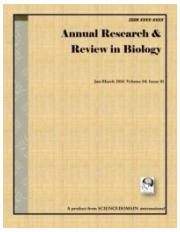

*Annual Research & Review in Biology*
*4(24): 4293-4304, 2014*

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# Transgenic Investigation of Canola (*Brassica napus L.*)

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author SF designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author EA managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.*

***Original Research Article***

***Received 29th May 2014***
***Accepted 25th July 2014***
***Published 8th August 2014***

## ABSTRACT

Investigating transgenic characteristics of Californium, Jura, Elvis and Orkan varieties of canola has been carried out in this study. These plants are widely cultivated in Turkey. The Canola varieties were grown in an established tissue culture and the total DNA isolated. The 35S and pNOS promoter region of the transgene were scanned using classic and multiplex PCR techniques. The transfer of transgenic characteristic to the NAD gene region confirms the accuracy of the PCR technique. This study, has determined that Californium, Jura, Elvis and Orkan varieties of canola consist of transgenic characteristics and all the four varieties have multiple insertion region in terms of CaMV 35S and Pnos promoters.

*Keywords: Canola; transgenic; geneticly modified organisms; pNOs promoter; 35S promoter; multiplex PCR.*

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## 1. INTRODUCTION

Rapeseed is a valuable oil plant containing 45% oil per seed; first cultured in India in 2000 B.C. and cultivated in Belgium in 13th century. It is an amphidiploid species developed by crossing of cabbage and rape. With summer and winter varieties rapeseed contains high amounts of erucic acid and glucosinolate. Because erucic acid and glucosinolate are hazardous to animal and human health new rapeseed varieties containing low erucic acid and glucosinolate levels were developed through classical breeding techniques and named “canola”, short for “Canadian Oil Low Acid” [1-3].

Today, canola ranks second worldwide in oil plant cultivation following soya bean. Rapeseed, containing 40-45% oil per seed, is used commonly in human nutrition and has an important place in biodiesel production. Moreover; as a result of improved oil quality and quantity and incorporation of properties like herbicide resistance, canola has taken its place among transgenic plants cultivated worldwide. In recent years, studies have been conducted on canola extensively to incorporate herbicide resistance and improve oil quality. Studies have focused especially on glyphosate (N-phosphonomethyl glycine), glyphosate ammonium and bromoxynil herbicide tolerance. Studies on resistance against viruses, tolerance against soil salinity, heavy metals and low temperatures are in progress.

Fats are made up of smaller units called fatty acids. Canola oil is rich in two fatty acids that are essential in your diet because your body can't make them: i) Alpha-linolenic acid (ALA) is an essential omega-3 fatty acid. It protects against heart attacks and strokes by helping to lower bad cholesterol. ii) Linoleic acid (LA) is an essential omega-6 fatty acid. It's important for the brain and for the growth and development of infants. The ratio of omega-6 to omega-3 in canola oil is 2:1, which is nutritionally ideal. Both of these fatty acids are polyunsaturated. Canola oil also contains very high levels of heart-healthy monounsaturated fatty acids, which lower bad cholesterol (LDL) and help control blood glucose. Compared to all other vegetable oils the market, canola oil has the lowest levels of the fats that are "bad" for human health: Saturated fats raise the bad cholesterol (LDL) in your body and have been linked to coronary heart disease. Canola oil has the lowest saturated fat level of all vegetable oils. Olive oil contains twice as much saturated fat as canola oil. Trans fats raise bad cholesterol and lower good cholesterol (HDL). While all processed oils contain very small levels of trans fatty acids, canola oil is defined as zero trans fat by government regulatory authorities in North America [4-6]. Transfats can be increased when vegetable oils are partially hydrogenated to make them more solid. Be wise: Choose canola oil and non-hydrogenated soft margarines instead of solid fats, such as partially-hydrogenated margarine, shortening, lard and butter. High-oleic canola provides additional opportunities to choose products that are free oftrans fat. Organisms that have been made to incorporate new properties, otherwise impossible to be acquired naturally, through transfer of genes from one species to another are called genetically modified organisms (GMO) [7]. With the “Green Revolution” in the 1950s; problems such as soil contamination, lessening of water sources resulting from efforts to increase yield and efficiency through over-irrigation, mineral fertilization, pesticide use and plant breeding made GMO seem like a savior. DNA, RNA, protein and metabolite based techniques are used in studies involving gene transfer to organisms. The most widely accepted methods in determination of genetically modified organisms are DNA based methods, because DNA molecule exhibits high stability under some food production processes. The most widely used DNA based method is polymerase chain reaction (PCR) [1,8].

The parallel amplification of the transgene and endogenous reference gene (housekeeping gene) using PCR technique provides highly reliable results [9-15]. This study aimed to perform transgene analysis of the *Californium, Jura, Elvis* and *Orkan* varieties of canola that have not undergone transgene analysis previously using primers specific to the 35S and *pNOS* promoter regions, which are used widely in transgene technology also compare between the efficiency of classical PCR and multiplex PCR in transgene analysis of canola varieties using primers specific to 35SCaMV and pNOS promoters.

## 2. MATERIALS AND METHODS

The *Jura, Orkan, Californium* and *Elvis* varieties of canola (*Brassica napus* L.) were obtained from Black Sea Agricultural Research Institute-Samsun/Turkey. The certified reference materials (Bt11 0%, Mon810 2%) were obtained from IRRM (Institute for Reference Materials and Measurements). Surface sterilization was performed initially to take the canola seeds into tissue culture. The seeds were treated with 20% (v/v) sodium hypochlorite solution for 20 minutes following treatment with 70% (v/v) ethyl alcohol for 5 minutes. The surface sterilized canola seeds were taken into Murashige-Skoog (MS) [16] medium. The media were placed inside plant growth chamber and tissue cultures were established under 400W of light, 70% humidity and 16/8 hours day/light cycle respectively. The genomic DNA isolations of canola plantlets and certified corn materials were performed using “peq Gold Plant DNA Mini Kit” (cat. No:12-3846-01) For DNA isolations 200mg of leaf from canola plantlets and 50mg each of 0% and 2% certified transgenic corn materials were used. Canola plantlets were crushed in sterile mortars using liquid nitrogen; 800µl of P1 buffer and 10µl of β-mercapto-ethanol were added after the powders were transferred into separate 2 ml centrifuge tubes. The powders of 0% and 2% corn that were transferred into centrifuge tubes were mixed with 800µl of P1 buffer and 10µl of β-mercapto-ethanol. Then the tubes were held in water bath at 65ºC for 10 minutes. Afterwards 140µl of P2 buffer was added and centrifuged at 10,000 rpm for 10 minutes at room temperature. The supernatants were transferred into new centrifuge tubes. 0.7 volumes of isopropanol were added into the solutions and supernatants were discarded following centrifugation at 10,000rpm for 2 minutes at room temperature. The pellet was left to dry at room temperature for 1 minute. The pellets were resuspended in 300µl of sterile distilled water previously heated to 65ºC, and the tubes were incubated at room temperature for 2 minutes following 4µl of RNase addition. Then 150µl of P3 buffer at 37ºC and 300µl of pure alcohol (99.5%; v/v) were added. The 750µl solutions were taken into the seperatory colons and centrifuged at 10,000 rpm for 1 minute at room temperature. The supernatant accumulated below the colons were discarded; the pellets were eluted with 750µl of DNA wash buffer and centrifuged at 10,000 rpm for 1 minute at room temperature. The elution with wash buffer was repeated once. Accumulation tubes were centrifuged at 12,000 rpm for 2 minutes at room temperature to dry the membranes and the colons were placed in fresh accumulation tubes. 100µl elution (solvent) buffer was added and tubes were centrifuged at 10,000 rpm for 1 minute at room temperature. This step was repeated once and DNA’s were stored in 200µl elution buffer at -20ºC. Classical and multiplex PCR techniques were used to perform transgene analysis of the *Elvis, Californium, Orkan* and *Jura* varieties of canola cultivated in Turkey in addition to 0% and 2% certified corn materials. PCR was performed at a total volume of 50µl using G-Storm Gradient PCR machine. Primers specific to *35S* (227bp) and *pNOS* (167bp) promoter regions were used to perform transgene analysis of the canola varieties and certified corn materials (Table 1).

**Table 1. Sequences of 35S, pNOS and NAD primers**

| Primers | SEQUENCE (5′-3′) | Weight (bp) | Tm (Cº) |
|---|---|---|---|
| AM35S F | AAGGGTCTTGCGAAGGATAG | 227 | 60 |
| AM35S R | AGTGGAAAAGGAAGGTGGCT | | 60 |
| pNOS F | GGAACTGACAGAACCGCAACG | 167 | 66 |
| pNOS R | TGGAACGTCAGTGGAGCATTT | | 62 |
| NAD5 F | TAGCCCGACCGTAGTGATGTTAA | 813 | 68 |
| NAD5 R | ATCACCGAACCTGCACTCAGGAA | | 70 |

It is stated in the product catalogs that the 0% Bt11 corn contains the *35S* promoter, *tNOS* and *zSSIIb* genes; the 2% MON 810 corn contains the *35S, tNOS* and *zein* genes. In addition to this promoter regions primers specific to *NAD* (813bp) gene were used to analyze the standard gene (housekeeping gene) expression (Demeke et al.). Fermentas PCR Master Mix (2X) was used to amplify the NAD gene and the two promoter genes included in the transgene analysis. PCR ingredients are given in Table 2 and classical PCR cycles are given in Table 3.

**Table 2. Classical PCR ingredients**

| Ingredients | Concentration (µl) |
|---|---|
| Basic Mix | 25 |
| Primers | 1 |
| Genomic DNA | 2 |
| Distilled water | 21 |

**Table 3. Classical PCR cycles**

| | Time (minute) | Temperature (ºC) |
|---|---|---|
| Initiation denaturation | 5 | 95 |
| Denaturation | 0.5 | 95 |
| Annealing | 0.5 | 60 |
| Longation | 1 | 72 |
| Last longation | 3 | 72 |
| Total cycles | 35 | |

While a single set of primers was added into the reaction tubes for classical PCR, 3 primer sets belonging to 35S, *pNOS* and *NAD* genes were added into each reaction tube for multiplex PCR. Qiagen Multiplex PCR Kit (Cat. No: 206143) was used; the reaction components are given in Table 4 and PCR cycles are given in Table 5.

**Table 4. Multiplex PCR Ingredients**

| Ingredients | Concentration (µl) |
|---|---|
| Distilled water | 14 |
| Q solution | 5 |
| Primer mix (*pNOS, AM35S, NAD5*) | 5 |
| DNA | 1 |
| Basic mixture | 25 |

**Table 5. Multiplex PCR cycles**

| | Time (minute) | Temperature (ºC) |
|---|---|---|
| Initiation denaturation | 15 | 95 |
| Denaturation | 0.5 | 94 |
| Annealing | 1.5 | 57 |
| Longation | 1.5 | 72 |
| Last longation | 10 | 72 |
| Total cycles | 30 | |

The classical PCR and multiplex PCR analysis of 35S*, pNOS* and *NAD* regions were performed in agarose gel electrophoresis system. For the analysis of classical and multiplex PCR amplification products obtained using specific primer sequences 2% (v/v) agarose gel was cast. To prepare the gel, 0.8gr agarose was measured and dissolved in 40ml 1X TAE buffer by heating in microwave. The agarose solution was cooled down to 60ºC and cast in gel cassette after addition of 2µl ethidium bromide (10mg/ml) to visualize the amplified DNAs under UV light. Following the polymerization of the gel 10µl each of the 2 types of PCR products were loaded into the wells of the agarose gel. To sink the products into the wells inside the TAE buffer and visualize the migration of PCR products in the gel each 10 µl PCR product was mixed with 2µl bromophenol blue and loaded into the wells. The DNA's loaded into the gel were run at a constant voltage of 80V for 1hour. The amplification products were visualized and images were recorded using gel visualization device (Avegen, X-lite 200) following electrophoresis.

## 3. RESULTS AND DISCUSSION

Seeds from *Californium, Jura, Elvis* and *Orkan* varieties of canola plant were grown in vitro in MS medium inside plant growth chamber. Plantlets of appropriate size for DNA isolation (5-7 cm) were obtained from all the tissue cultured seeds at the end of 7 days. The genomic DNA isolates required for classical and multiplex PCR were obtained from plantlets grown in tissue culture and certified corn materials intended as control group using peq Gold Plant DNA Mini Kit. Isolated DNA amounts were determined by spectrophotometric measurements. Transgene analysis of *Californium, Jura, Elvis* and *Orkan* varieties of canola plant and of 0% and 2% certified corn materials was performed with respect to *35S* and *pNO S*promoter regions using classical and multiplex PCR techniques. Primers specific to the 35S (227bp) and *pNOS* (167 bp) promoter regions, commonly used in GMO's, were used for transgene analysis in the study. In addition to these two primer sets primers specific to the *NAD* (813bp) region were used to verify the accuracy of the PCR process and the quality of amplification products. *35S* and *pNOS* promoter regions of 0% and 2% certified corn materials were amplified using classical PCR method. At the end of the classical PCR 35S and *pNOS* amplification products were detected for both 0% and 2% corn materials (Figs. 1 and 2).

Amplification products of *NAD* region, which were used to determine the product quality, were detected for all 4 varieties (Fig. 3).

*35S* and *pNOS* promoter regions of *Californium, Jura, Elvis* and *Orkan* varieties of canola were scanned with classical PCR for transgene analysis. At the end of PCR, no amplification products were observed in any of the 4 varieties (Fig. 4).

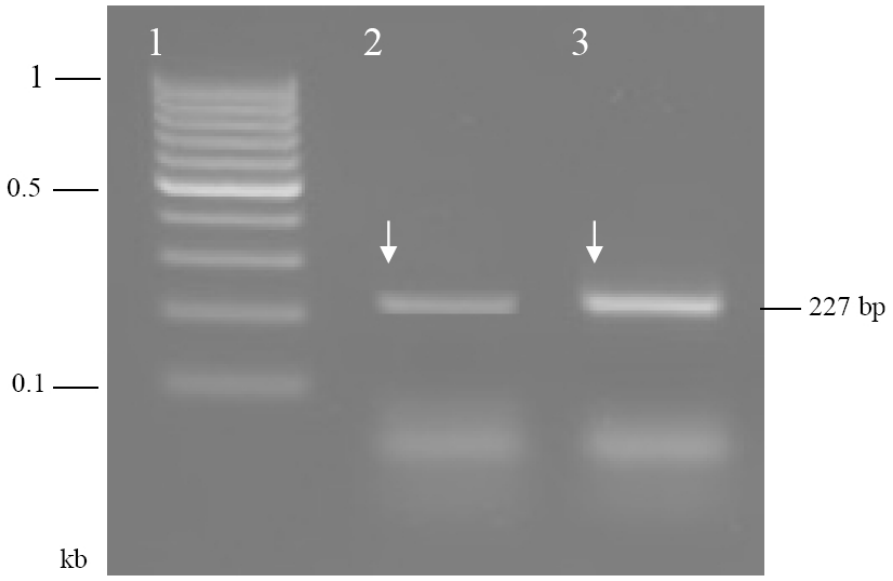


**Fig. 1. PCR yields of certificated maize *35S* promoter in %0 ve % 2 (%2 w/v) (1: 100 bpstandart DNA, 2: %0 certificated maize, 3:%2 certificated maize)**

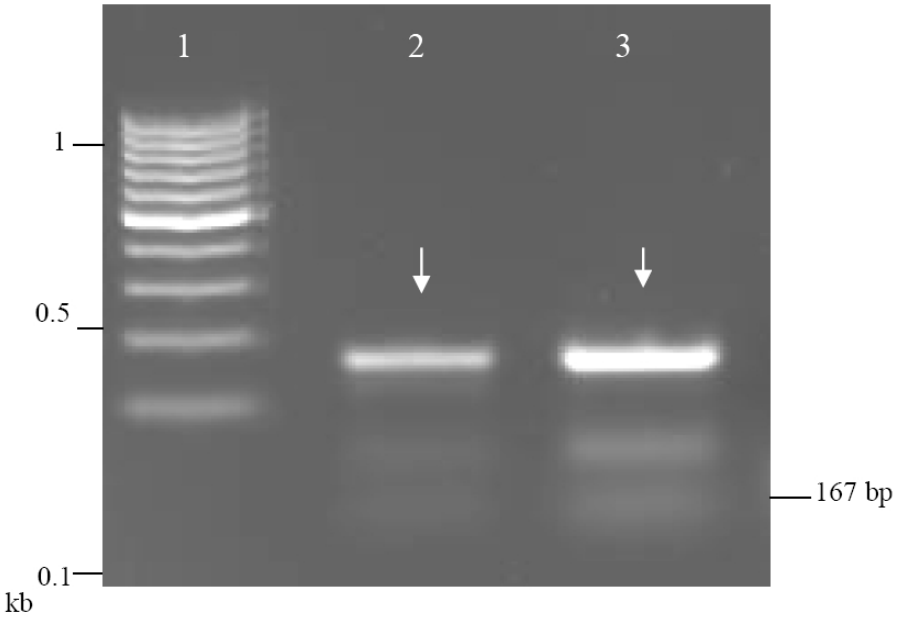


**Fig. 2. PCR yields of *pNOS*promotor in %0 ve% 2 certificated maize (%2 w/v) (1: 100bp standart DNA, 2: %0 certificated maize, 3:%2 certificated maize)**

For transgene analysis of canola varieties together with 0% and 2% corn varieties multiplex PCR method was used in addition to classical PCR. At the end of multiplex PCR,

amplification products of both *35SCaMV* and *pNOS* regions were detected in agarose gel electrophoresis for all canola varieties (Fig. 5).

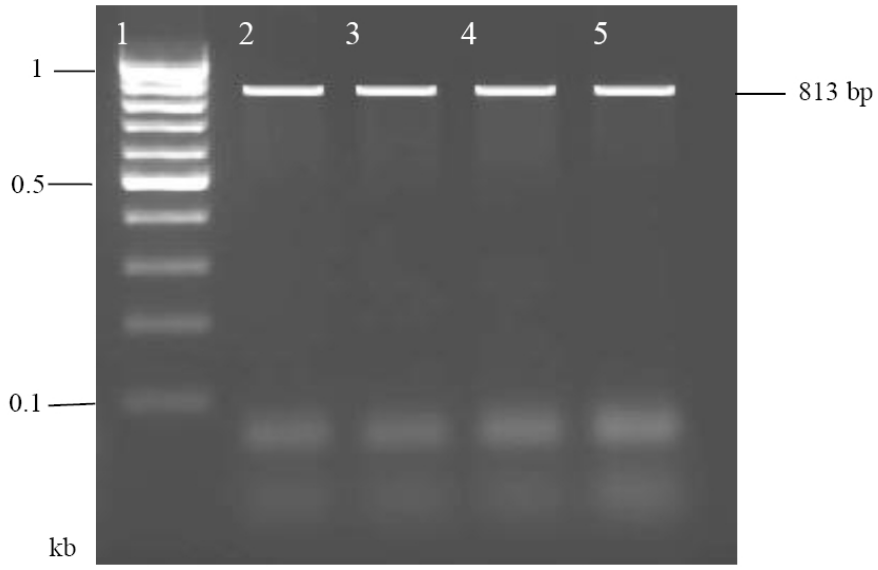


**Fig. 3. PCR results of NAD primers (1: 100 bpstandart DNA, 2: Californium, 3: Jura, 4: Elvis, 5: Orkan)**

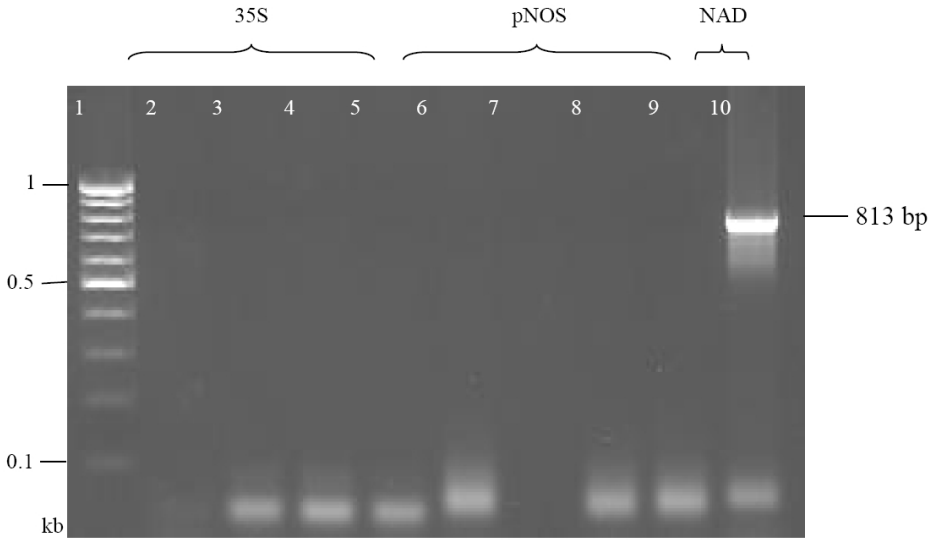


**Fig. 4. Classical PCR results of *35SCaMV, pNOS* and *NAD* (1: 100bp standart DNA, 2, 6 and 10: Californium, 3 and 7: Jura, 4, and 8: Elvis, 5 and 9: Orkan)**

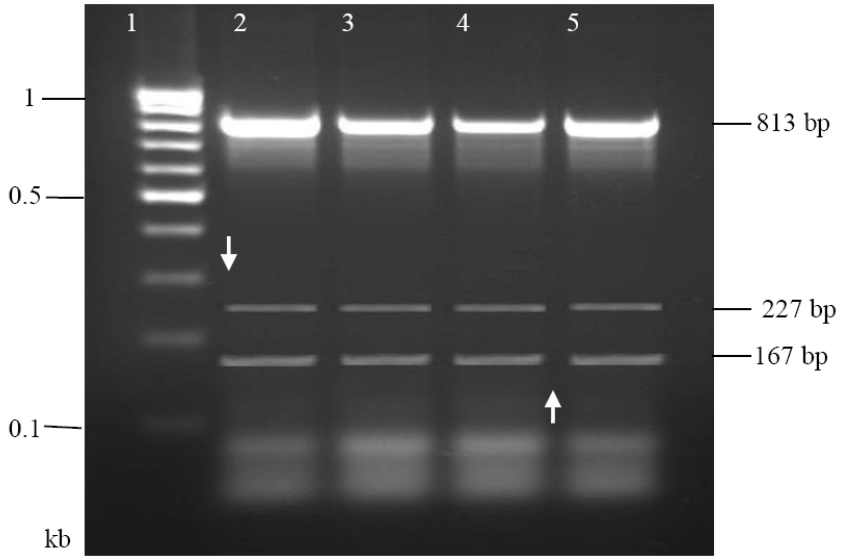


**Fig. 5. Multiplex PCR results of *35SCaMV* and *pNOS*(1: 100 bpstandart DNA, 2: Californium, 3: Jura, 4: Elvis, 5: Orkan)**

This study aimed to perform transgene analysis of *Californium, Jura, Elvis* and *Orkan* varieties of canola (*Brassicus Napus* L.) cultivated in Turkey using PCR techniques. With this purpose tissue cultures of canola varieties were established initially. Genomic DNA isolations were performed from the certified corn materials and plantlets grown in MS medium. Then transgene analysis of *Californium, Jura, Elvis* and *Orkan* varieties of canola was performed with regard to 35S and pNOS promoter regions, which are used frequently in transgene technology, using classical and multiplex PCR techniques. *35S* and *pNOS* promoter regions of both 0% and 2% certified corn materials, which are used as control groups in transgene analyses, were also amplified using classical PCR method (Fig. 1 and 2). No data were found in literature to support our findings. In his transgene analysis of soya and corn using PCR methods Turhan [17] used "Roundup Ready" 0%, 0.1%, 0.5%; MON810 0%, 0.1%, 0.5%; Bt11 0%, 0.1%, 0.5%; Bt176 0%, 0.1%, 0.5% standard materials obtained from IRMM as positive and negative controls. Turhan [17] also used 35S primers during the transgene analysis of 0% Bt11 material and obtained no amplification products. Nikolic et al. [18] used the Roundup Ready soya bean, Bt11 corn and H7-1 sugar beet obtained from IRRM as reference material in their study on the presence of genetically modified soya bean in labeled or non-labeled food samples. They used 0% soya bean material as negative control in their study. In this study 35S as promoter region and *NOS* as terminator region was scanned in transgene analyses of the samples but no amplification products were detected. Park et al. [19] used PCR methods for transgene detection in corn grown around ports, warehouses, and feed production facilities where major animal feed and seed import occurs in Republic of North Korea. They have used 5% Bt11 corn provided from IRMM as positive control and shown the amplification products belonging to this sample in agarose gel electrophoresis. It is stated in products catalogs of certified materials from IRMM that 0% corn material may contain transgenic products up to 0.12g/kg. The detection of *35S* and *pNOS* amplification products from 0% and 2% corn materials in agarose gel electrophoresis is in accordance with the information provided by IRMM. The amplification of *35S* and *pNOS* promoter regions from 0% and 2% corn materials indicate multiple gene insertion meaning more than one gene was integrated into the plant genome (Figs. 1 and 2). Particle bombardment and Agro

bacterium mediated gene transfer are used widely in multiple gene transfer. Wu et al. [20] transferred genes to cotton using A. *tumefaciens* and included 2 insect resistance genes (API-B and Bt29K) in the vector they designed. They have shown that the multiple gene transfer was successful using PCR and "southern blot hybridization" methods. The findings and literature search shows that multiple gene transfers in plants are commonly and successfully performed [2,21,22]. In our study we have made use of the housekeeping gene *NAD* to verify the accuracy of PCR method and quantify the amplification products; all four canola varieties used in the study were found to produce amplification products (Fig. 3). When the PCR conditions are met *NAD* primers bind to the template DNA inside the tube and amplify the region containing the gene. With this property, *NAD* is used as control in PCR. Demeke et al. [23] used the *NAD* gene, the gene used in our study as a housekeeping gene, to verify the accuracy of the PCR method used in the transgene analysis and quantify the PCR products; obtained positive results. Wu et al. [20] studied the *TT51-1* gene in rice, which provides resistance against insect pathogens, using PCR technique. They have used *PLD* gene region as housekeeping gene and obtained positive results. Demeke and Ratnayaka [23] studied the canola varieties cultivated in Canada and scanned the GT73 gene using classical, multiplex and RT-PCR methods. They have shown the accuracy of the PCR experiments using Fat-A gene (76 bp) in multiplex PCR and *hmg* gene (99 bp) in RT-PCR as housekeeping genes. In another study, Nikolic et al. [18] used RT-PCR method to determine the transgene quantities in food samples of soya bean and lectin gene as the housekeeping gene. They have detected amplification products of lectin gene in agarose gel electrophoresis. In our study we have performed transgene analysis of the *Californium, Jura, Elvis* and *Orkan* varieties of canola using PCR methods; *35S* and *pNOS* promoter regions were scanned in the canola varieties. 35S and pNOS promoter regions weren't amplified with classical PCR in all 4 varieties (Fig. 4). In contrast to our PCR findings Lee *et al*. [24] performed transgene analysis of MS8 and RF3 canola varieties with classical PCR method using 35S and NOS primer sets and amplified the *35S* and *NOS* regions successfully. Aydın [25] performed transgene analysis of processed and non-processed corn samples using classical PCR method. In the analysis of GMO corns collected from stores in different regions of Turkey 10 non-processed and 18 processed corn products were scanned with regard to transgenes. The samples were analyzed with PCR technique with regard to *35S, NOS* terminator and kanamycin resistance genes. Aydın [25] showed that all non-processed materials contained foreign genetic elements. He also reported that 6 of the 10 non-processed corn materials were Bt11 corn and that GMO analysis is possible with corn material and corn starch where it is not possible with corn flakes, corn chips and popcorn. In our study, multiplex PCR method in addition to classical PCR method was used in transgene analysis. With this purpose genomic DNAs isolated from *Californium, Jura, Elvis* and *Orkan* varieties; primer sets belonging to *35S, pNOS* regions and *NAD* primer were mixed in a single reaction tube and multiplex PCR was carried out. In contrast to results from classical PCR, multiplex PCR analyses showed DNA bands corresponding to *35S* and *pNOS* regions in all 4 varieties (Fig. 5). Forte et al. [26], who used utilized PCR method in their study, used *zein, lectin, 35S* and *tNOS* primers for the transgene analyses of corn and soya bean seeds obtained from Monsanto and Novartis; they have stated that multiplex PCR method provides proper results in GMO detection and is more favorable than classical and quantitative PCR, which are much faster and much more expensive. In their study performed on genetically modified soya bean, corn, canola and rice, Zhou et al. [27] reported that multiplex PCR is a fast and reliable method. In their study on canola, which pioneered our work, Demeke et al. [23] used both classical and multiplex PCR methods. They have acquired the seeds used in their study from Grain Research Laboratories (GRL) and worked on *GT73, HCN92* and *OXY235* gene regions, which provides herbicide resistance. In addition to *35S, pNOS, CAN2, 4EPSPS, BXN* gene regions they have used *NAD* gene region as the housekeeping

gene and they have shown with both classical and multiplex PCR methods that the DNA isolated from canola seeds contained transgenic elements. Multiplex PCR results have shown that the canola varieties cultivated in Turkey are transgenic with regard to *35S* and *pNOS* promoter regions and that all 4 varieties contain multiple gene insertions with regard to *35S* and *pNOS* promoter regions; as the case is with certified corn materials in our study. When they scanned the Exceed, Independence and Innovator varieties cultivated in Canada with regard to *HCN92* gene region (herbicide tolerance against glufosinate ammonium) using PCR technique, Demeke et al. [28] observed the presence of bands corresponding to *35S* and *pNOS* regions, which were also determined in our study, and reached the conclusion that these varieties contained multiple gene insertions. Although data on the presence of transgenes in varieties cultivated in Turkey could not be obtained using classical PCR method, the detection of multiplex PCR amplification bands corresponding to *35S* and *pNOS*regions *in Californium, Elvis, Orkan* and *Jura* varieties revealed that the classical PCR method used in our study was not suitable for transgene analysis. It is considered that the lack of findings in the varieties that were scanned for transgenes using classical PCR method could be due to low transgene levels. The studies conducted with analytical methods aim to determine the presence and quantity of transgenes in food and feed and to evaluate food safety. PCR technique, which is a commonly used method, is preferred because of its speed and sensitivity in determining the target DNA. However, false positive results have been reported by Liu et al. [29] due to primers binding to unintended regions. In spite of its possible dangers to human and animal health and ecological environment, demand for GMO has been increasing. In some countries labeling of the products of GMO plants that are cultivated widely worldwide is mandatory prior to consumer access [27,29-32] Based on the findings from our transgene analyses of *Californium, Jura, Elvis* and *Orkan* varieties of canola cultivated in Turkey, which have not undergone transgene analysis previously, it was concluded that the 4 varieties are transgenic with regard to *35S* and *pNOS* promoter regions. This transgene analysis study is the first one conducted on *Californium, Jura, Elvis* and *Orkan* varieties of canola.

## 4. CONCLUSION

In some countries labeling of the products of GMO plants that are cultivated widely worldwide is mandatory prior to consumer access. Based on the findings from our transgene analyses of *Californium, Jura, Elvis* and *Orkan* varieties of canola cultivated in Turkey, which have not undergone transgene analysis previously, it was concluded that the 4 varieties are transgenic with regard to *35S* and *pNOS* promoter regions. This transgene analysis study is the first one conducted on *Californium, Jura, Elvis* and *Orkan* varieties of canola.

## ACKNOWLEDGEMETS

This work was supported by the Research Fund of The University of Istanbul, Project no: T-2422.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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