 2005. Over 100 genetically modified plants have already been approved by regulatory agencies in different countries. Due to public perception of GMOs as controversial, legislation requires traceability and detection of GMOs in some countries. In more than 15 countries, compulsory labeling is required for products that contain GMOs or derived product above a certain threshold [3]. Therefore the demand for analytical methods has increased, not only in countries with labeling requirements, but also in those that want to export to countries with restrictions [1].

Two kinds of PCR strategies are currently used for GMO detection; conventional PCR and quantitative PCR (also named real-time PCR or qPCR). In the case of conventional PCR, the product of the amplification is usually loaded onto an electrophoretic gel and separated by means of an electric field. Amplified bands are recognized to be the right ones their molecular weight as compared to appropriate standards. In the case of quantitative PCR the reaction is followed kinetically. The “primers” which are employed by both methods are short single-stranded DNA pieces (usually 20–25 bases in length), whose nucleotide sequence is complementary to the nucleotide sequence of the borders of a target sequence. In the presence of the thermostable enzyme Taq polymerase, the primers act as starters for a polymerization reaction the consequence of which is a new copy of each DNA strand. A typical amplification pattern encompasses many cycles (30–50 typically) consisting of a denaturation step (to denature DNA), an annealing step (where primers find their correspondent complementary sequence on the template) and an elongation step (where Taq polymerase copies the complementary strand). Depending on the number of cycles applied, even a very small initial DNA template (few picograms) is amplified million/billion-fold and made detectable [4].

In quantitative PCR, an additional item called probe, a piece of single-stranded DNA, is added to the reaction. A fluorochrome, chemically linked to the probe, is a key point for this kind of approach: during each cycle of amplification a fluorescent signal is emitted and measured by the instrument. The signal is proportional to the amount of DNA amplified at that time, enabling the operator to follow the reaction kinetics [8]. A “threshold cycle” (Ct) represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. This enables the standardized comparison of the different samples’ amplification kinetics and the calculation of the initial amount of DNA. For this reason qPCR is also used as a quantitative method, while the conventional PCR is usually considered a qualitative method only. Under certain circumstances it can also be considered and used as a semi-quantitative method [5].

Target sequences can be the specific genes introduced (e.g. the Bt gene, the herbicide resistance gene, etc.), a regulatory sequence (e.g. promoter and terminator), the marker genes and/or a sequence spanning between a regulatory sequence and a gene. The marker genes are necessarily associated to the heterologous genes of interest and are needed to verify the successful transformation. Sometimes they are silent or semi-silent (e.g. ampicillin resistance) but their sequence is still in the plant genome. The targets mostly considered as universal are the 35S promoter, which comes from the cauliflower mosaic virus and that is used as a strong constitutive transcription promoter in most GMO constructs, and the nos (nopaline synthase) terminator, which comes from *Agrobacterium tumefaciens*. At the moment, there are few constructs and consequently transgenic plants that do not contain the 35S as a promoter [4].

Instead of using a single method for GMO detection, it was discussed in several reviews that a set of unified methods can be used for detection starting with qualitative or conventional PCR [6, 7]. If no GMOs are detected with a validated qualitative method, the products would be evaluated for the presence of protein. If no protein is detected, the product is presumed not detectable. If the qualitative PCR shows a positive result, the product is considered as ‘non-approved GMO’, and a validated Q-PCR is used to detect the level of GMO. If the GMO level is above an established threshold, the product is labeled as ‘non-approved GMO’, but if below the threshold, the product need not be labeled [7]. The greatest uncertainty of using DNA-based assays, as for protein-based methods, is that not all products derived from GM foods (e.g. refined oil) contain enough DNA. In addition, heating and other processes associated with food production can degrade DNA [4]. In this study, the efficiency of three different PCR based methods; reverse-transcription PCR (RT-PCR), real-time PCR (qPCR) and

conventional PCR were compared in reference with the transgene copy numbers assessed by Southern blot hybridization in detection of genetic modification in lentil, a common food legume of Mediterranean and Middle East.

2. MATERIALS AND METHODS

2.1 Conventional PCR Analysis and Agarose Gel Electrophoresis

For genomic DNA isolation, 1g of plant tissue was ground to fine powder inside a pre-cooled mortar by the help of liquid nitrogen. DNA isolations were done by using Favorgen® Plant DNA isolation kit (Favorgen, Taiwan) according to the kit manual. DNA concentration in each sample was equalized to 200ng/µl by dilution. The PCR analysis was performed by using *gus* gene primers with sequences “ggtgggaaagcgcgttacaag” and “tggcggaagcaacgcgtaaac” for forward and reverse, respectively. Primers for *gus* gene amplifies 1203 base pair product which is almost equal to the complete *gus* gene sequence including the intron region.

For visualization of PCR products, 1 % gels were prepared by dissolving 1 gram agarose in 100ml of 0.5X TBE buffer. 5µl of ethidium bromide solution was added to 50ml of agarose gel solution. After seting the gel, DNA samples and size markers (Fermentas SM331, SM371) were loaded into wells by mixing with 6X loading buffer at a final concentration of 1X. Power supply was adjusted to 75-100 V and the gel was run for 2-4 hours. Finally the bands were visualized under UV light and photographed by using Vilbert-Lismart gel documentation system.

2.2 Quantitative PCR Analysis

The analysis was performed by using Roche LightCycler instrument and LightCycler GMO Screening Kit. Total RNA was isolated according to De Graff (1988) by using TRIZOL reagent (a mono-phasic solution of phenol and guanidine isothiocyanate). All the equipment was DEPC treated before the isolation. All the reagents except TRIZOL and chloroform were prepared by using DEPC-treated water. Total cDNA was prepared by using Fermentas First Strand cDNA Synthesis Kit with oligo(dT)18 primers according to the instruction manual by using 3µg template RNAs which were extracted according to De Graff [8].

PCR set-up was prepared according to the Roche suppliers’ instructions [9]. LightCycler GMO Screening Kit (Roche) which is an optimized kit for the flourometric detection of 35S promoter of cauliflower mosaic virus (CaMV) and the 3’ untranslated region (terminator) of the nopaline synthase (NOS) gene of *A. tumefaciens* was used for the real-time detection. Also as a reference and control for DNA extraction efficiency, a primer set for a plant-specific gene is included in the kit. One set of 35S-specific probe is labeled at the 5’ end with Light-Cycler–Red 705 and one NOS-specific probe is labeled with LightCycler–Red 640. The other set of 35S- and NOS-specific probes are labeled at the 3’ end with fluorescein. After hybridization to the template DNA, two probes for each target come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, fluorescein, the donor fluorophore, is excited by the light source of the LightCycler Instrument and part of the excitation energy is transferred to LightCycler–Red, the acceptor fluorophore. The emitted fluorescence is then measured by the instrument. For the detection of the reference plant gene, a specific pair of probes, one labeled with LightCycler–Red 640 and the other with fluorescein, are utilized [9].

T-DNA of pTJK136 binary vector contains the *gus* gene which is driven by the 35S promoter and ended by nos terminator. Therefore, both of the fluorescent labeled probes of GMO detection kit were suitable for the detection of transgene and used for the analysis of transgenic nature of T0 generation.

2.3 Reverse Transcription PCR

Total RNA samples were isolated according to the method of De Graff [8] and first strand cDNAs were synthesized by using the template RNAs and Fermentas First Strand cDNA Synthesis Kit. Synthesized cDNA strands were directly used in the reverse transcription PCR (RT-PCR) experiments by the use of same primer sets designed for genomic DNA PCR analysis for the amplification of *gus* cDNAs.

2.4 Southern Blot Analysis

Southern blot analysis was performed by using DIG-High Prime DNA Labeling and Detection Starter Kit II, Roche Applied Sciences, according to the instructions manual. The kit is composed of Klenow enzyme which allows random-primed labeling of DNA templates with DIG-11-dUTP, Anti-digoxigenin-AP conjugate, blocking solution and hybridization granules to be used in the preparation of hybridization buffer. Detection was achieved by the use of CDP-Star reagent from New England Biolabs, MA, USA. Probe was prepared by random primed labeling reaction following the PCR amplification of a partial *gus* gene by the use of primers having the sequences “ctgtcggctttaacctctctttag” and “agtgaagatccctttcttgttacc” for forward and reverse, respectively. Primers for the *gus* gene amplifies 656 base pair product which corresponds to a non-conserved region of the gene which was determined according to “Conserved Domain Search” tool of National Center for Biotechnology Information (NCBI). All the steps of gel preparation and southern blotting were performed according to Brown [10].

3. RESULTS AND DISCUSSION

Southern blot analysis was performed on a non-transformed control plant and five transgenic lentil plants to determine copy number of the transgene (Figure 1, a). While non-transformed control plant did not exhibit any signal, a single hybridization signal of different molecular sizes was obtained in lines B3 (9.5 kb), C4 (18.5 kb) and D5 (6.6 kb). Two lines, A1 and A2 exhibited the same banding pattern shown in Figure 1. The analysis confirmed that this two sister lines were derived from a single transformation event and regenerated from the same explant.

PCR analysis was performed to detect *gus* sequence integrated into the plant genome. Primers were designed to amplify whole gene sequences except the promoter and terminator regions. Amplification of the *gus* gene with an intron region resulted in a 700bp product (Figure 1, b).

LightCycler GMO Screening Kit (Roche), which is optimized for the flourometric detection of 35S promoter of cauliflower mosaic virus and the 3' untranslated region of the nopaline synthase (NOS) gene of *A. tumefaciens*, was used for the qPCR analysis. The kit contains one set of 35S-specific probe which is labeled at the 5' end with Light-Cycler–Red 705 and one NOS-specific probe labeled with LightCycler–Red 640. The other set of 35S- and NOS-specific probes are labeled at the 3' end with fluorescein. The emited fluorescence during energy transfer between the two fluorophores, which comes in close proximity after hybridization to template DNA, is measured by the instrument. To be able to measure the kinetics of the reaction in the early phases of PCR, provides a distinct advantage over traditional PCR detection which can only measure the amount of product at the end (plateau) phase.

The qPCR analysis was performed on cDNAs of 7 putative independent transformants which belong to T_0 progeny and a non-transformed control lentil plant. The *gus* gene in T-DNA region of pTJK136 vector construct is driven by 35S promoter and terminated by nos. Therefore, both of the fluorescent labeled probes of Roche LightCycler GMO detection kit were suitable for the detection of the cDNA of *gus* gene prepared from putative transformants. Figure 1 (d) shows the NOS terminator amplification signals of 7 GUS positive plants which are collected before cycle 26. Non- transformed control lentil cDNA responded on the 36th cycle which is 8 cycles behind the reference positive control cDNA and 10 cycles later than the signal of putative transformants. Similar amplification curves were obtained from the qPCR for 35S promoter (Figure 1, e). cDNAs from 7 putative independent transformants gave amplification signals earlier than that of reference positive control cDNA and signal of non-transformed lentil control cDNA appeared only at the 38th cycle.

Reverse transcription PCR was performed on cDNAs synthesized from total RNAs of 7 *gus* gene expressing lines from T_0 generation. *Gus* primers amplified 1100 bp products which corresponded to intronless *gus* gene sequence of complementary DNA (Figure 1, c).

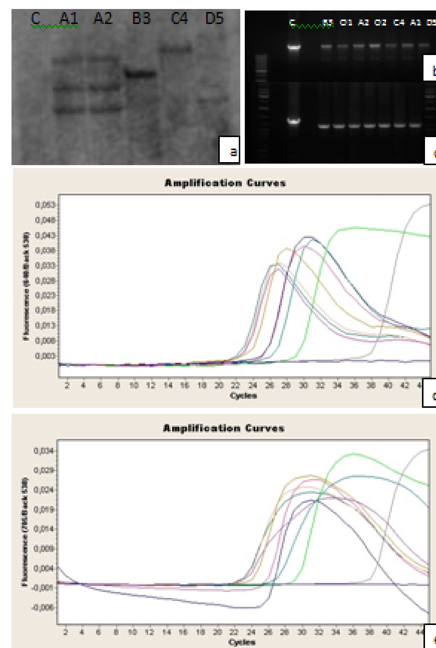

Figure 1. Molecular analysis for detection of *gus* genein transgenic lentil plants. Southern hybridization (a), conventional PCR (b), reverse transcription PCR (c), real time PCR analysis for the amplification of nos terminator (d) and 35S promoter controlling *gus* gene (e).

Table 1. Comparison of signal strengths obtained by different methods for the detection of transgene presence in 7 transgenic lentil lines.

Sample name		Control	B3	O1	A2	O2	C4	A1	D5
qPCR representation			Dark green	Green	Light pink	Dark blue	Purple	Pink	Yellow
Copy number of the gene			1	0	3	0	1	3	1
Order of qPCR signal			1	2	1	2	1	2	1
Mean gray value	RT-PCR	1	0.61	0.53	0.58	0.6	0.64	0.52	0.50
	conventional PCR	1	0.52	0.38	0.52	0.56	0.35	0.41	0.31
Amount of DNA (ng)	RT-PCR	80	48	42	46	48	50	42	39
	conventional PCR	80	42	30	42	45	28	33	25

When three PCR techniques were compared in terms of amplification signal strengths, no correlation between conventional PCR and qPCR was detected (Table 1). Also neither PCR technique showed any correlation with copy numbers of the gene, which were detected by southern hybridization. qPCR results were consistent with RT-PCR results to some extent, however no correlation with copy numbers of the gene was observed, which is quite unexpected when the sensitivity of the technique in detection of small concentration differences were considered.

4. CONCLUSION

In this study three different PCR techniques namely, conventional PCR, quantitative PCR and reverse transcription PCR were used to detect and amplify transgene signals in transgenic lentil plants. All three methods were successful in detecting marker genes in transgenic plants, however, no significant correlation in amplification signal strengths was detected by the use of different techniques. In processed food systems, where the detection of DNA sequences is harder and quantification is very important, use of an array of different detection and quantification systems appears to be necessary for the accuracy of GMO content information.

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