DETECTION OF GENETICALLY MODIFIED INSECT RESISTANT TOMATO VIA POLYMERASE CHAIN REACTION

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

DETECTION OF GENETICALLY MODIFIED INSECT RESISTANT TOMATO VIA POLYMERASE CHAIN REACTION

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Tomato, which is one of the most important component of human diet, has been genetically modified to develop some properties like delayed ripening and insect resistance. In order to give a choice to the consumer, it is necessary to detect and label GM foods.

This study was carried out to detect genetically modified tomato samples purchased from different food markets of Turkey. PCR method was used to detect genetically modified insect resistant tomatoes. The DNAs of collected samples were isolated according to CTAB DNA extraction protocol and the amplification capacity of isolated samples were checked with patatin specific control PCR. Screening tests of tomatoes were done by targeting 35S promoter, Nos terminator and NptII kanamycin resistance gene with four primer sets. It was aimed to detect Cry1A and Cry1Ac genes with three PCR systems, in order to identify insect resistant samples.

From twenty-eight samples, twenty-two gave positive amplification signal in NptII specific PCR system and results were confirmed with sequence analysis. Additionally, we observed seventeen and ten DNA fragments with Cry1A-F/Cry1A-R and Cry1Ac-F/Cry1Ac-R primer sets respectively, it is necessary to confirm these results with DNA sequencing.

Key words: Genetically modified organisms, GMO detection, insect resistant tomato, PCR

ÖZ

GENETİK OLARAK DEĞİŞTİRİLMİŞ BÖCEK DİRENÇLİ DOMATESLERİN POLİMERAZ ZİNCİR REAKSİYONU İLE TESPİT EDİLMESİ

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İnsan besinin önemli bir bileşeni olan domates, bozulmayı geciktirme ve böcek direnci gibi özellikler kazandırmak için genetik olarak değiştirilmiştir. Tüketiciye seçim hakkı vermek için, GM gıdaların tespiti ve etiketlenmesi gerekmektedir.

Bu tez çalışması, Türkiye'nin farklı gıda marketlerinden alınan örneklerde, genetik olarak değiştirilmiş domatesleri tespit etmek için gerçekleştirilmiştir. Genetik olarak değiştirilmiş böcek dirençli domateslerin tespiti için PZR metodu kullanılmıştır.

Toplanan örneklerin DNAları CTAB DNA izolasyon metoduna uygun olarak izole edilmiş ve patatin genine özel PZR sistemi ile amplifikasyon özellikleri kontrol edilmiştir. Domateslerin nitel (kantitatif) tarama testleri dört primer seti ile 35S promotör, Nos terminatör ve NptII kanamicin direnç geni hedeflenerek yapılmıştır. Böcek dirençli örneklerin belirlenebilmesi için üç PZR sistemi ile Cry1A ve Cry1Ac genlerin tespit edilmesi amaçlanmıştır.

Yirmi sekiz örnekten, yirmi biri NptII' ye özel PZR sisteminde pozitif amplifikasyon sinyali vermiş ve sonuçlar sekans analizi ile doğrulanmıştır. Ek olarak, sırasıyla Cry1A-F/Cry1A-R ve Cry1Ac-F/Cry1Ac-R primer setleriyle, on yedi ve on DNA bandı belirlenmiştir, sonuçların DNA sekansı ile doğrulanması gerekmektedir.

Anahtar kelimeler: Genetik olarak değiştirilmiş organizmalar, GDO tespiti, böcek dirençli domates, PZR

To My Family; Güner, Handan, Can

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Acc: Aminocyclopropane carboxylate synthase

ACC: 1-aminocyclopropane 1-carboxylic acid

accd: 1-aminocyclopropane 1-carboxylic acid deaminase

µg : Microgram

µl : Microliter

bp : base pair

Bt: Bacillus thuringiensis

CaMV: Cauliflower mosaic virus

CBP: Cartagena Biosafety Protocol

CTAB: cetyltrimethylammonium-bromide

Cry proteins: Crystal proteins

DNA: Deoxyribonucleic acid

ds : double stranded

EC: European Commission

ECB: European Corn Borer

EDTA: Ethylenediamineetetraacetic acid

ELISA: Enzyme linked immunosorbent assay

EMBL: European Molecular Biology Laboratory

EPA: Environmental Protection Agency

EtBr: Ethidium Bromide

EU: European Union

FDA: Food and Drug Administration

GMO: Genetically Modified Organisms

GMF: Genetically Modified Foods

GM: Genetically Modified

Kan: Kanamycin

kb : kilobase

LB medium: Luria Bertoni medium

LDR: ligation detection reaction

MAFF: The Ministry of Agriculture, Forestry and Fisheries

MHLW: The Ministry of Health, Labor and Welfare

min : minute

mM : Milimolar

mRNA : Messenger RNA

NCBI: National Centre for Biotechnology Information

ng : Nanogram

Nos: Nopaline synthase

NptII: Neomycin phosphotransferase

PCR : Polymerase Chain Reaction

PEG: Polyethylene glycol

PEPC: phosphoenolpyruvate carboxylase

PG: polygalacturonase

pmol : Pico mole

PVP: Polyvinylpyrollidone

QC-PCR: Quantitative Competitive -Polymerase Chain Reaction

rDNA : recombinant DNA

Rpm : Rotation per minute

RT-PCR: Real Time-Polymerase Chain Reaction

sam-k: S-adenosylmethionine hydrolase encoding gene

USDA: US Department of Agriculture

UV : Ultra Violet

v/v : volume/volume

w/v : weight/volume

X^{TM}: Trade Mark

CHAPTER I

INTRODUCTION

1.1 Genetically Modified Organism (GMO)

Recent advances in molecular biology has opened new avenues for production of plants with new genetic properties which often referred to as genetically modified (GM) crops. In brief genetically modified organisms are the organisms that have the ability to synthesize some additional proteins which confer new properties on them.

The first genetically modified organisms (GMOs) were created in the early 1970s using recombinant DNA technology, and the first GM plants were produced in 1983. By the late 1980s, GM crops which are virus resistant tobacco and tomato were on sale in China, but they did not become widespread until 1994. The early examples of GM crops were delayed ripening "Flavr Savr" tomato, insect-resistant corn that was introduced in 1995 and herbicide tolerant soybean that was introduced in 1996 (Hails & Kinderlerer, 2003). The transgenic corn MaisGard™ from Monsanto (USA) was approved in the United States in 1996 and cultivated in 1997 on

approximately 1 million hectares (Zimmermann *et al.*, 1998). During the eight-year period 1996 to 2003, global area of transgenic crops increased 40 fold, from 1.7 million hectares in 1996 to 67.7 million hectares in 2003, with an increasing proportion grown by developing countries (Figure 1.1)(James, 2003).

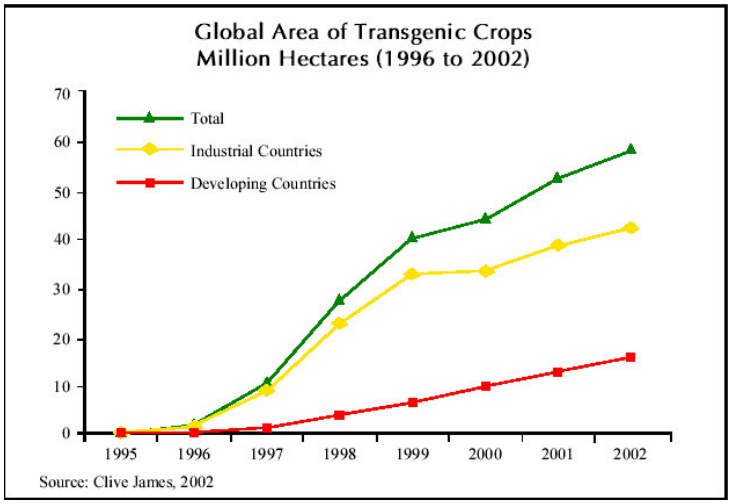


Figure 1.1; Global Area of Transgenic Crops (1996 to 2002), (James, 2003)

The most widely adopted GM traits are herbicide tolerance, insect resistance and a combination of these. In 2001, 13 countries contributed to the increased planting of GM crops: eight industrialized nations and five from the developing world (Thomson, 2003).

1.1.1 Advantages of Genetically Modified Crops

Since the world population exceeded 6 billion in 2000 and is expected to reach approximately 8.5 billion by 2025, to meet the increasing needs of the growing human population, it will be necessary to produce 50% more food by 2025. Genetic engineering techniques gives opportunity to find a solution for this problem.

Crops modified with recombinant DNA techniques have different improved properties. Genetic engineering can be used to increase levels in foods of minerals and naturally occuring anti-oxidant vitamins (caroteniods, flavonoids, vitamins A and E). Antioxidant and lycopene found in tomatoes with elevated levels can slow or shut down biological oxidation, a damaging chemical reaction, that appears to promote the development of some cancers (Gachet *et al.,* 1999) and coronary heart diseases (Fraser *et al.,* 2002). The nutritionally enhanced crops will help to reduce malnutrition, and will enable developing countries to meet their basic dietary requirements. Golden rice has been genetically modified to produce a vitamin A precursor which would prevent malnutrition, blindness, and anemia in people of developing countries. Genetic engineering can be used to modify oils to achieve a reduction in the levels of fatty acids which are responsible for cholesterol production in the body. GM can also be used to increase the levels of unsaturated fatty acids in some commonly used oils such as canola, soybean, sunflower and peanuts.

The Flavr Savr tomato is the first genetically engineered crop that was bio-engineered to have a longer shelf-life by having delayed ripening and softening processes (Gachet *et al.,* 1999) and whole food approved by the FDA (Food and Drug Administration, USA)(Bruderer & Leitner, 2003).

The carbohydrate content of some food crops has been increased by genetic engineering. Tomatoes with high solid content have been produced and this is useful to food processors for making tomato paste and sauce. Potato has been genetically modified to have high solid content, which makes it useful for making French fries. The high solid potatoes have been produced by Monsanto Corporation that absorbs less oil during processing into French fries (Gachet *et al.,* 1999, Bruderer & Leitner, 2003). The modification of the potato results in decreases in cooking time, costs and fuel use. This leads to better tasting French fries that provides economic benefit to the food processor.

Genetic engineering can be used to increase crop yield and reduce crop loss by making plants tolerant to pest, weeds, herbicides, viruses, insects, salinity, pH, temperature, frost, drought and weather (Taylor, 1997).

Some tropical crops such as banana, which are consumed raw when ripe, have been bio-engineered to produce proteins that may be used as vaccines against diseases like hepatitis and rabies in developing countries (Gachet *et al.,* 1999). These vaccines in edible foods will be beneficial in developing countries where such foods are grown and distributed at low cost.

Genetically modified plants have been produced in several major crop plants such as maize, wheat, barley, rice, cotton, potato, tomato and soybean. Soybean, cotton and maize are the most widely used crops in transgenic technologies (Figure 1.2). A series of genes governing agronomically important traits have been transferred through various transformation techniques.

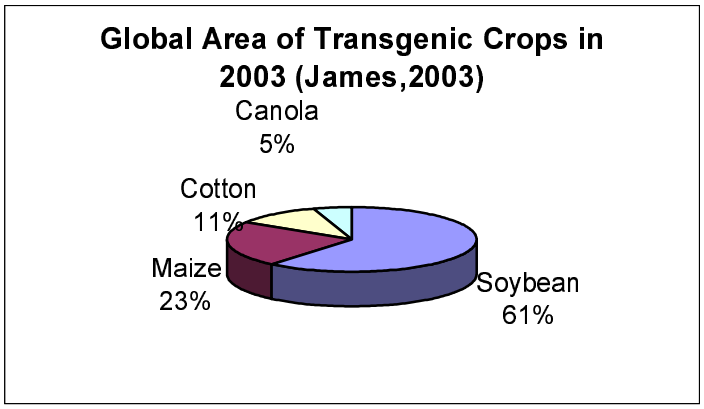


Figure 1.2; Global Area of Transgenic Crops in 2003, (James, 2003)

1.1.2 Methods used for production of GMOs

A number of methods are now available to produce genetically modified plants,

· *Agrobacterium*-mediated gene transfer and,

· Direct gene transfer methods; Polyetylene glycol (PEG) mediated gene transfer, Electroporation, Microinjection and Microprojectile bombardment.

Agrobacterium tumefaciens is a soil bacterium which infects a wide range of plant species and is capable of transferring a piece of DNA (T-DNA) into the genome of

host plants. The genes inserted into the T-DNA region through Ti plasmids are co-transferred and integrated into the host genome.

Agrobacterium tumefaciens has played a major role in the development of plant genetic engineering. It accounts for about 80% transgenic plants produced so far (Wei *et al.,* 2000). This soil bacterium naturally infects only dicotyledonous plants and many economically important plants, including cereals have remained inaccesible for genetic manipulation (Querci *et al*., 2004). But recent studies have shown that *Agrobacterium*-mediated gene transfer methods can be also used in transformation of agronomically important monocotyledonous like maize, wheat and rice (Babu *et al.*, 2003).

Both *A. tumefaciens*-mediated gene transfer and direct DNA transfer methods have been used to produce transgenic plants with new genetic properties. Several genes for insect resistance have been transferred through genetic engineering techniques. Use of delta (δ)-endotoxin coding sequences originating from *Bacillus thuringiensis* (Bt) and use of plant derived genes, such as those encoding enzyme inhibitors or lectins are the two main approaches to produce such genetically engineered plants (Babu *et al.*, 2003).

1.1.3 Genetically Modified Tomatoes

The commercial cultivated tomato is identified botanically as *Lycopersicon esculentum* and it is a member of the Solanaceae family that includes potatoes, peppers, tobacco and many other plants (Jaccaud *et al.,* 2003). The cultivated tomato is thought to have originated in Mexico, with the cherry tomato probably being the direct ancestor of the modern cultivated forms (GM Database, 20.08.2004, www.agbios.com, Long life tomato-PG tomato, 20.08.2004, http://www.hasdenbosh.nl/algemeen/projecten/tomaat/lesniv1/pg_tomaat_1_zeneca.html).

Tomatoes are a valuable source of food minerals and vitamins. Beside their low calorie values, they are also rich in an anti-oxidant called lycopene, a carotenoid that has been found to protect cells from oxidants that have been linked to cancer. Lycopene has been linked to risk reduction for a number of types of cancers, including prostate, lung and stomach, pancreatic, cervical, colorectal, oral and esophageal cancers.

Today, tomatoes are grown commercially in 159 countries, with a combined production of over 98 million metric tones in 2000. The major producers of tomatoes in 2000 were China, Egypy, India, Italy, Turkey and The United States.

By using the recombinant DNA technology, genetically modified tomatoes with variable improvements such as delayed ripening, virus resistance and insect

resistance have been produced (Table 1.1). As seen on Table 1.1 genetic improvement studies of tomato plant mostly focused on the delayed ripening property. The first approved genetically modified crop was Flavr Savr™ tomato which contains an artificially introduced gene. The Flavr Savr™ –gene, is transcribed into a messenger RNA (mRNA) that is the antisense to mRNA from the polygalacturonase gene. The complementary *in vivo* base pairing of these two molecular species results in inhibition of the expression of the polygalacturonase gene, with loss of the enzymatic activity. Polygalacturonase degrades cell wall pectin. Its inhibition increases the shelf-life of the tomatoes and prevents them from becoming soft (Meyer & Candrian, 1996). B, Da, F transgenic line has also been engineered by insertion of PG gene (Bruderer & Leitner, 2003).

In the case of 1345-4 line delayed ripening of the fruit is achieved by the introduction of aminocyclopropane synthase (Acc) gene in the sense orientation, resulting in tomato plants which exhibit significantly reduced levels of ACC synthase and ethylene biosynthesis. Another approved delayed ripening tomato line 8338 has also been genetically altered by reducing the activity of ACC (1-aminocyclopropane 1-carboxylic acid) with the introduction of accd (1-aminocyclopropane 1-carboxylic acid deaminase) gene. Event 35 1 N has been genetically engineered by introducing the sam-k gene encoding the enzyme S-adenosylmethionine hydrolase. This enzyme alters the ethylene biosynthetic pathway and delay the ripening of tomato on vine (Bruderer & Leitner, 2003).

Table 1.1; Approved GM Tomatoes (Bruderer & Leitner, 2003)

Event	Company	Traits	Approval
117,1046,1202,1208	Nivot	Virus resistance	Japan
1345-4	DNA Plant Technology	Delayed ripening	Canada,USA
35 1 N	Agritope	Delayed ripening	USA
405,707	Nivot	Virus resistance	Japan
5345	Monsanto	Insect resistance	Canada, USA
8338	Monsanto	Delayed ripening	USA
B, Da, F	Zeneca	Delayed ripening	Canada, USA
China tomato 1	Peking University	Virus resistance	China
China tomato 2	CCAU	Delayed softening	China
Flavr Savr	Calgene	Delayed softening	Canada, Japan, Mexico, USA
ICI9, ICI13	Zeneca	Delayed softening	Japan
Japan tomato 1	NIAES Planttech Research Institute	Virus resistance	Japan
N°4-7	Hokkaido Nat. Agr. Station	Virus resistance	Japan

1.1.4 Insect Resistance

There are an estimated 67,000 pest species worldwide that damage agricultural crops, of which approximately 9,000 species are insects and mites. Worldwide insecticide use on major crops that demonstrated in Figure 1.3 is 8.110 million US $. At this point, use of insect resistant crops are increasing the productivity as well as providing important benefits to farmers, the consumer and the environment. *Bacillus thuringiensis* (*Bt*) is the only one among the several insect control strategies enabled through biotechnology.

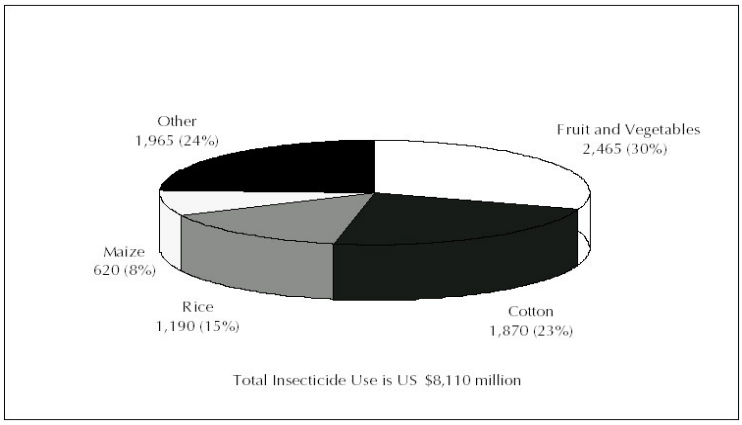


Figure 1.3; Worldwide insecticide use on major crops (US$ million),(Krattiger, 1996)

The use of *Bt* as a biopesticide was discovered in the first decade of this century when larvae of flour moths died suddenly. Research into their deaths led to the discovery that the presence of *Bt* was responsible for the death. However, it took 50 years before *Bt* became a widely used biopesticide with its registration in the USA in 1961.

Bacillus thuringiensis, commonly known as *Bt*, is a gram-positive bacterium that occurs naturally in the soil around the world. It was first discovered in Japan in 1902 and characterized by Berliner in Thüringen (Germany) in 1912. It was known by bacteriologists that some strains of *B. thuringiensis* kill certain insects and the toxic substances responsible for this insect death are insecticidal crystalline inclusions synthesized during sporulation. The crystalline structure of the inclusion is made up of protoxin subunits, called δ-endotoxins. Most *B. thuringiensis* strains produce several crystalline proteins (Cry proteins), each of which shows a rather narrow host range. *Bt* toxin genes earlier were classified into four types, based on their specificity

and sequence homology (Krattiger, 1996, Sharma *et al*., 2000). These four classes and their host ranges are as follows:

· CryI (active against *Lepidoptera*),

· CryII (*Lepidoptera* and *Diptera*),

· CryIII (*Coleoptera*) and

· CryIV (*Diptera*).

The *Bt* δ-endotoxins are now known to constitute a family of related proteins for which 140 genes have been described.

When certain insects ingest either the bacterium or the protein produced by the bacterium (δ-endotoxin), these proteins are solubilized in the insect midgut and are activated by gut proteases that cleave the protein into a smaller polypeptide, the toxin. This toxin binds to the surface of epithelial cells in the midgut, inducing lesions that destroy the cells and lead to the death of the insect (Jouanin *et al*., 1998). The *Bt* protein is not harmful to mammals, birds or fish, nor to beneficial insects. Mammals, including humans, do not have δ-endotoxin receptors in their guts and all *Bt* proteins tested so far are degraded within 20 seconds in the presence of mammal digestive juices (Krattiger, 1996).

The commercial use of *Bt* biopesticide is limited by high production costs and the instability of the crystal proteins when exposed to the field (Vaeck *et al.,* 1987,

Krattiger, 1996). Transgenic plant systems provide an entire plant protection, especially against insects such as borers, infect plant parts that sprays often can not reach. The first results concerning the transfer of *B. thuringiensis* genes in tobacco and tomato were published in 1987 (Fischhoff *et al.*)(Table 1.2) and many more crops soon followed (Jouanin *et al.,* 1998).

Maize is one of the most widely consumed insect resistant crop; Bt11, Mon810 and Event 176 maize lines containing Cry1Ab have approvals for consumption as food and feed. Bt11 transgenic maize has been engineered to express the Cry1Ab delta-endotoxin insecticidal protein that was known to be effective against certain lepidopteran insects, including European Corn Borer (ECB) (Bruderer & Leitner, 2003).

Table 1.2; Insect-resistant transgenic plants expressing B. thuringiensis toxins (Fontes *et al.,* 2002).

Crop plant	Toxin	Target insect	Reference
Canola	cry1Ac	Thrichoplusia ni (Hübner) (Lep.)*, Spodoptera exigua (Hübner), Heliothis virescens (Fabr.), Helicoverpa zea (Boddie) (Lep.)	Stewart *et al*. (1996b)
	cry1Ac	P. xylostella	Ramachandran *et al.* (1998)
Cotton	cry1Ab	H. virescens, H. Zea	Perlak *et al.* (1990)
	cry1Ac and cry2Ab	S. exigua, Pseudoplusia includens (Walker) (Lep.)	Adamczyk *et al.* (2001)
Corn	cry1Ab	Ostrinia nubilalis (Hübner) (Lep.)	Koziel *et al.* (1993)
	cry9c	O. nubilalis	Jansem *et al.* (1997)
Potato	cry1Ab	Phthorimaea operculella (Zeller) (Lep.)	Peferoen (1992), Rico *et al.* (1998)
	cry1Ab	Heliothis armigera (Hübner)	Chakrabarti *et al.* (2000)
	cry3Aa	L. decemlineata	Adang *et al.* (1993), Perlak *et al.* (1993) Coombs *et al.* (2002)
Rice	cry1Ab	Chilo supressalis Walker (Lep.)	Fujimoto *et al.* (1993)
	cry1B	C. supressalis Cnaphalocrosis medinalis Guenée (Lep.)	Wunn *et al.* (1996)
	cry1Ac, cry2A	C. medinalis, Scirpophaga incertulas Walker (Hom.)* Nilaparvata lugens Stål (Hom.)	Maqbool *et al.* (2001)
	cry1Ab and cry1Ac	C. supressalis	Cheng *et al.* (1998)
Soybean	cry1Ac	H. virescens, H. zea, P. includens	Stewart *et al.* (1996a)
Tobacco	cry1Aa	Manduca sexta (L.) (Lep.)	Barton *et al.* (1987)
	cry1Ab	M. sexta	Vaeck *et al.* (1987)
	cry1Ab and cpTI	M. sexta	Perlak *et al.* (1991)
	cry1Ab	M. sexta	Williams *et al.* (1993)
	cry1Ac	H.virescens, H. zea, S. litoralis	McBride *et al.* (1995)
	cry1C	S. litoralis	Strizhov *et al.* (1996)
	cry2A	H. armigera	Selvapandiyan *et al.* (1998)
	cry2A	H. virescens, H. zea, S. exigua	Kota *et al.* (1999)
Tomato	cry1Ab	H. virescens	Fischolff *et al.* (1987)
	cry1Ac	H. armigera	Mandaokar *et al.* (2000)

*(Lep: Lepidoptera; Col: Coleoptera; Hom: Homoptera)

The first publications reported a very low level of expression of the *cry* genes in comparison to other genes transferred to plants. A critical factor following transformation is the desired expression of the insecticidal gene. Bt genes are Adenine–Thymine rich while plant genes tend to have a higher Guanine–Cytosine content. The expression of insecticidal proteins have been enhanced by increasing Guanine–Cytosine content of their encoding genes without changing the amino acid sequence. Most changes are made to the third codon thereby minimising changes in the amino acid sequence and increasing the expression Bt toxin by 10 to100 fold (Sharma *et al.,* 2000). Since then Bt genes have been transferred to many crops including cotton, maize, rice and potato. Fischhoff *et al.* (1987) constructed a chimeric gene (A gene or DNA sequence not found in nature but has been constructed in a laboratory.) containing the CaMV 35S promoter that provides the contitutive expression of inserted gene and the *B. thuringiensis* protein coding sequences. The transgenic tomato plants expressed the *Bt* toxin gene. Transgenic plants showed little feeding damage by the larvae of *Heliothis virescens Fabricius* (tobacco budworm) (Babu *et al*., 2003). Field trials have been initiated with the two types of genes, and more convincing results were obtained with the synthetic genes (Jouanin *et al.,* 1998).

1.1.4.1 Insect Resistant Tomato

Tomatoes are subject to damage from many insects, nematodes and fungal, viral, bacterial pathogens. Tobacco budworm (*Heliothis virescens*) and tomato fruitworm (*Helicoverpa zea*) are destructive pests of tomato.

Tomato engineered with the insecticidal Cry1Ab gene(s) from *B. thuringiensis* has been reported to provide protection to the plants against tomato fruit worm (Fischhoff *et al*., 1987; Vaeck *et al*., 1987). And then the potential use of transgenic tomato plants, expressing a Cry1Ab gene from *B.thuringiensis*, to control *H. armigera* under Indian conditions was evaluated by Kumar & Kumar, (2004)(Figure 1.4).

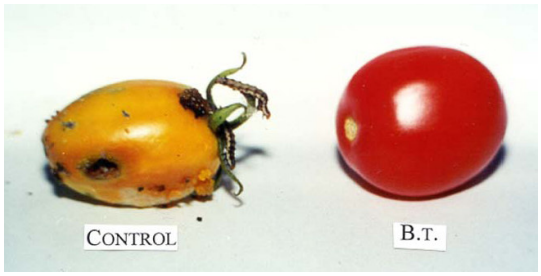


Figure 1.4; Damage caused by *H. armigera* to the fruit of transgenic and non-transgenic tomato plants in the laboratory (Kumar & Kumar, 2004)

Delannay *et al.* (1989) evaluated transgenic tomatoes expressing Bt insect control protein under field conditions in 1987 and 1988. The transgenic plants showed very limited feeding damage after infestation with the tobacco hornworm (*Manduca sexta*) whereas control plants showed heavy feeding damage and were almost completely defoliated within two weeks. Significant control of tomato fruit worm and tomato pinworm was also observed.

Insect resistant tomato line 5345 was developed via *Agrobacterium*-mediated transformation to express the insecticidal protein, Cry1Ac, encoded by the *cry1Ac*

gene from the soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* strain HD73 (Figure 1.5). The nucleotide sequence of the *cry1Ac* gene was modified by site-directed mutagenesis to contain plant preferred codons in order to maximize protein expression in plant cells (Bruderer & Leitner, 2003)

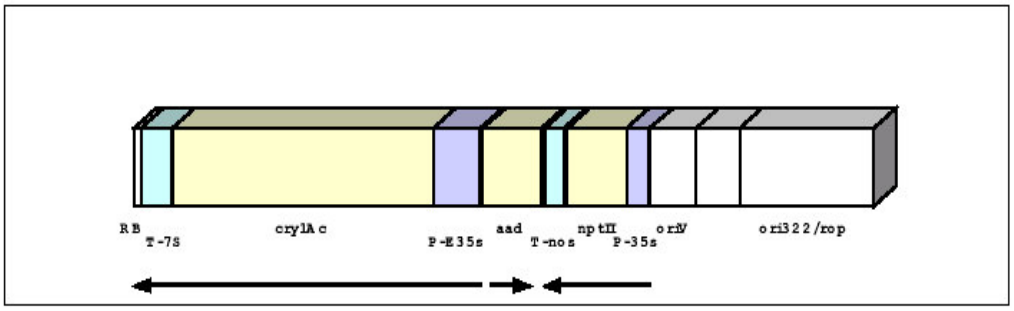


Figure 1.5; Genetic construct of insect resistant tomato line 5345 (Bruderer & Leitner, 2003)

Tomato line 5345 was field tested in the United States from 1994 to 1997. Comparisons with non-transformed tomato lines determined that the agronomic traits for line 5345 were all within the range of unmodified tomato lines. Compositional analyses were conducted to compare transgenic line 5345 tomato fruit with fruit obtained from control non-transgenic plants grown under the same conditions. The measured parameters included total solids, protein, carboyhdrates, calories, vitamin A, vitamin C, and folic acid. There were no statistically significant differences both for any of these compared parameters and for organoleptic properties between insect-resistant 5345 tomatoes and control tomatoes. Tomato line 5345 was approved as food in Canada in 2000, both as food and feed in the United States in 1998 (GM Database, 20.08.2004, www.agbios.com).

The potential for toxicity and allergenicity of the Cry1Ac protein was assessed by an examination of its physicochemical characteristics and amino acid sequence homology to known protein toxins and allergens. Unlike known protein allergens, the Cry1Ac protein was readily digested under conditions simulating the gastric and intestinal environment, and it was denatured upon treatment. There were no amino acid sequence homologies with known allergens or protein toxins (Canada Food Program, 17.01.2004, http://www.hc-sc.gc.ca/food-aliment/mh-dm/ofb-bba/nfi-ani/e_2000_tomato.html, U.S. Environmental Protection Agency, 17.01.2004, http://www.epa.gov/fedrgstr/EPA-IMPACT/1998/April/Day-09/i9376.htm).

1.2 Detection of Genetically Modified Organisms

By the time genetically modified crops began to find more place in supermarket shelves as food, detection and labelling of these GM crops gain much more importance. Labelling of genetically modified crops provides an opportunity to consumers that they prefer to buy a GM food or not. Since it was highly controversial in development of GMOs due to its potential effect to human health and the ecological environment, it is an important issue that by true and informative labelling public perception can be turn to positive.

Different detection methods based on nucleic acids and proteins are used for GMO detection. The basis of every type of GMO detection technology is to exploit the difference between the unmodified variety and the transgenic plant.

1.2.1 DNA Based Detection Methods

In DNA based detection methods, it is necessary to have some previous sequence information about the GM construct. These might be plasmid vector sequences, commonly used polylinkers, selectable markers, promoters and terminators.

The most commonly used DNA based GMO detection techniques are Southern blot and PCR analysis. Beside these techniques there are some microarray based detection systems under development, Bordoni *et al.* (2004) performed amplification and quantification of cry1Ab gene from Bt-176 transgenic maize by ligation detection reaction (LDR) combined with a universal array approach. DNA based technologies have some advantages over protein based methods like sensitivity and specificity.

The Southern Blot method involves fixing isolated sample DNA onto nitrocellulose or nylon membranes, probing with double-stranded (ds)-labeled nucleic acid probes specific to the GMO, and detecting hybridization radiographically, fluoremetrically or by chemiluminescence. However, because only one probe is used, and no amplification is carried out, this method is considered less sensitive than PCR, which employs DNA of two primers (Ahmed, 2002).

1.2.1.1 Polymerase Chain Reaction

PCR is a widely used technique in molecular biology. The detection of the introduced genetic elements at the level of the deoxyribonucleic acids (DNA) using the polymerase chain reaction (PCR) has been shown to be the most useful method of identifying genetically modified foodstuffs (Zimmermann *et al*., 1998). It has a high potential of increased sensitivity and specificity when compared to protein based methods.

Using the PCR method to identify GM products, a primer is designed based on a regulatory sequence or structural gene in the inserted fragment. These designed primers possess some specific characteristics and can be used for product screening and product-specificity detection. The PCR products need to be further confirmed by the following methods: specific cleavage of the amplified product by restriction endonuclease digestion, hybridization with a DNA probe specific for the target sequence, direct sequencing of the PCR product and nested PCR in which two sets of primer pairs bind specifically to the amplified target sequence (Lin *et al*., 2000, Ahmed, 2002).

The major limitations for PCR-based detection of DNA derived from genetically modified organisms are access to information about applicable PCR primers and access to DNA suitable for reliable analysis (Holst-Jensen *et al.,* 2002). Some problems could arise in developing a PCR method for the detection of GMO in foods; (1) Processed food: In highly processed or fermented foods, genes could be

altered or proteins could be denatured, making detection difficult. (2) Varieties of GM products: a great quantity with different varieties of GM-crops are cultivated. (3) Mixture of GM products: different varieties of GM-crops are mixed either artificially or naturally. (4) Shortage of the information regarding the gene of sequence of the inserted gene, which could result in difficulty in designing a suitable primer. (5) Trade to countries where GMF are unregulated could create a problem in GMF detection (Lin *et al.,* 2000).

The first detection method specifically developed for the identification of a commercialised genetically engineered plant was demonstrated for detection of the Flavr Savr^{TM} tomato. The Flavr Savr^{TM} –gene, the *kan^{r}* gene that confers resistance to kanamycin and the cauliflower mosaic virus promoter CaMV35S, the detection of these three foreign sequences could be achieved by developing two PCR assays. One primer pair amplifies a 173 bp fragment from *kan^{r}*. The second primer pair consists of an oligonucleotide hybridizing to the promoter sequence, the other being complementary to the Flavr Savr^{TM}- gene. The resulting amplicon is 427 bp long. When fresh tomatoes from different countries, a sample of canned tomato and a Flavr Savr^{TM} tomato obtained from Calgene were analysed, only the genetically engineered tomato yielded the two specific amplicons (Meyer, 1995).

1.2.1.1.1 Qualitative PCR methods for GMO detection

Qualitative PCR-based GMO tests can be grouped into at least four categories corresponding to their level of specificity as illustrated in Figure 1.6. Each category corresponds to the composition of the DNA fragment that is amplified in the PCR.

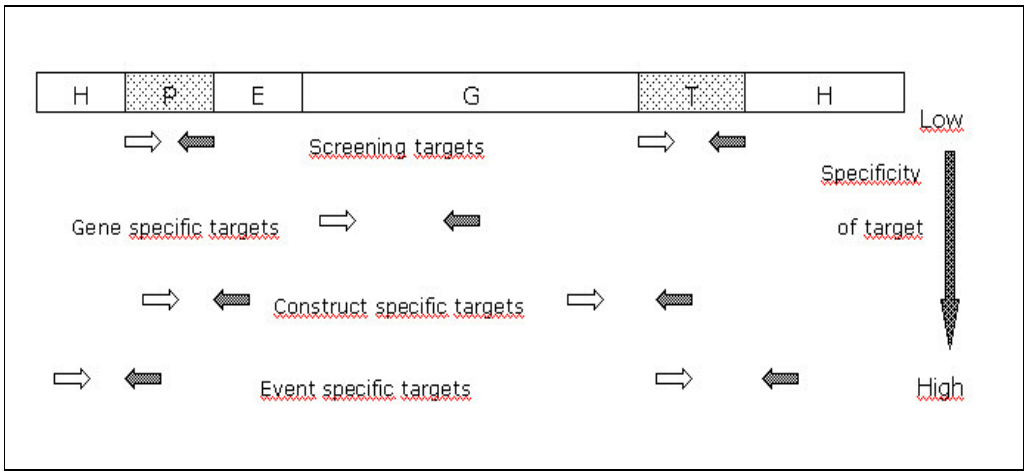


Figure 1.6; Scheme of qualitative PCR systems (Holst-Jensen *et al.,* 2002) (H, host genome; P, promoter; E, enhancer; G, gene of interest)

The choice of target sequence is the only most important factor controlling the specificity of the PCR method. The target sequence is normally a part of the modified gene construct like a promoter, a terminator, a gene or a junction between two of these elements. However, the elements may originate from wildtype organisms, they may be present in more than one GMO, and their copy number may also vary from one GMO to another (Holst-Jensen *et al.,* 2002).

1.2.1.1.1.1 Screening PCR

The majority of GM plants have been transformed with constructs containing the Cauliflower Mosaic Virus (CaMV) 35S promoter and *Agrobacterium tumefaciens* nopaline synthase terminator. Because of this reason, they are the mostly targeted genes in screening PCR systems. The other most commonly used GMO screening targets are the antibiotic resistance genes like neomycin/kanamycin that used in most of the vector systems.

The detection limits of the 35S-PCR system represents the decisive factor for GMO labeling in Switzerland. The detection limits of the 35S- and NOS-PCR system were determined in a collaborative study that revealed up to twenty-fold differences in sensitivity between different laboratories. Furthermore, the 35S-PCR was found to be more sensitive than the NOS-PCR (Hübner *et al.,* 1999a).

One of the most important problem of GMO screening system is the false results. The presence of one of the screening targets (35S promoter , Nos terminator, *bla*-ampicillin resistance gene or *nptII*-kanamycin resistance gene) may not necessarily imply the presence of GMO-derived DNA. The source of P-35S or T-35S could be naturally occurring CaMV (Holst-Jensen *et al.,* 2002) , and it is generally believed that *Agrobacterium* or other soil bacteria containing one or more of the targets are present in soil. Antibiotic resistance markers used in laboratory construction of plant expression systems may be eliminated from genetically modified plants prior to their

application in the field (MacCormick *et al.,* 1998). As a further step, identification of GMO with gene specific targets could minimize the risk of false detection.

1.2.1.1.1.2 Gene specific PCR

PCR methods targeting the gene of interest are more specific than screening PCR systems. Inserted gene that gives a proper characteristic to the donor organisms is the target of gene specific PCR system. Hupfer *et al.* (1997) described a detection method for insect-resistant Bt maize that targeted a truncated version of Cry1Ab gene. Characterization and confirmation of the PCR products were done both by Southern hybridization and DNA sequencing.

1.2.1.1.1.3 Construct specific PCR

Target of these construct specific PCR systems are junctions between adjacent elements of the gene construct, like the promoter and the gene of interest. Four construct specific PCR systems that specifically detect Bt11, Event176, Mon810 and Liberty maize lines were developed by Yamaguchi *et al.* (2003). For each maize line, specific primer sets that cover the junctions between elements are used and the results of construct specific PCRs were compared with ELISA technique results.

1.2.1.1.1.4 Event specific PCR

The only unique signature of a transformation event, within the limitations of present day technology, is the junction at the integration locus between the recipient genome and the inserted DNA. Because of this reason event specific PCR provides unique detection.

Zimmermann *et al.* (1998) evaluated a specific and sensitive nested PCR-method for the detection of transgenic corn MaisGard™. This nested PCR sytem amplifies a unique therefore specific sequence that lies between the 35S promoter and the corn-specific heat shock protein 70 (hsp70). So far, none of the other approved genetically modified crops contains this particular combination of genetic elements. Another event specific PCR system was developed by Windels *et al.* (2003) that targeted the junction between the CBH-351 insert DNA and the plant genomic DNA of StarLink maize.

Multiplex-PCR technique is another approach used for GMO detection. By using this method it is possible to amplify different target genes in one PCR reaction. Detection of nine soybean, six maize, seven potato and two rice samples were done by multiplex-PCR. After the amplification of samples with biotinylated screening and identification primers, the multiplex PCR products and membrane bound oligonucleotide probes were hybridized. This multiplex polymerase chain

reaction/membrane hybridization assay improves detection efficiency obviously, reduces false negative or false positive results, and improves the result accuracy of GMOs detection effectively (Su *et al.,* 2003).

1.2.1.1.2 Quantitative PCR methods for GMO detection

Swiss Food Regulation and the EU Novel Food Regulation is based on a PCR detection system specific to the 35S promoter and the Swiss government revised its food regulations, introducing a threshold value of 1% GMO content as the basis for food labelling (Querci *et al.,* 2004). Since qualitative PCR based detection systems can lead to interlaboratory differences, these systems are not suitable for the control of labeling limits. Reliable quantitative methods are the basis for the control of limits of GMO for the labeling of GMO-containing food (Hardegger *et al.,* 1999). The enforcement of such threshold values requires quantitative detection systems such as quantitative competitive (QC-PCR), real-time PCR or immunochemical detection of modified proteins using ELISA systems (Hübner *et al.,*1999b).

The quantitative competitive PCR was first described in the early 1990s and is now used for a wide range of applications (Hardegger *et al.,* 1999). Internal DNA standards and the target DNA which have very similar features and amplifiability are co-amplified in a single reaction tube.

Hardegger *et al.* (1999) developed and evaluated a 35S promoter and Nos terminator specific QC-PCR that gives the possibility to detect and quantify about 90% of the GM plants with the same method. Hübner *et al.* (1999a) developed QC-PCR systems for the quantitative detection of Roundup Ready™ soybean (RRS) and Maximizer maize (MM) in food samples. The internal DNA standarts consisted of cloned PCR fragments of RRS and MM DNA carrying internal insertions of 21 and 22bp, respectively. Zimmermann *et al.* (2000) have developed and extended a quantitative PCR system to overcome two difficulties of "conventional" PCR systems used in GMO analysis. This quantitative PCR has some advantageous properties on two topics: different copy numbers of integrated constructs in different GMOs and different GMOs containing identical transgenic constructs. By using inverse PCR, Zimmermann *et al*. (2000) characterized the genomic sequence at the 5'-site of the integrated transgenic sequence in the Bt-11 corn genome and developed a novel and ambigous quantitative PCR system covering the integration border at the 5' site of the transgene.

Real-time PCR, which is a reliable quantitative PCR technique, has some advantages over conventional quantitative methods. This method gives the opportunity to take DNA concentration data proportional to PCR cycle number in real-time and permit the differentiation between specific and non-specific PCR products by probe hybridization or melt curve analysis of PCR products (Ahmed, 2002). Conventional PCR relies on end-point measurements, when often the reaction has gone beyond the exponential phase because of limiting reagents. Other PCR-based techniques, such as

quantitative competitive PCR and real-time PCR have been recently developed which address the problem of establishing a relationship between the concentration of target DNA and the amount of PCR product generated by the amplification. For relative GMO concentrations in food mixtures, the quantification of a GM-marker has to be normalized to a plant specific reference gene. The accurate relative quantification might be achieved by a combination of two absolute quantification reactions: one for the GMO-specific gene and a second for the plant reference gene. With the assumption that GMO material has been submitted to the same treatment as the non-GMO material, the measurement can be expressed as genome/genome% (g/g%) or weight/weight% (w/w%) (Bonfini *et al.,* 2001).

1.2.1.1.2.1 Limit of Quantification (LOQ)

Limit of quantification can be calculated that if samples are distributed in a binomial manner, 299 maize kernels are needed to detect 1% GMO with a confidence of 95%. In other words, if the test result is negative, then there is 95% probability that the true concentration is 1% or less. If the DNA is extracted from e.g. homogenised maize chips it might well happen in the final reaction mixture for DNA amplification, the total genome copy number is below the critical particle size and hence, that the relationship between sample size at the beginning and at the experiment has been lost.

The DNA amount in the unreplicated, haploid nuclear genome of an organism is referred to as its C value. *Zea mays ssp. mays*, for instance, has ten chromosomes in its haploid nuclear complement. The DNA content of the unreplicated haploid complement (n=10) is known as the 1 C value, but C value can not be necessarily correct for all lines of same species, arising from the intra-specific variations in nuclear DNA. The estimated 1 C total nuclear DNA value ranges from about 2.45 pg (2.364 Mb) to 3.35 pg (3.233 Mb). In 1994, over 120 estimates of DNA C values for more than 100 genotypes of *Zea mays ssp. mays* from 25 sources were known. Based on the 1 C values 100 ng of DNA may contain approximately 3.8 X 10^4 copies of maize genome. This corresponds to 38 genome copies for maize if only 0.1 % of 100 ng DNA is of GMO origin. Consequently theoretical detection limit (which is equivalent to one copy of unreplicated haploid genome) in a 100 ng DNA reaction is 0.003% in the case of maize (Bonfini *et al.,* 2001).

1.2.2 Protein Based Detection Methods

The protein based test method uses antibodies specific to the protein of interest. Both Western blot and enzyme-linked immunosorbant assay (ELISA) techniques have been used for the analysis of protein products of Monsanto's transgenic RR soybean (Ahmed, 2002, Duijn *et al.,* 1999).

The Western blot is a highly specific method that gives the opportunity to detect a certain protein by using the antibody specific to that protein, it involves the

hybridization of proteins bound to a filter. It provides qualitative results suitable for determining whether a sample contains the target protein below or above a predetermined threshold level. However, this method is considered more suited to research applications than to routine testing (Ahmed, 2002).

ELISA detects or measures the amount of protein of interest in a sample that may contain other dissimilar proteins. Immunoassay techniques with antibodies attached to a solid phase, have been used in two formats: a competitive assay in which the detector and analyte compete to bind with capture antibodies, or a two-site (double antibody sandwich) assay in which the analyte is sandwiched between the capture antibody and the detector antibody. Antigen-antibody product generates a color that can be easily visualised and quantified based on a comparison of a standart curve of the protein of interest.

A variation on ELISA, using strips rather than microtiter wells, led to development of lateral flow strip technology. Immobilize double antibodies, specific for the expressed protein, are coupled to a color reactant and incorporated into a nitrocellulase strip, which, when placed in a plastic ependorf vial containing an extract from plant tissue harboring a transgenic protein, leads to an antibody sandwich with some of the antibody that coupled to the color reagent. This colored sandwich flows to the other end of the strip through a porous membrane that contains two captured zones, one specific for the transgenic protein sandwich and another specific for untreated antibodies coupled to the color reagent. The presence of only

the control line on the membrane indicates a negative sample, and the presence of two lines indicates a positive result. Commercially available lateral flow strips are currently limited to few biotechnology-derived protein-producing GM products (Ahmed, 2002).

The method of detecting expressed protein was simple, highly specific and easy to quantitate, although the sensitivity was low and frequently it failed to detect the fully processed products (Anklam *et al.,* 2002). Another drawback of protein based Detection method is, genetically modified products might be produced only during certain developmental stages or in certain plant parts and such GMOs are therefore unlikely to be readily detected with these methods. In Table 1.3 there is a comparison of DNA based and protein based methods with their summarized properties.

Table 1.3; Summary of methods that specifically detect rDNA produced by GM foods (Ahmed, 2002)

Parameter	Protein-based			DNA-based			
	Western blot	ELISA	Lateral flow strip	Southern blot	Qualitative PCR	QC-PCR and limiting dilution	Real-time PCR
Ease of use	Difficult	Moderate	Simple	Difficult	Difficult	Difficult	Difficult
Needs special equipment	Yes	Yes	No	Yes	Yes	Yes	Yes
Sensitivity	High	High	High	Moderate	Very high	High	High
Duration	2 d	30-90 min	10 min	6 h-30 h	1.5 d	2 d	1 d
Cost/sample (US$)	150	5	2	150	250	350	450
Gives quantitative results	No	Yes	No	No	No	Yes	Yes (with high precision)
Suitable for field test	No	Yes	Yes	No	No	No	No
Employed mainly in	Academic labs	Test facility	Field testing	Academic labs	Test facility	Test facility	Test facility

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GM, genetically modified; QC-PCR, quantitative-competitive PCR; rDNA, recombinant deoxyribonucleic acid;

Although protein based detection methods like ELISA and lateral flow strip provides an easy and practical GMO detection, PCR based methods are accepted as more sensitive than conventional protein based detection tests (Gachet *et al.,* 1999). Since proteins are thermo-sensitive molecules and they may be expressed only in specific parts of the plant like leaves, they are not reliable in all conditions.

1.3 Impacts of GM foods on human health and the environment

The critics of genetic engineering of foods have concerns for safety, allergenicity, toxicity, carcinogenicity, altered nutritional quality of foods and also for the environment (Kuiper & Kleter, 2003).

1.3.1 Human health concerns

The main concerns about adverse effects of genetically modified (GM) foods on health are: inherent toxicity of the novel gene and their products, the potential to express novel antigenic proteins or alter levels of existing protein allergens, the potential for unintended effects resulting from alterations of host metabolic pathways or over expression of inherently toxic or pharmacologically active substances and the potential for nutrient composition in the new food occur differing significantly from a conventional counterpart (Malarkey, 2003).

The first allergenicity scenario of GM foods occured in 1996 when the 2S albumen protein from Brazil nut (rich in cystein and methionine) was transferred into soybean to improve the nutritional content of soybean for cattle feed. 2S albumen was found to be a major Brazil nut allergen and the newly expressed protein in transgenic soy retained its allergenicity. Patients allergic to Brazil nuts and not to soybean showed an IgE mediated response towards GM soybean (Lack, 2002).

1.3.1.1 Safety assessment principles for GM foods

An approach has been developed for the safety assessment of GMFs, which is based on the concept of "Substantial Equivalence". The concept of substantial equivalence embodies the idea that conventional foods can serve as comparators for the properties of GMFs, since conventional foods are considered as safe, based on a history of safe use. The comparison takes the agronomical, morphological, genetic and compositional properties of the GMF and the traditionally produced food into account, and establishes the degree of equivalence between the GMF and the traditional counterpart. Identified differences are the subject of further toxicological, analytical and nutritional investigation (Kuiper & Kleter, 2003). The principle is that if a new food or food component is found to be substantially equivalent to an existing food or food component by chemical analysis, then it can be treated in the same manner with respect to safety, bearing in mind that establishment of substantial equivalence is not a safety or nutritional assessment in itself but an approach to compare a potential new food with its conventional counterpart (Moseley, 2002).

Present approaches to the detection of expected and unexpected changes in the composition of GMF crops are primarily based on measurements of a limited selection of single compounds (targeted approaches). In order to increase the possibility of detecting secondary effects, new profiling methods, using gene expression technologies, proteomics and metabolics, should be further developed and validated (non-targeted approach)(Kuiper & Kleter, 2003).

1.3.2 Environmental concerns

The impact of genetically modified crop to environment could be direct, through increased invasiveness, or indirect, via an alteration of agronomic practice. Ecological risks might be classified into three groups: (1) Those concerning genome organization within plant; foreign genes might alter nutritional value of foods in unpredictable ways by decreasing levels of some nutrients while increasing levels of others. (2) The escape of transgenes into wild relatives (the likelihood and the consequences); there is a concern that deliberately breeding antibiotic resistance into widely consumed crops may have unintended consequences for the environment as well as for humans and animals consuming the crops. Also genetically modified crops having herbicide and insect resistance could cross-pollinate with wild species, and unintentionally create hard to-eradicate super-weeds. (3) The impact on nontarget species in the wider ecosystem (Hails, 2000, Uzogara, 2000).

1.4 Public Perception of Genetically Modified Crops as Food

The first major controversy associated with gene technology in Europe took place in the late 1980s when genetically modified foods were not at commercialisation stage, but industrial applications of gene technology were developed to the production and marketing stages.

Novel foods resulting from the application of recombinant DNA technology have been on sale in British supermarkets since 1994. The cheese made using chymosin from genetically modified microorganisms and tomato paste from tomatoes modified to delay ripening were labelled (Moseley, 1999), in the latter case with large lettering drawing attention to the fact that the paste was made from genetically modified tomatoes. There appeared to be very little hostility to the products and labelling allowed consumers a choice between genetically modified and traditional varieties. Public concern was triggered by the introduction particularly of the soya products from genetically modified plants into about 60% of manifactured foods without appropriate labelling. Failure to consult the public about policy development leads to a loss of public confidence in science and technology.

The introduction of genetically modified foods would elicit a public backlash predates the introduction of such items into our diet. At the ethical level there are concerns about scientists “playing God” by tinkering with the stuff of life, that genetic manipulation breaches the natural barriers and boundaries between species which nature has set up through the process of evolution, and that genetic manipulation distorts mankind’s relationship with the rest of nature. There are also concerns that the technology is an expensive one and will not be available to ”poor” farming communities and may even distort the economies of third world countries (Moseley, 1999).

1.5 Regulations on GMO's

A number of genetically modified organism(GMO) have been approved for human consumption but concerns over safety persist in the public. Allergenicity and toxicity, which are caused by proteins, are the major concerns (Kuiper & Kleter, 2003). In order to improve a positive impact on consumers, it is necessary to give true and satisfactory information. Regulations on GM foods and labelling are inevitable to provide confidence of consumers.

1.5.1 Regulations in USA

Genetically modified crops were first commercially introduced in the United States in 1996 and have been rapidly adopted by farmers. It has been estimated that 24% of the corn, 63% of the soybeans and 64% of the cotton growing in the United States in 2001 are GM varieties, it is also estimated that 70% to 85% of processed foods on supermarket shelves in the United States today contain one or more ingredients potentially derived from GM crops.

GM crops are regulated through a coordinated framework developed in 1992 and administered by three agencies- the US Department of Agriculture (USDA) that ensures the products are safe to grow, the Environmental Protection Agency (EPA) that ensures the products are safe for the environment and the Food and Drug Administration (FDA) that ensures the products are safe to eat (Harlander, 2002).

Fifty-one products have been reviewed by FDA, including several varieties of corn, soybeans, canola, cotton, rice, sugar beets, potatoes, tomatoes, squash, papaya and flax. Because FDA considers these crops "substantially equivalent" to their conventional counterparts, no special labeling is required.

A cornerstone of the evaluation process is the concept of "substantial equivalence". Regulatory agencies compare GM crops to their conventional counterparts. Labelling of substancially equivalent GM crops are not required in Unites States. However some companies have decided to voluntarily label their products as non-genetically modified.

1.5.2 Regulations in European Union

In contrast to the position in United States, there is a mandatory labelling of GM foods and food ingredients in the European Union.

In the European Union (EU), the safety of genetically modified (GM) food is controlled by the Novel Foods and Novel Food Ingredients Regulation 258/97/EC which came into effect on 15th May 1997 and introduced a mandatory premarket safety assessment for all novel foods. In this regulation, a novel food is defined as a food or food ingredient which has not been used for human consumption to a significant degree within the European Community (Moseley, 2002, Querci *et al.,* 2004). A later Regulation (1139/98/EC) serves as a model for labelling within the EU and for the requirements for food labelling required by 258/97/EC. In these

regulations there is a de minimus value, below which a declaration is not required. This value is usually arrived at pragmatically, taking account of the level of contamination likely, the degree to which contamination represents a health hazard and the availability of robust methods of detection available to enforce legislation. In the case of GM foods , labelling provisions have been amended by Commission Regulation 49/2000 to provide a 1% adventitious contaminaton level below which the presence of GM material does not have to be declared as long as their presence in the product is accidental or technically unavoidable. Regulation 50/2000 deals with specific labelling requirements for foodstuffs and food ingredients containing additives and flavourings derived from GMOs (Moseley, 2002).

Before a novel food can be approved under the regulation, it must satisfy 3 criteria that it must not: present a danger to the consumer, mislead the consumer, or differ from a food it is intended to replace such an extent that its normal consumption would be nutritionally disadvantageous to the consumer.

Norway and Switzerland which are not the members of EC, has been set the labelling limit of GMOs in food as 2% (Hardegger *et al.,* 1999).

1.5.3 Regulations in Japan

Thirty-five transgenic crop plants such as herbicide-tolerant soybean, cotton, canola, insect resistant corn, cotton and potatoes were authorized and marketable in Japan until 2001.

The Ministry of Agriculture, Forestry and Fisheries (MAFF) and The Ministry of Health, Labor and Welfare (MHLW) are the regulatory agencies of Japan. The Japanese labeling system for GMO is thus regulated by these 2 independent organizations, although the subject and contents in the regulations are virtually the same.

In Japanese system, food are classified into 3 groups; those using GMOs, those using non-GMOs and those for which GMO use is not segregated during their production and distribution. Labeling is compulsory for using GMOs and not segregated foods, yet it is optional for non-GM foods. The threshold level of unintended mixing of GMO is 5% in the cases of soy and maize (Hino, 2002).

1.5.4 Regulations in Turkey

The Ministry of Agriculture is the regulatory agency of Turkey in the issue of genetically modified crops and the required regulations are in progress (TAGEM-Tarım İşletmeleri Genel Müdürlüğü, 20.04.2004, www.tagem.gov.tr). Public perception arising from the groups and newspapers can force the regulatory agencies to prepare the required regulations.

Cartagena Biosafety Protocol (CBP) which seeks to protect biological diversity from the potential risks posed by resulting from modern biotechnology has been signed by Turkey on 24.05.2000. Food law (No 5179) which was focusing on controlling of all commercialised foods was implemented on 27 May 2004.

1.6 Objectives of this study

Turkey is one the fourth biggest tomato producer country in the world and Turkey import tomato seeds mostly from the Israel and then from USA, Holland and France. Despite the fact that detection and labelling of genetically modified foods is a controversial issue, there is not any study or regulation that have been developed in Turkey so far. The aim of this thesis study is to analyze and evaluate tomato samples collected arbitrarily from Turkey food market and check these samples whether they contain genetic modification or not.

The preferred detection method is a DNA based technique, Polymerase Chain Reaction (PCR). Since there is not any available construct and sequence information about genetically modified insect resistant tomato which Turkey imports, Monsanto's transgenic tomato line 5345 was taken as a model, and focused on the target genes of this genetically modified insect resistant tomato line. In order to detect three screening targets and an identification target gene, 8 primer sets were designed that were specific to 35S promoter, Nos terminator, NptII kanamycin resistance gene, Cry1A genes (including Cry1Ab and Cry1Ac) and specifically Cry1Ac insect resistance gene.

DNAs from tomato samples collected from Turkey food market were isolated by CTAB method. Extracted DNA samples were amplified with screening and identification primers.

CHAPTER II

MATERIALS AND METHODS

2.1. Food materials

Raw tomato samples were purchased from different outdoor markets and supermarkets of Turkey. In order to make a comparison, samples from Belgium, Spain, America and China were also included. As positive controls, genetically modified tomato seeds which contains 35S promoter, Nos terminator and NptII resistance gene were kindly obtained from Dr. Chris Bowler, Laboratory of Molecular Plant Biology Stazione Zoologica- Italy.

2.2. DNA isolation

CTAB DNA isolation protocols both from fresh tissue and from dry tissue were used for DNA extraction.

2.2.1. DNA isolation from fresh tissue

DNA isolation from fresh tomato tissues were done according to the CTAB extraction procedure which provides easy and rapid DNA extraction (Doyle & Doyle, 1990).

Freshly taken tomato pulps were homogenized with liquid nitrogen by using mortar and pestle. 5-7.5 ml CTAB isolation buffer (2% (w/v) CTAB (Applichem), 1.4 M NaCl (Merck), 20 mM EDTA (Sigma), 100 mM Tris-HCl (Sigma) pH:8.0 and 1% (w/v) PVP-40 (Sigma)) with freshly added 1% (v/v) β-mercaptoethanol (Applichem) was preheated in a 50 ml falcon tube to 60°C in a water bath. 0.5-1 g homogenized sample and the preheated buffer were mixed and placed at 60°C for 30 min. After incubation samples were extracted with an equal volume of chloroform-isoamylalcohol (Applichem, (24:1), (v:v)), mixed gently but thoroughly and centrifuged (MSE Mistral 1000) at 6000 rpm for 10 min. The aqueous phase was removed with a pipet to a new ependorf, 2/3 volumes of cold isopropanol (Delta Kimya) was added and mixed gently to precipitate the nucleic acids.

After centrifugation at 500- 1000 rpm for 1-2 min, the supernatant was discarded and 10-20 ml washing buffer (75% v/v ethanol (Delta Kimya), 10mM ammonium acetate (Applichem)) was added. The pellet was washed for minimum 20 minutes and centrifuged at 6000 rpm for 10 minutes. The supernatant was poured off carefully and allowed pellet to air dry at room temperature. The dried pellet was resuspended in 0.5 ml TE buffer (10 mM Tris-HCl (Sigma), 1 mM EDTA (Sigma)).

2.2.2. DNA isolation from dry tissue

DNA extraction from positive control tomato seeds was performed by CTAB DNA isolation procedure (Querci *et al.,* 2004).

100 mg of homogeneous sample was transferred into a sterile 1.5 ml microcentrifuge tube, 300 µl of sterile deionised water and 500 µl of CTAB-buffer pH 8.0 (20 g/l CTAB (Applichem), 1.4 M NaCl (Merck), 0.1 M Tris-HCl (Sigma), 20 mM Na_2EDTA (Sigma)) were added to microcentrifuge tube and mixed with a loop after each addition. 20 µl Proteinase K (20 mg/ml, MBI Fermentas) was added, mixed and placed at 65°C for 30-90 min. After incubation 20 µl RNase A (10 mg/ml, MBI Fermentas) was added, mixed and again kept at 65°C for 5-10 min. After this second incubation samples were centrifuged (Hettich Zentrifugen Mikro 12-24) for 10 min at about 16,000xg and the supernatant was transferred to a microcentrifuge tube containing 500 µl chloroform (Applichem), mixed for 30 s and again centrifuged for 10 min at about 16,000xg until phase separation occurs. 500 µl of upper layer was transferred into a new microcentrifuge tube containing 500 µl chloroform (Applichem), mixed and centrifuged for 5 min at 16,000xg.

The upper layer was transferred to a microcentrifuge tube and 2 volumes of CTAB precipitation solution pH 8.0 (5 g/l CTAB (Applichem), 0.04 M NaCl (Merck)) was added, mixed by pipetting. After an incubation for 60 min at room temperature, samples were centrifuged for 5 min at 16,000xg, the supernatant was discarded and

the precipitate was dissolved in 350 µl NaCl (1.2 M, (Merck)). 350 µl chloroform (Applichem) was added and mixed for 30 s, centrifuged for 10 min at 16,000xg until phase separation occurs, the upper layer was transferred to a new microcentrifuge tube, 0.6 volumes of isopropanol (Delta Kimya) was added and mixed.

After a centrifugation for 10 min at 16,000xg, the supernatant was discarded. 500 µl of 70% ethanol (Delta Kimya) solution was added and mixed carefully, centrifuged for 10 min at 16,000 xg. The supernatant was again discarded, the pellet was dried and DNA was re-dissolved in 100 µl sterile deionised water.

2.2.3. Concentration determinaton of isolated DNA by spectrophotometry

DNA samples were diluted 1/10 in 50µl of double distilled water and absorbance values were measured at 260 nm and 280 nm in Perkin Elmer EZ201 spectrophotometer.

DNA absorbs UV light at 260 nm, but it is also required to know the absorbance values of proteins at 280 nm in order to evaluate the purity of DNA samples. Pure DNA should have a A_{260}/ A_{280} ratio of approximately 1.8. If there is contamination with protein and aromatic substances, the A_{260}/ A_{280} value will be below 1.6 (Pauli *et al.,* 2000, Querci *et al.,* 2004) and the A_{260}/ A_{280} value above 2 indicates possible contamination with RNA (Querci *et al.,* 2004).

Concentrations of DNA samples were determined according to the equation given below:

Conc. of DNA (µg/µl)= A_{260} value x dilution factor x 50 µg/µl DNA/ A_{260}

1 A_{260}=50µg/µl DNA (Querci *et al.,* 2004).

2.3. Designing the PCR primers

Targeted sequences were obtained from NCBI and EMBL GenBanks (NCBI GenBank, 05.01.2004, www.ncbi.nlm.nih.gov/Pubmed ,EMBL GenBank, 05.01.2004, www.ebi.ac.uk/index.html) Primers used to detect NptII kanamycin resistance gene, Cry1A and the Cry1Ac genes were designed by using the software programmes ClustalW (ClustalW homology search programme, 24.09.2004, http://www.ebi.ac.uk/clustalw) and Primer3 (Primer3 primer design programme, 17.05.2004, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). ClustalW programme was used to check homologous regions of sequences found from NCBI and EMBL gene banks and the Primer3 programme was used to design specific primers that could match to these homologous regions. All oligonucleotides used in this study are listed in Table 2.1 and they were synthesized by Iontek (Istanbul/Turkey).

Table 2.1; Primer sets used in this study

Detection	Target	Primer sequences (5' to 3')	Product size	Reference sequences *	Reference for primer sets and related sequences
Control	Patatin gene	PatGc-F CTC ATT Agg CAC Tgg CAC T PatGd-R gTA AgA ACT TgC TgC ACT AgT C	124 bp	X03932	Jaccaud et al., 2003
Screening	35S Promoter	35S-F CCC CgT gTT CTC TCC AAA 35S-R CCA AAg ATg gAC CCC CAC	264 bp	CMV7626	This study
		35S-1-F gCT CCT ACA AAT gCC ATC A 35S-2-R gAT AgT ggg ATT gTg CgT CA	195 bp	AF078810	Tozzini et al., 2000- Jaccaud et al., 2003- Lin et al., 2000
	Nos terminator	Nos1-F gAA TCC TgT TgC Cgg TCT Tg Nos3-R TTA TCC TAg TTT gCg CgC TA	180 bp	U12540	Hardegger et al., 1999- Smith et al., 2004
	NptII resistance gene	Kan-F TTg CTC CTg CCg AgA AAg Kan-R gAA ggC gAT AgA Agg CgA	459 bp	AF274974	This study
Identification	Cry gene	Cry-F CAA CTg CCT gAg CAA CCC Cry-R AAg CAC gAA TCC AgC ACC	136 bp	U63372	This study
	Cry1A gene	Cry1A-F ggT TCC TTA Tgg CCg CTT Cry1A-R CAT gCC CTT TCA CgT TCC	645 bp (for Cry1Ac-Aa) and 567 bp (for Cry1Ab)	AF254640, AF059670, AY122057, M73248, M35524,	This study
	Cry1Ac gene	Cry1Ac-F CAg AAA AAg Cgg AAC ggT Ag Cry1Ac-R gAA TCg ggg TTA CAg AAg CA	415 bp	AF148644, AY122057, U87397, U87793, M11068	This study

*Reference sequences can be obtained from NCBI and EMBL GenBanks, 05.01.2004

(www.ncbi.nlm.nih.gov/Pubmed , www.ebi.ac.uk/index.html)(F, Forward; R, Reverse)

2.4 Amplification of target genes by Polymerase Chain Reaction (PCR)

All amplification reactions were performed in 30µl volume, using Techne Progene and Techne 412 thermocyclers. The DNA amount used in PCR amplifications were 5 ng for control PCR and 20 ng for screening and identification PCR's.

In order to obtain sensitive and specific detection, different cycling conditions like annealing temperatures, denaturation, annealing and extension times, cycle numbers were checked. PCR parameters including $MgCl_2$ and primer concentrations were optimized by performing the same PCR with variable concentrations of each parameters. PCR cycling conditions mentioned in below tables 2.2, 2.3, 2.4, 2.5, 2.6, 2.7 are all optimized parameters.

2.4.1 Control PCR

The amplification capacity of extracted tomato DNAs were checked by targeting the patatin gene. Primer set specific to patatin gene has been previously designed by Jaccaud *et al.* (2003) against the sequence NCBI Genbank accession no X03932 (Table 2.1).

PCR reaction mix components with following final concentrations were collected in a 200 µl sterile PCR tube: 1X PCR Buffer (MBI Fermentas), 1.5mM $MgCl_2$ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 20 pmol PatGc forward primer, 20 pmol PatGd reverse primer, 1 unit *Taq DNA Polymerase* enzyme, and ddH_2O up to 30 µl. Cycling conditions were mentioned in Table 2.2 for patatin control PCR.

Table 2.2; PCR conditions for patatin

	Temperature	Time
Initial denaturation	98°C	2 min
Denaturation	95°C	30 sec
Annealing	53°C	30 sec
Extension	72°C	40 sec
Number of cycles	35	
Final extention	72°C	3 min
	4°C	∞

2.4.2 Screening PCRs

Screening of genetically modified tomatoes targets three genes; 35S promoter, Nos terminator and NptII kanamycin resistance gene.

2.4.2.1 35S Promoter Specific PCR

Two different 35S promoter specific primer sets were used to screen genetically modified tomatoes. The first primer set 35S-F/35S-R were designed against the sequence EMBL GenBank accession no. CMV7626 and were expected to produce an amplicon of 264bp (Table 2.1).

The second primer set 35S-1-F/35S-2-R, that is commonly used in screening of many different kinds of genetically modified crops, produce an amplicon of 195 bp (Table 2.1).

PCR reaction mix components with following final concentrations were collected in a 200 µl sterile PCR tube: 1X PCR Buffer (MBI Fermentas), 1.5mM $MgCl_2$ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 1 unit *Taq DNA Polymerase* enzyme, for first primer set 60 pmol 35S forward primer, 60 pmol 35S reverse primer and for second primer set 30 pmol 35S-1 forward primer, 30 pmol 35S-2 reverse primer, PCR mix was adjusted to 30 µl with ddH_2O. Cycling conditions for 35S screening PCRs are as follows (Table 2.3);

Table 2.3; PCR conditions for 35S promoter

	Temperature	Time
Initial denaturation	94°C	2 min
Denaturation	94°C	45 sec(35SF/35SR), 30 sec(35S-1-F/35S-2-R)
Annealing	56°C	1 min
Extension	72°C	1 min
Number of cycles	40(35SF/35SR), 30(35S-1-F/35S-2-R)	
Final extention	72°C	10 min(35SF/35SR), 5 min(35S-1-F/35S-2-R)
	4°C	∞

2.4.2.2. Nos Terminator Specific PCR

The primer set, NOS1/NOS3, specifically targets the *Agrobacterium tumefaciens* Nos terminator region, produces an amplicon of 180 base pairs (bp) in length (Table 2.1).

PCR reaction mix components with following final concentrations were collected in a 200 µl sterile PCR tube: 1X PCR Buffer (MBI Fermentas), 3mM $MgCl_2$ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 60 pmol forward NOS1 primer, 60 pmol reverse NOS3 primer, 1 unit *Taq DNA Polymerase* enzyme, and ddH_2O up to 30 µl. PCR cycling conditions were illustrated in Table 2.4.

Table 2.4; PCR conditions for Nos terminator

	Temperature	Time
Initial denaturation	94°C	2 min
Denaturation	94°C	45 sec
Annealing	54°C	1 min
Extension	72°C	1 min
Number of cycles	30	
Final extention	72°C	5 min
	4°C	∞

2.4.2.3 NptII Resistance Gene Specific PCR

The primer set Kan-F/Kan-R was designed against the sequence NCBI GenBank accession no. AF274974 and were expected to produce an amplicon of 459 bp (Table 2.1).

PCR reaction mix components with following final concentrations were collected in a 200 µl sterile PCR tube: 1X PCR Buffer (MBI Fermentas), 1.5mM $MgCl_2$ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 30 pmol Kan-F primer, 30 pmol Kan-R

primer, 1 unit *Taq DNA Polymerase* enzyme, and ddH_2O up to 30 µl. PCR cycling conditions were demonstrated in Table 2.5.

Table 2.5; PCR conditions for NptII gene

	Temperature	Time
Initial denaturation	94°C	2 min
Denaturation	94°C	45 sec
Annealing	54°C	1 min
Extension	72°C	1 min
Number of cycles	40	
Final extention	72°C	10 min
	4°C	∞

2.4.2.3.1. Re-amplification of NptII Specific PCR Results & Sequencing

1 µl of positive NptII PCR product was re-amplified to obtain enough sample for automatic sequencing. PCR reaction was done at same conditions with KanF/KanR primer set. Re-amplified PCR product was custom sequenced with 5µmol KanR primer.

2.4.3 Identification PCRs (Gene specific target)

Three different primer sets were used for identification PCR experiments. The first primer set Cry-F/Cry-R was designed against the sequence NCBI GenBank accession no. U63372, to produce an amplicon of 136 bp (Table 2.1).

The second primer set Cry1A-F/Cry1A-R, that was designed against the sequences NCBI GenBank accession no. AF254640, AF059670, AY122057, M73248 and M35524. The expected amplicon length is 567 bp for Cry1Ab sequences and 645 for Cry1Ac sequences (Table 2.1) .

The third primer set Cry1Ac-F/Cry1Ac-R was designed against the sequences NCBI GenBank accession no. AF148644, AY122057, U87397, U87793 and M11068, it was expected to produce an amplicon of 415 bp for Cry1Ac (Table 2.1). The advantage of that primer pair is that, reverse part of the primer showed homology only to Cry1Ac sequences, providing more specificity (Figure 3.12).

PCR reaction mix components with following final concentrations were collected in a 200 µl sterile PCR tube: 1X PCR Buffer (MBI Fermentas), 1.5mM (for Cry primer set) and 3mM (for Cry1A and Cry1Ac primer sets) $MgCl_2$ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 50 pmol forward primer, 50 pmol reverse primer, 1 unit *Taq DNA Polymerase* enzyme, and ddH_2O up to 30 µl. Cycling conditions for identification PCRs were illustrated in Table 2.6.

Table 2.6; PCR conditions for Cry1A and Cry1Ac genes

	Temperature	Time
Initial denaturation	94°C	2 min
Denaturation	94°C	45 sec
Annealing	56°C (Cry primers) 53°C(Cry1A and Cry1Ac primers)	1 min
Extension	72°C	1 min
Number of cycles	40	
Final extention	72°C	10 min
	4°C	∞

2.4.3.1. Re-amplification of Cry1A and Cry1Ac Specific PCR Products & Sequencing

1 µl of positive Cry1A and Cry1Ac PCR products were re-amplified by taking the samples from gel with tips, in order to obtain enough sample for sequencing.

For re-amplification, PCR reaction mix components with following final concentrations were collected in a 200 µl sterile PCR tube: 1X PCR Buffer (MBI Fermentas), 1.5 mM (for Cry1A and Cry1Ac primer sets) $MgCl_2$ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 30 pmol forward primer, 30 pmol reverse primer, 1 unit *Taq DNA Polymerase* enzyme, and ddH_2O up to 70 µl. PCR cycling conditions were shown in Table 2.7.

Table 2.7; PCR conditions for re-amplification of Cry1A and Cry1Ac genes

	Temperature	Time
Initial denaturation	94°C	2 min
Denaturation	94°C	45 sec
Annealing	54°C	35 sec
Extension	72°C	1 min
Number of cycles	30	
Final extention	72°C	5 min
	4°C	∞

After the re-amplification step PCR products were directly sent to automatic sequencing with 5 µmol primer. In order to cope with the automatic sequencing

problems of Cry1A and Cry1Ac PCR products. We tried sequencing of these Cry1A and Cry1Ac amplicons after a cloning step. Re-amplified bands were loaded on 1.0% agarose gel. The bands corresponding to expected molecular weights were cut using a lancet and prepared for cloning.

2.5. Visualization on agarose gels

PCR products and restriction digestion results of λ DNA with PstI were analysed on 1.0% (NptII, Cry1A, Cry1Ac) or 2.0% (Patatin, 35S promoter, Nos terminator) agarose (Prona Basica Le) gels prepared and run in 1X TAE. PstI digested λ (lambda) DNA and 100 bp DNA Ladders (MBI Fermentas) were used as DNA markers. All gels were run at 75V (BioRad) and stained with ethidium bromide. PCR products were visualized under UV light.

2.5.1. Digestion of Lambda (λ) DNA with PstI

In order to prepare a suitable marker, λ bacteriophage DNA was digested with *Pst*I restriction endonuclease. The reaction was prepared by mixing the followings and incubated at 37°C for 3 hours: 50 µL λ DNA (0.3 mg DNA/ml, MBI Fermentas), 5 µL *Pst*I enzyme (10 unit/µl, MBI Fermentas), 7 µL O^+ Buffer with BSA and 8 µL dd H_2O.

Fragment sizes of the λ/*Pst*I digestion are as follows (in base pairs):
11501, 5077, 4749, 4507, 2838, 2556, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, 216, 211, 200, 164, 150, 94, 87, 72, 15.

2.6. Cloning of Cry1A and Cry1Ac PCR products

Fermentas InsT/Aclone™ PCR product cloning kit was used to clone 645 bp Cry1A and 415 bp Cry1Ac PCR fragments.

2.6.1. Ligation of re-amplified fragments to pTZ57R/T plasmid vector

DNA bands that were cut from agarose gel were extracted from agarose by sequential freezing and thawing in liquid nitrogen. 6.5 µl of extracted DNA was directly ligated to pTZ57R/T plasmid vector with a final volume of 10 µl in a PCR tube. Following components were combined; 6.5 µl extracted DNA, 1 µl plasmid vector pTZ57R/T, 1 µl 10X Ligase Buffer, 0.5 µl T_4 DNA Ligase enzyme (5u/µl) and 1 µl PEG 4000 solution. Reaction mixture was incubated overnight at 22°C (approximately 18 hours).

2.6.2. Preparation of E.coli competent cells

A single colony of *E.coli* Dh5-α cells was inoculated into 2 ml TransformAid™ C-Medium. Cells were incubated at 37°C with moderate shaking at 250 rpm for overnight. 1/10 volume of overnight grown culture was added to pre-warmed TransformAid™ C-Medium and the tubes were incubated in a shaker at 37°C for 20 min.

2.6.3. Transformation of E.coli competent cells with ligation products

TransformAid™ T-Solution was prepared by mixing equal volumes of T-Solution (A) and T-Solution (B), the prepared T-Solution was kept on ice. 1.5 ml of fresh culture was dispensed into a microcentrifuge tube and centrifuged at maximum speed for 1 min at room temperature. The supernatant was discarded and the pelleted cells were resuspended in 300 µl of TransformAid™ T-Solution, the tubes were incubated on ice for 5 min. After centrifugation for 1 min at room temperature, supernatant was removed. The cells were resuspended in 120 µl of TransformAid™ T-Solution and the tubes were incubated on ice for 5 min. 2.5 µl ligation product was transferred to a new microcentrifuge tube and kept on ice for 2 min. After 2 min incubation on ice, 100 µl resuspended cells were added to microcentrifuge tube containing the ligation products and the mixture was incubated on ice for 5 min. 100 µl of mixture was spread on pre-warmed LB-Ampicillin agar plates. Plates were incubated at 37°C overnight. After the incubation, white colonies were selected among grown colonies and these colonies were transferred to 300 µl LB medium containing sterile 1.5 ml tubes. Selected colonies were PCR amplified.

2.6.4. PCR amplification of colonies

1X PCR Buffer (MBI Fermentas), 0.2 mM dNTP (MBI Fermentas), 0.8 pmol M13 Forward and Reverse primers (TIB Molecular Biology), 1.2 mM $MgCl_2$ (MBI Fermentas), 1 unit *Taq DNA Polymerase* (MBI Fermentas), 1 µl of selected colonies

and sterile PCR water up to 25 µl final volume combined in a sterile PCR tube. Amplification reaction was performed at conditions mentioned in Table 2.8.

Table 2.8; PCR conditions for M13 primers

	Temperature	Time
Initial denaturation	94°C	2 min
Denaturation	94°C	30 sec
Annealing	55°C	30 sec
Extension	72°C	1 min
Number of cycles	30	
Final extention	72°C	3 min
	4°C	∞

PCR products were run on 1.0% agarose gel to detect positive and false positive colonies. Insert containing recombinant cells were selected and used in plasmid isolation step.

2.6.5. Plasmid isolation from colonies

Plasmids were isolated using QIAGEN QIAprep Spin Miniprep Kit according to the manufacturer's protocol.

2 ml of overnight grown bacterial culture was centrifuged for 10 min at 15,000 rpm. Supernatant was poured off, tube was inverted and blotted on a paper towel to remove the excess media. 250 µl of Buffer P1 was added to tube and cell pellet was

completely resuspended by vortexing. After adding 250 µl of Buffer P2, the tube was mixed by gently inverting four times, incubated at room temperature for maximum 5 min. N3 solution of 350 µl was added and mixed by inverting 4 times. Sample tubes were centrifuged at 15,000 rpm for 10 min at room temperature. A spin column, provided by the manufacturer, inserted into collection tube. Cleared lysate, the upper phase was transferred to the spin column and centrifuged at 15,000 rpm for 1 min at room temperature.

After the centrifugation, lower phase in the collection tube was discarded and collection tube re-inserted. 500 µl of Buffer PB was added and centrifuged at 15,000 rpm for 1 min. The collection tube was emptied and the spin column was re-inserted.

Column Washing solution Buffer PE of 750 µl was added to spin column, centrifuged at 15,000 rpm for 1 min at room temperature, flowthrough discarded and the collection tube re-inserted again and to remove the residual wash buffer completely additional 1 min centrifugation was involved. The contents of spin column was transferred to a new sterile 1.5 ml tube, the plasmid DNA was eluted by adding 50µl of Buffer EB and centrifugating at 15,000 rpm for 1 min. Spin column assembly was removed and harvest stored at -20°C.

2.6.6. Determination of DNA integration into the plasmid

The integration of DNA into the plasmid and the size of the insert was evaluated by double digestion of plasmid DNA by *Eco*RI and PstI restriction enzymes. 7 µl purified plasmid, 1 µl NE 3 Buffer (New England Biolabs), 1 µl *Eco*RI enzyme (New England Biolabs) and 1 µl PstI enyzme (New England Biolabs) were combined in a PCR tube with a final volume of 10 µl. Mixture was incubated at 37°C for overnight. After the incubation, samples were run on 1.0% agarose gel.

2.7 Sequencing reaction

The plasmids were automatic sequenced by using T7 primers (Fermentas, 14.09.2004, http://www.fermentas.com/profiles/kits/pdf/instaclone1214.pdf)

2.8 Sequence and Homology Analysis

DNA sequencing results were both compared with the target sequences that were used for primer design, and were analysed with the BLAST programme in order to control the sequence homology (BLAST sequence analysis programme, 23.09.2004, http://www.ncbi.nlm.nih.gov/BLAST/).

Additionally the Cry1A, Cry1Ac forward and the reverse primer pairs were checked for their specificity by BLAST analysis.

CHAPTER III

RESULTS AND DISCUSSION

3.1 DNA isolation for GMO detection analysis

Quality and purity of nucleic acids are some of the most critical factors in meaningful PCR analysis. Therefore, extraction of pure and high quality DNA is necessary for our experiments. In this study cetyltrimethylammonium-bromide (CTAB) method which had been first developed by Murray and Thompson in 1980 (Querci *et al.,* 2004), was used. CTAB method has been known as a suitable protocol for the elimination of polysaccharides and polyphenolic compounds.

Like many other plant species, tomato tissues contain high levels of polyphenolic compounds. When cells are disrupted, these compounds can come into contact with nuclei and other organelles. In their oxidized forms, polyphenols covalently bind to DNA giving it a brown color and making it useless for most research applications (Peterson *et al.,* 1997). At the beginning of this thesis study, contamination of extracted DNAs with polyphenolic compounds, caused inhibition of PCR amplification reactions. In order to increase the yield, CTAB protocol was modified by adding 1% PVP (Doyle & Doyle, 1990).

According to the type of starting material, two kinds of CTAB isolation protocol from fresh tissue and tomato seeds were used, the extracted DNA samples were checked with spectrophotometer. DNA concentrations and obtained A_{260}/ A_{280} values were summarized in Table 3.1. The reason for differences in concentration values was probably due to handling during sample homogenization with liquid nitrogen. A_{260}/ A_{280} value below 1.6, shown in samples 21 and 23, indicates a contamination with proteins and aromatic substances (Pauli *et al.,* 2000), these two samples and sample 25 that exhibited a critical A_{260}/ A_{280} value were checked with all our DNAs in patatin PCR to control their amplification capacity before elimination.

Table 3.1; Concentration of samples

Sample No:	Sample ID:	Conc: Ng/µl	WL:260	WL:280	260/280
1	Ankara (Eryaman)	4,5	0,090	0,059	1,847
2	Ankara (Etimesgut)	5,5	0,110	0,073	1,716
3	Ankara (Yıldız)	28,1	0,562	0,439	1,777
4	Ankara (Yıldız)	4,4	0,118	0,075	1,772
5	Ankara (Armada)	5,5	0,073	0,055	1,922
6	Ankara (Etimesgut)	4,0	0,027	0,021	1,862
7	Ankara (Çankaya)	9,4	0,251	0,186	1,654
8	Ankara (Bilkent)	9,7	0,194	0,150	1,725
9	Ankara (Yıldız)	9,5	0,190	0,142	1,702
10	Spain 1	4,5	0,090	0,059	1,847
11	Spain 2	6,7	0,135	0,100	1,638
12	Belgium 1	7,5	0,075	0,056	1,882
13	Belgium 2	5,7	0,153	0,111	1,806
14	Çanakkale	5,5	0,073	0,055	1,922
15	Ayaş	4,0	0,027	0,021	1,862
16	Eskişehir	8,5	0,085	0,061	1,789
17	Isparta	15,1	0,151	0,103	1,705
18	Antalya	8,1	0,081	0,054	1,790
19	Afyon	12,2	0,122	0,085	1,789
20	China 1	32,2	0,129	0,110	1,620
21	China 2	4,0	0,080	0,076	1,222
22	Antalya-Kalkan 1	12,7	0,254	0,184	1,767
23	Antalya-Kalkan 2	7,8	0,052	0,048	1,567
24	Antalya-Kalkan 3	19,2	0,128	0,115	1,696
25	Antalya-Kalkan 4	3,5	0,069	0,049	2
26	USA 1	18,8	0,094	0,069	1,795
27	USA 2	20,8	0,139	0,105	1,642
28	Mersin 1	7,5	0,150	0,109	1,717
29	Mersin 2	6,2	0,165	0,103	1,784
PC	Positive Control (Italy)	Not examined			

3.2 PCR Amplifications

Eight different PCR systems were performed with each sample, one to evaluate the quality of the DNA and at the same time to check the presence of PCR reaction inhibitors. The four PCR systems were developed for the detection of screening targets commonly used in genetically modified crops. And the other three PCR systems were performed to identify the insect resistant genetically modified tomatoes by targeting Cry genes (Table 2.1).

3.2.1 Patatin Specific Control PCR

In order to avoid the arising of false negative result due to the presence of PCR inhibitors like proteins, polysaccharides and polyphenolic compounds, it is highly recommended to perform a control experiment to test PCR inhibitors.

To assess the detection of potato DNA, a nested PCR system has been designed for patatin gene by Jaccaud *et al.* (2003). Patatin is a major storage protein gene in potato tubers encoded by a multigene family. Studies of Jaccaud *et al.* (2003) have shown that potato, tomato and tobacco DNA' s generated a 124 bp amplified fragment (Appendix A), whereas no fragments were amplified from soya, maize, and rapeseed samples. Therefore, these primers have been found to be specific for Solanacea (Jaccaud *et al.*, 2003), the botanical family to which tomato, tobacco, and potato are belonging. The PCR with the primers Pat-F/Pat-R determines if amplifiable tomato DNA is present in the sample. All DNAs extracted from collected tomato samples were tested with this PCR system. Except the sample 21 and the

maize DNA with lane designation C which was used as negative control, all other samples showed a very intense 124 bp amplicon that indicates their amplification capacity for further detection analysis (Figure 3.1). The sample 21 which did not produce 124 bp amplicon, was eliminated. The A_{260}/ A_{280} value obtained from spectrophotometric measurements also indicates protein and aromatic substance contamination of sample 21 DNA. By detecting the 124 bp fragment it was observed that extracted DNA samples have the required amplification capacity.

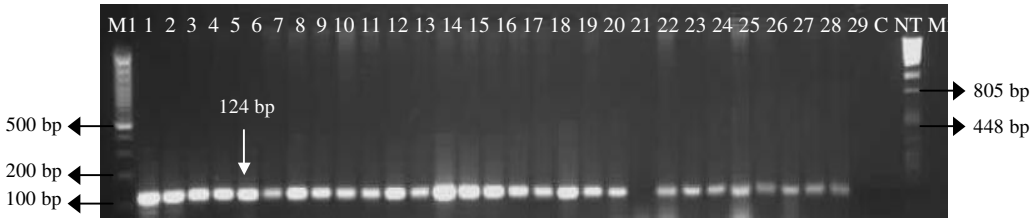


Figure 3.1; Patatin specific PCR results: lineM1;100bp molecular weigth marker, line 1-29 samples, line C; negative control maize DNA, line NT; no template, line M2; pstI digested lambda (λ) DNA

3.2.2 Screening PCR systems for the detection of genetically modified tomatoes

By using these PCR systems, it was aimed to detect three screening targets that are commonly used in GMO detection. In detection studies, such methods are accepted only as an indication of presence of a genetic modification, and the presence of such targets should be further assessed by specific PCR detection methods.

The antibiotic resistance gene *NptII* derived from *Escherichia coli* (Bruderer & Leitner, 2003), and regulatory elements such as the *Nos* terminator from *Agrobacterium tumefaciens* (Lin *et al.,* 2000), and the 35S promoter from the

cauliflower mosaic virus (Lin *et al.,* 2000) have been used for general screening of genetically modified crops. However, according to the USDA/APHIS decision documents, it has been stated that, p35S CaMV was not used in all of the transgenic lines that were developed (Smith *et al.,* 2004). Therefore, it should be notified that these targets are not necessarily be the main targets for detection of all GM plants.

3.2.2.1 35S Promoter spesific PCR system

One of the most important factors for achieving the desired expression levels of a transgene is the choice of the promoter that regulates transcription of the transgene. Many of the approved transgenic crops contain a copy of the constitutive 35S promoter (P-35S) from the CaMV or one of the derivatives of this promoter like the enhanced and duplicated 35S promoter regions. The P-35S has been widely used in the screening detection methods. At total 56% of genetically modified crops contains CaMV35S promoter which provides constitutive expression of inserted gene of interest (Bruderer & Leitner, 2003). A comparison of P-35S sequences available from public sources shows that they are not identical and there are different sequence mutants of P-35S fragments in different GM crops (Bruderer & Leitner, 2003).

Therefore, two different 35S promoter specific primer sets were used in this study; The first primer set 35S-F/35S-R was designed against the 35S promoter sequence with an EMBL GenBank accession no. CMV7626. It was not observed any band both in unknown samples and in nematode resistant tobacco DNA that was used as positive control (data not shown), resulting from the non-homology between the

sequences CMV7626 and common 35S promoter region of Cauliflower mosaic virus AF078810 (Appendix B).

In order to solve this non-homology problem, a new validated primer set 35S-1/35S-2 that allows the amplification of a 195 bp fragment from the 35S promoter of the cauliflower mosaic virus (P-35S) was used. In this pcr system amplification signal was observed only in the positive control genetically modified tomato (Figure 3.2).

Figure 3.2; 35S promoter (AF078810) specific PCR results with 35S-1/35S-2 primer set, line M1; 100bp molecular weight marker, line PC; positive control; line NT; no template, line 1-29; samples and line M2; pstI digested lambda (λ) DNA

3.2.2.2 NOS Terminator specific PCR system

The Nos terminator (T-Nos), isolated from the nopaline synthase gene of *Agrobacterium tumefaciens* is commoly used in transgenic crops for the termination of transcript of trait genes. 37% of approved genetically modified crops contain *A. tumefaciens* Nos terminator region (Bruderer & Leitner, 2003).

The validated primer set Nos-1/Nos-3 allows the amplification of a 180 bp fragment (Appendix C). In our 28 unknown tomato samples, it was not observed any band

indicating the presence of Nos terminator region, the only amplification signal was from the positive control genetically modified tomato (Figure 3.3).

Figure 3.3; Nos terminator (U12540) specific PCR results with Nos-1/Nos-3 primer set, line M1; 100bp molecular weight marker, line PC; positive control; line NT; no template, line 1-29; samples and line M2; pstI digested lambda (λ) DNA

3.2.2.3 NptII gene specific PCR system

More than 40 distinct genes have been used for generation of currently approved transgenic crops. The most frequently used transgene is NptII, originating from the *E.coli* transposon 5. NptII gene is used as a marker gene in the construction of transgenic plants, the expression of this gene confers resistance to the antibiotic kanamycin. The purpose of inserting the NptII gene into crops with any other transgene is to have an effective method for indicating the success of genetic modification.

In screening of genetically modified tomatoes, detecting the NptII gene has an extraordinary importance. Nearly all approved genetically modified tomato lines including 1345-4, 35 1 N, 5345, 8338, B-Da-F and Flavr Savr contains NptII kanamycin resistance gene (Bruderer & Leitner, 2003)(Table 3.4). Although the three regions; 35S promoter, Nos terminator and NptII are the most widely used

screening targets, kanamycin resistance gene provides the most general detection for tomato among those regions. It gives the opportunity to screen different GM tomatoes with different modified characteristics like delayed ripening and insect resistance.

Therefore, the tomato samples were tested for the presence of NptII gene by means of the primer pair Kan-F/Kan-R which generates an amplicon of 459 bp in length (Appendix D). 22 samples (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 18, 19, 24, 25, 26, 28 and 29) out of 28 produced the expected 459 bp amplification signal (Figure 3.4).

Figure 3.4; NptII specific (AF274974) PCR results with Kan-F/Kan-R primer set, line M1; 100bp molecular weight marker, line PC; positive control tomato sample; line NT; no template, line 1-29; samples and line M2; pstI digested lambda (λ) DNA

In order to confirm the results of NptII PCR products, we sent 60µl of PCR product to automatic sequencing. After DNA purification from agarose gel, band was sequenced with 5µmol primer by IONTEK (Istanbul/Turkey). The result of the sequence analysis was compared with the targeted sequence AF274974 by ClustalW programme, obtained data indicated a 93% homology (Figure 3.5).

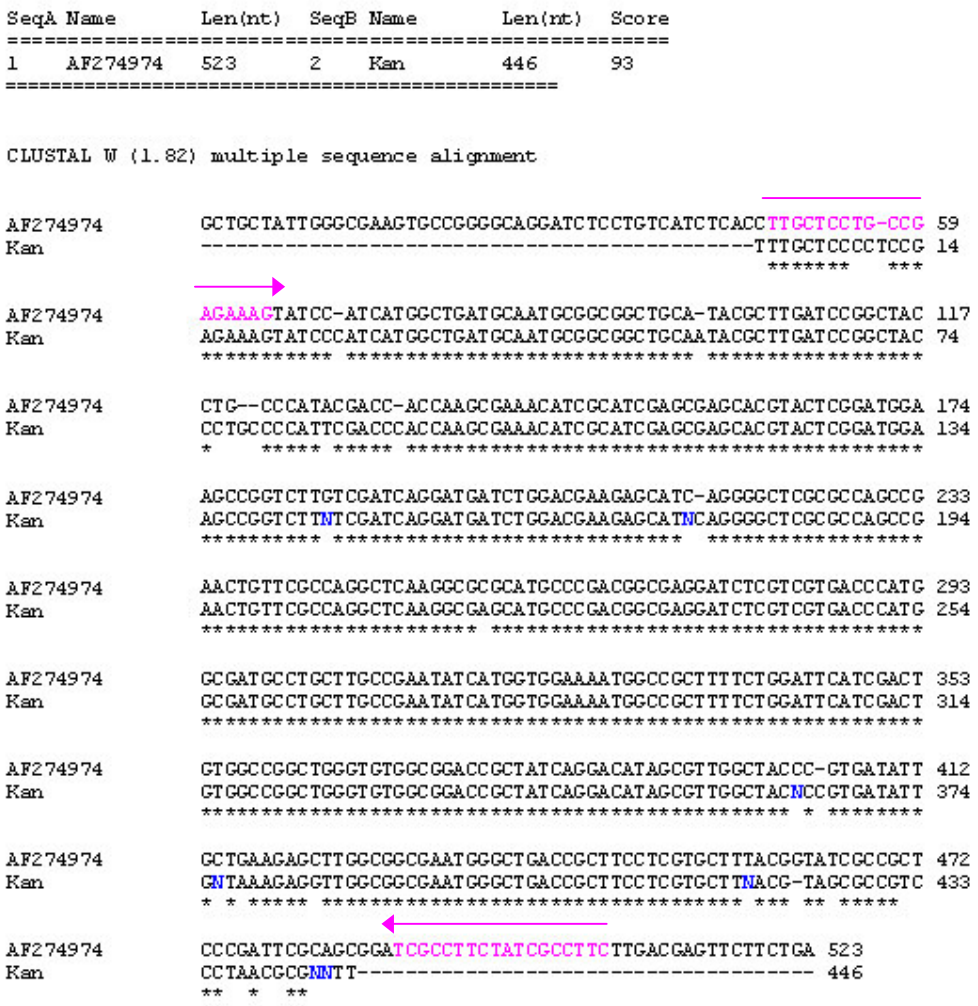


Figure 3.5; ClustalW analysis result of the obtained NptII sequence and the target sequence AF274974 (*, indicates the homologous base pairs)

Nucleotide and amino acid sequence alignments of sequenced NptII result was checked with nucleotide-nucleotide and protein-protein BLAST programmes in order to identify this sequence, obtained results exhibited high homology with NptII DNA and protein sequences found in GenBank database.

3.2.3 Identification PCR systems specific to genetically modified insect resistant tomatoes (Gene specific target system)

The variants of δ endotoxin gene from *Bacillus thuringiensis* are most frequently used genes in transgenic crops after NptII. The Cry genes, used in GM crops are all synthetic and modified, in some cases truncated forms of native genes are used, in order to optimise gene expression in the host organism. For the expression of Bt gene in the higher plants, a recognisable promoter and a terminator sequence must bracket the Bt gene. Beside the most popular constitutive promoters; 35S and ubiquitin promoters, there are some other promoter regions like tissue specific promoters; PEPC (phosphoenolpyruvate carboxylase) and maize pollen specific promoter (Sharma *et al.,* 2000, Bruderer & Leitner*,* 2003) are widely used in GM crops.

There are some studies about insect resistant genetically modified tomatoes containing Cry1Ab and Cry1Ac genes in literature (Fischhoff *et al.,* (1987), Delannay *et al.,* (1989), Kumar & Kumar, (2004), Mandokar *et al.,* (2000)) but Monsanto's transgenic line 5345 is the one that has an approval from FDA in USA (Bruderer & Leitner*,* 2003).

At that point, in order to identify insect resistant GM tomato, it was aimed to detect Cry target gene. For this purpose, a primer pair was designed against the sequence U63372, which provides an amplicon of 136 bp in length. But, it was not observed

any result by using Cry-F/Cry-R primer set. The defectiveness of Cry-F/Cry-R primer set may be; the targeted Cry gene sequence U63372 which exhibits no homology with other Cry gene sequences, therefore primer pair could not detect that region (Appendix E).

As a second step by comparing the Cry1Ab and Cry1Ac sequences with ClustalW homology search programme, a new pair of primer was designed that is specific to conserved regions of Cry1 Ab and Cry 1Ac sequences. AF254640 and AF059670

Cry1Ab sequences; U89872, AF148644, M35524, M73248 and AY122057 Cry1Ac sequences; and U87397, U87793, M11068 Cry1Ac sequences that are specific to *Bacillus thuringiensis* subsp. *kurstaki* HD73 strain were found from EMBL, NCBI GenBanks. Primer pair Cry1A-F/Cry1A-R was designed targeting the homologous regions of this ten sequences. In primer design studies, it was observed that Cry1A-F/Cry1A-R primer set should produce 567 bp amplicon with Cry1Ab sequence and 645 bp amplicon with Cry1Ac sequences (Appendix F). The reason for non-homologous regions which cause the formation of Cry1Ab and Cry1Ac genes, is the insertion and deletions that occur spontaneously during the period of evolution.

In our 28 tomato sample, 17 of them produced 645 bp positive amplification signals. Expected PCR amplification signals were observed in samples 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19 and 24.

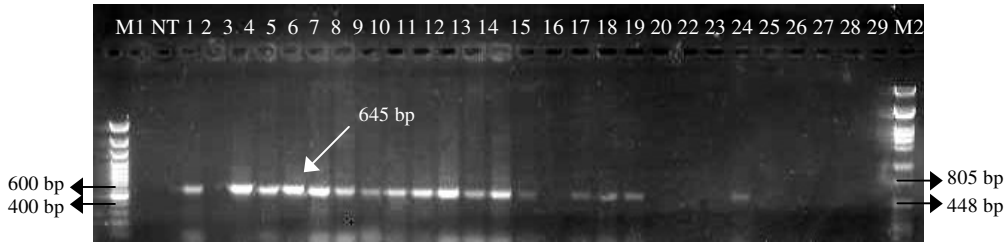


Figure 3.6; Cry1A specific PCR results with Cry1A-F/Cry1A-R primer set, line M1; 100bp molecular weight marker, line NT; no template, line 1-29; samples and line M2 pstI digested lambda (λ) DNA

In order to confirm the results of Cry1A PCR products, firstly 60µl of PCR product was sent for automatic sequencing. After DNA purification from agarose gel, bands were sequenced with 5µmol primer by IONTEK (Istanbul/Turkey). Arising from the quality of bands, the expected result from sequencing was not obtained. Sequencing trials of Cry1A PCR products were repeated for five times by using both reverse and forward primers and the obtained results were compared with the targeted sequence by ClustalW programme (Figure 3.7, 3.8, 3.9, 3.10, 3.11). Additionally the obtained results were compared with each other (Appendix G). The results of this comparison exhibited few homology among sequencing results, indicating a sequencing problem that might arise from primers.

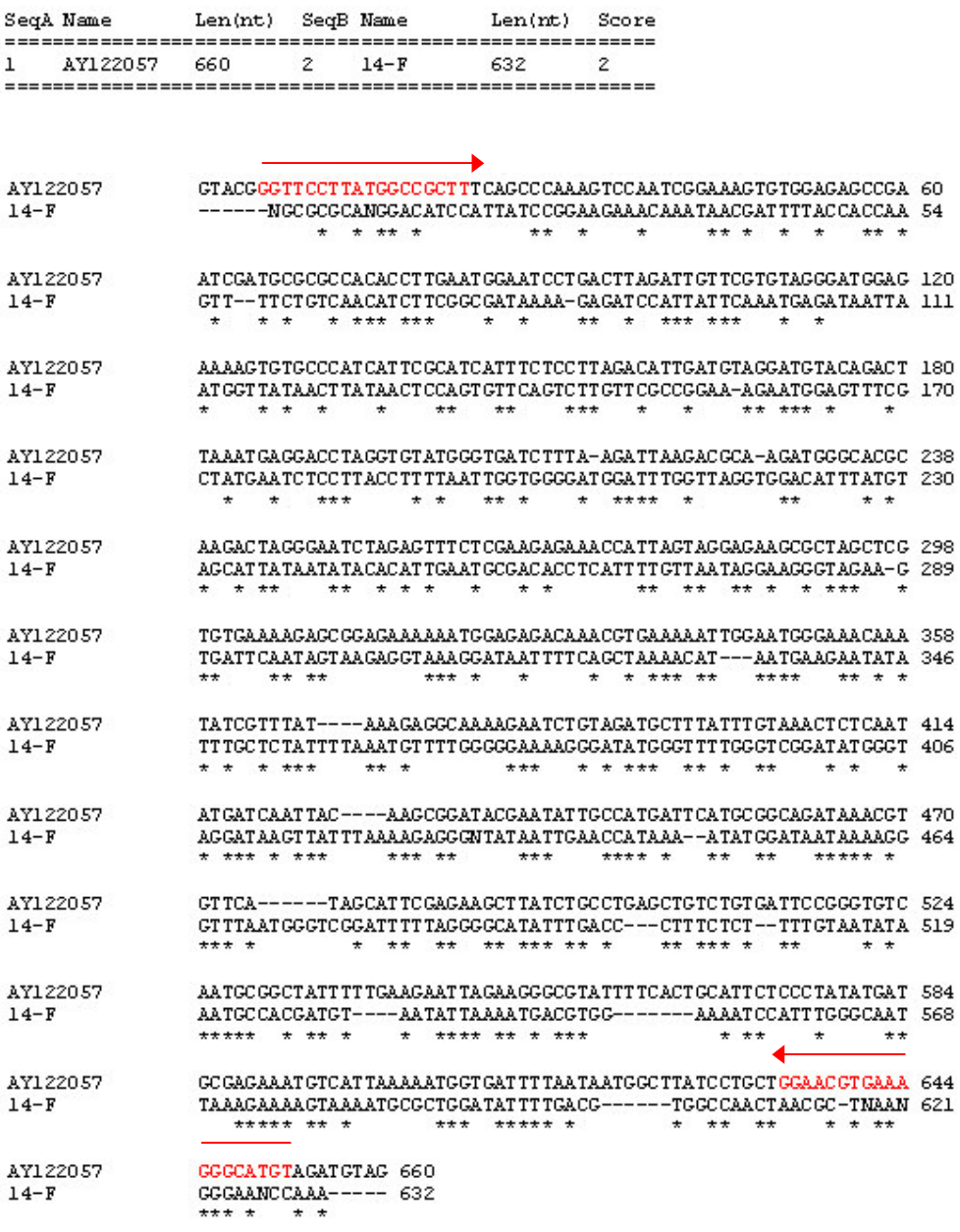
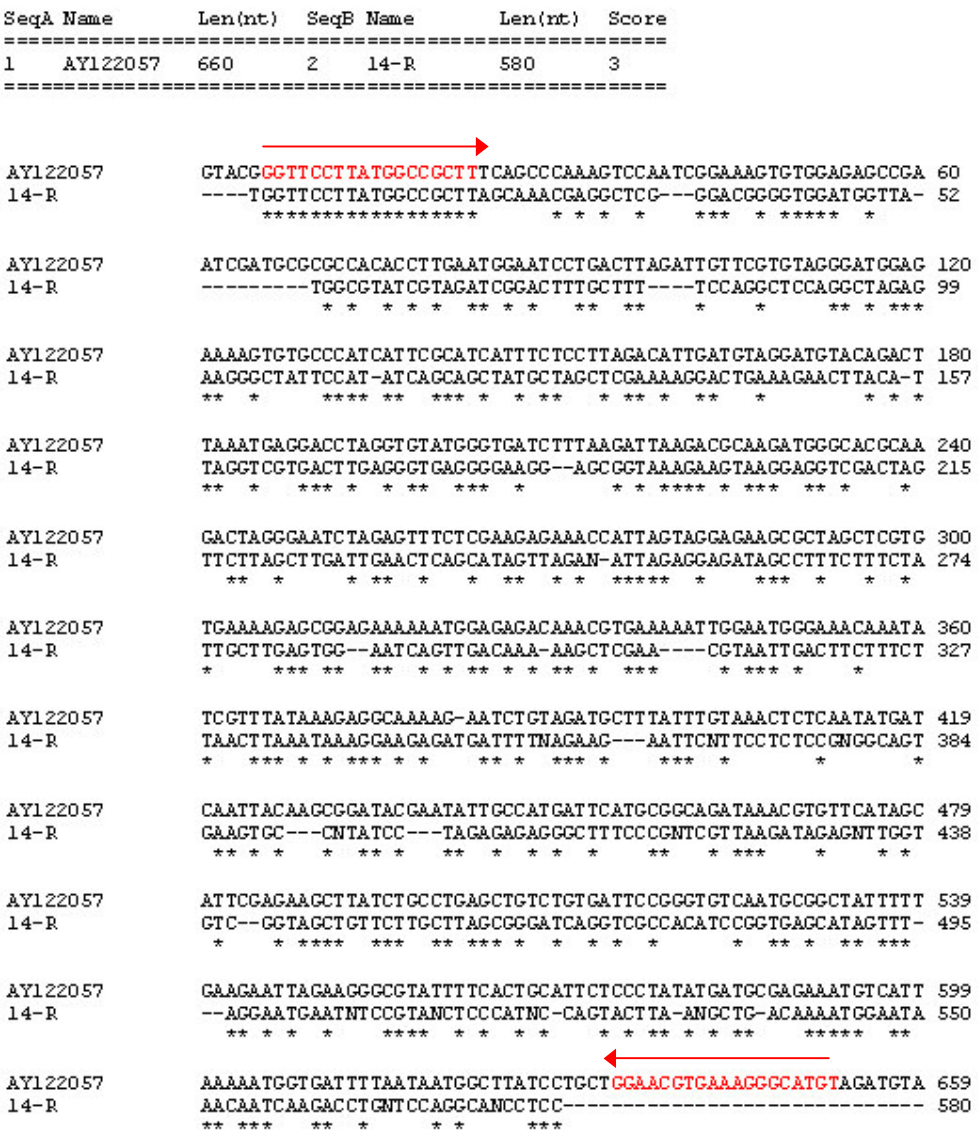


Figure 3.7; Sequence analysis results of sample 14 with Cry1A forward primer (*, indicates the homologous base pairs)



Fgure 3.8; Sequence analysis results of sample 14 with Cry1A reverse primer (*, indicates the homologous base pairs)

Figure 3.9; Sequence analysis results of sample 17 with Cry1A forward primer (*, indicates the homologous base pairs)

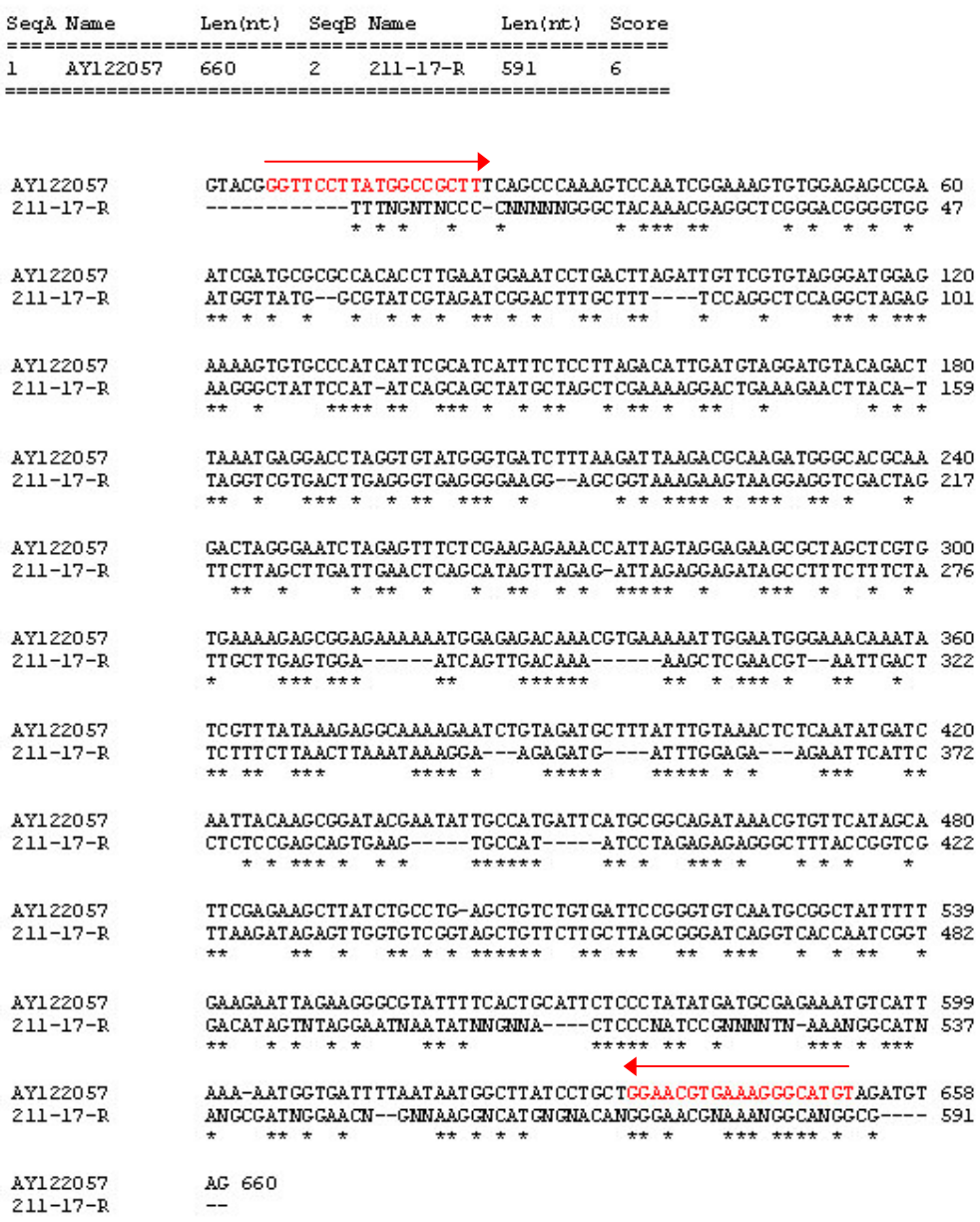


Figure 3.10; Sequence analysis results of sample 17 with Cry1A reverse primer (*, indicates the homologous base pairs)

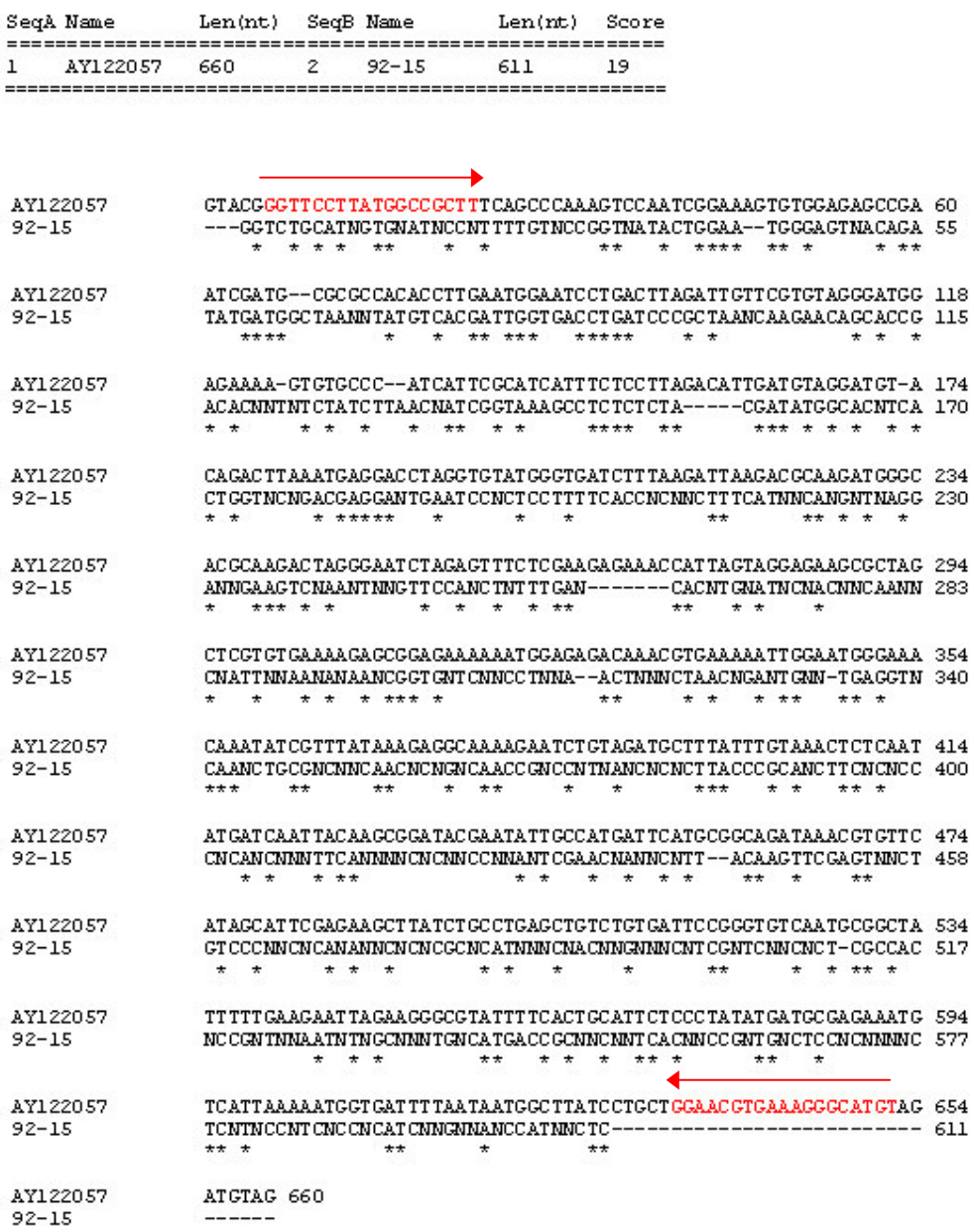


Figure 3.11; Sequence analysis results of sample 15 with Cry1A reverse primer (*, indicates the homologous base pairs)

In order to be ensure about specificity of primers to Cry1Ab and Cry1Ac genes, the forward and reverse primers were analysed by BLAST programme. The result of the analysis, summarized in the Table 3.2 unexpectedly showed that, Cry1A-F/Cry1A-R primer set was complementary to a wide range of Cry genes giving either 567 or 645 bp amplicons. Although this primer set was specific to cry genes, all the 645 bp products are not necessarily Cry1Ac genes. In addition, it was shown that some cry genes like Cry1Aa also produces an amplicon of 645 bp.

Table 3.2; Homology search of Cry1A forward and reverse primers

Cry1A-F ggT TCC TTA Tgg CCg CTT/ Cry1A-R CAT gCC CTT TCA CgT TCC			
Sequence accession no. & name	**From**	**To**	**Length**
AF288683 Bacillus thuringiensis delta-endotoxin Cry1 gene	2544	3189	645 bp
AF384211 Bacillus thuringiensis strain ly30 delta-endotoxin (cry1Aa) gene	2338	2983	645 bp
AF375608 Bacillus thuringiensis Cry1Ab16 (cry1Ab16) gene	2503	3070	567 bp
AY319967 Bacillus thuringiensis cry1A toxin gene	2341	2986	645 bp
AY122057 Bacillus thuringiensis insecticidal crystal protein Cry1Ac (cry1Ac)gene	2466	3111	645 bp
AY646166 Bacillus thuringiensis parasporal crystal protein gene	2341	2911	570 bp
AY225453 Bacillus thuringiensis Cry1Ac (cry1Ac) gene	2344	2989	645 bp
AF059670 Bacillus thuringiensis subsp. kurstaki plasmid pUCBtS93 crystal protein (cry1Ab) gene	2381	2948	567 bp
X56144 B. thuringiensis cryI gene for insecticidal crystal protein	2320	2965	645 bp
X53985 Bacillus thuringiensis BtX I gene for crystal protein	2449	3094	645 bp
Y09663 B.thuringiensis mRNA for delta-endotoxin	2418	3063	645 bp
AJ002514 Bacillus thuringiensis kurstaki cry218 gene	2731	3376	645 bp
Z22511 B.thuringiensis encoding crystal protein	2533	3178	645 bp
Z22510 B.thuringiensis encoding crystal protein	2362	3007	645 bp
Y09326 B.thuringiensis cry1M gene	2993	3638	645 bp
AJ130970 Bacillus thuringiensis gene encoding crystal toxin protein	2499	3144	645 bp
X13535 Bacillus thuringiensis cryA gene for parasporal crystal toxin	2338	2983	645 bp
X54939 B. thuringiensis cryIA(b) gene for insecticidal crystal protein	2413	2980	567 bp
AF358861 Bacillus thuringiensis crystal endotoxin Cry1Ab (cry1Ab) gene	3825	4392	567 bp
AF254640 Bacillus thuringiensis insecticidal protein P (cry1Ab) gene	2979	3546	567 bp
AF202531 Bacillus thuringiensis Cry032 (cry032) gene	2707	3352	645 bp
AB026261 Bacillus thuringiensis gene for BtT84A1 crystal protein	2490	3135	645 bp
D00348 Bacillus thuringiensis serovar aizawai gene	2410	3055	645 bp

continued

AF510713 Bacillus thuringiensis serovar sotto crystal protein Cry1Aa13 gene	2365	3010	645 bp
AF492767 Bacillus thuringiensis Cry1Ac gene	2341	2986	645 bp
AF154676 Bacillus thuringiensis subsp. kurstaki 135 kDa insecticidal protein gene	2338	2983	645 bp
U70725 Bacillus thuringiensis wuhanensis insecticidal crystal protein CryH2 (cry1Gb1) gene	2836	3481	645 bp
U94323 Bacillus thuringiensis protoxin Cry1Ea4 (cry1Ea4) gene	2707	3352	645 bp
AF358862 Bacillus thuringiensis cry1Db operon	4852	5497	645 bp
AF327926 Bacillus thuringiensis subsp. kunthalaRX27 insecticidal crystal protein BTRX27 gene	2496	3063	567 bp
AF327925 Bacillus thuringiensis subsp. kunthalaRX28 insecticidal crystal protein BTRX28 gene	2496	3063	567 bp
AF327924 Bacillus thuringiensis subsp. kunthalaRX24 insecticidal crystal protein BTRX24 gene	2496	3063	567 bp
U87397 Bacillus thuringiensis kurstaki crystal protein (CryIA(c)) gene	2496	3141	645 bp
U89872 Bacillus thuringiensis Cry1Ac delta-endotoxin gene	2731	3376	645 bp
U87793 Bacillus thuringiensis kurstaki insecticidal delta-endotoxin CryIA(c)gene	3319	3964	645 bp
U94191 Bacillus thuringiensis delta endotoxin gene	2413	2980	567 bp
U35780 Bacillus thuringiensis crystal toxin gene	3032	3677	645 bp
M73249 Bacillus thuringiensis gene	2344	2989	645 bp
M35524 B. thuringiensis delta-endotoxin gene	2580	3225	645 bp
M73248 Bacillus thuringiensis (cryIAc3) gene	2342	2987	645 bp
D17518 Bacillus thuringiensis cryIA(a) gene for insecticidal crystal protein	2418	3063	645 bp
D00117 Bacillus thuringiensis gene for delta-endotoxin	2503	3070	567 bp

Cry1Ac protein expressed in transgenic tomato lines exhibit highly selective insecticidal activity against a narrow range of lepidopteran pests. Insect resistant tomato line 5345 was developed by the insertion of the Cry1Ac gene from the soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (B.t.k.) strain HD73.

Focusing on the Monsanto's insect resistant tomato line 5345, a primer pair was designed specific to Cry1Ac gene by comparing the U89872, AF148644, M35524, M73248 and AY122057 Cry1Ac sequences and U87397, U87793, M11068 sequences that are specific to B.t.k HD73 strain (Appendix F). Sequences were found from EMBL, NCBI GenBanks. The ClustalW analysis of Cry1Ab, Cry1Ac and Cry1Ac-F/Cry1Ac-R primers indicated that the reverse primer showed homology only to the Cry1Ac sequences (Figure 3.12), providing a specific detection of Cry1Ac sequences including the B.t.k. HD73 strain.

```
U87397          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1637
89872U          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 2024
148644AF        ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1634
35524M          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1872
73248M          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1634
122057AY        ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1759
U87793          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1637
M11068          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1637
254640AF        ATCGGGTAAGAATTCGCTACGCTTCTACCACAAATTTACAATTCCATACATCAATTGACG 2265
059670AF        ATCGGGTAAGAATTCGCTACGCTTCTACCACAAATTTACAATTCCATACATCAATTGACG 1667
                **** **  *  * ** ** ******   **     * **  ** *  * * *****  *
```

Figure 3.12; Specificity of Cry1Ac reverse primer (*, indicates the homologous base pairs)

In Cry1Ac PCR results, beside the expected 415 bp amplicon, a second 197 bp unexpected fragment was observed in samples 2, 5, 6, 7, 8, 9, 15, 17, 18 and 19. In order to evalute the Cry1Ac PCR results, it is necessary to sequence these fragments.

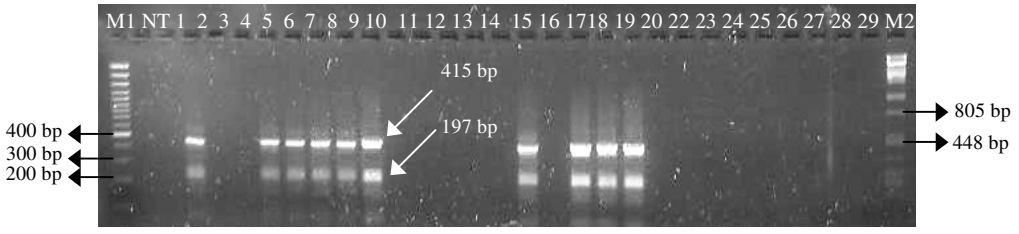


Figure 3.13; Cry1Ac specific PCR results with Cry1Ac-F/Cry1Ac-R primer set, line M1; 100bp molecular weight marker, line NT; no template, line 1-29; samples and line M2 pstI digested lambda (λ) DNA

The unexpected 197 bp fragment was taken from the gel by using tips and re-amplified. 60µl of obtained PCR product was sent to IONTEK (Istanbul/TURKEY) for automatic sequencing. As in the case of Cry1A PCR, the obtained result was not satisfactory with 41% homology, resulting from the quality of bands. The 197 bp resultant fragment contains 42 unidentified bp's N's.

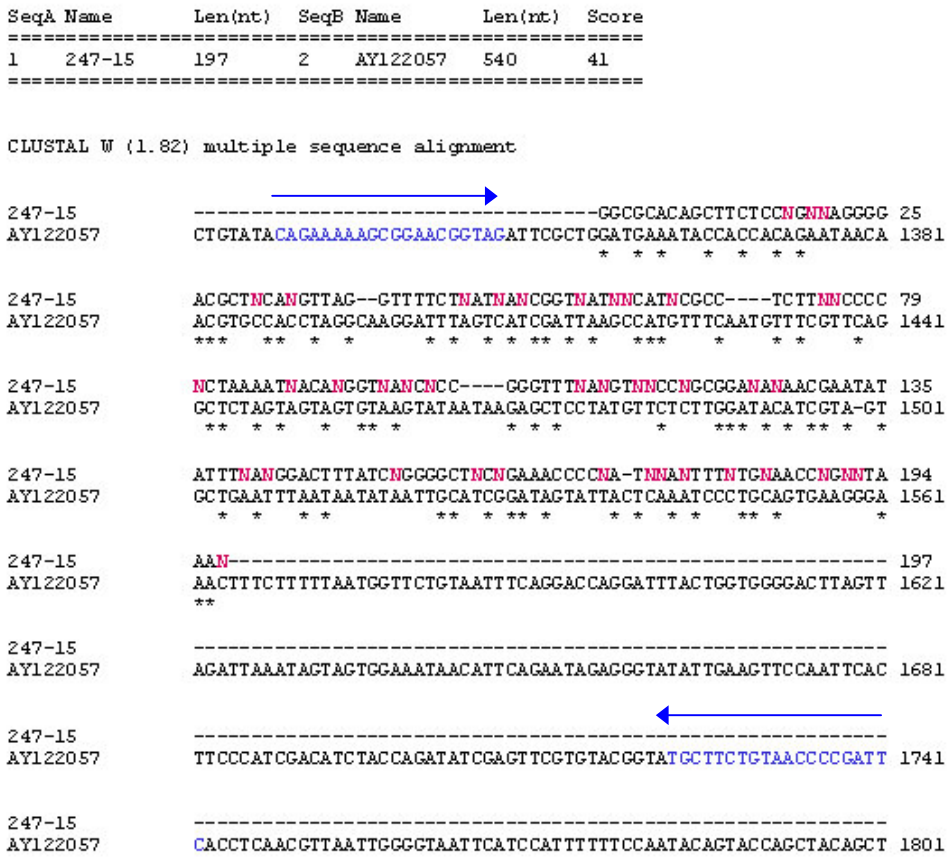


Figure 3.14; ClustalW analysis of Cry1Ac PCR result 200 bp and targeted sequence AY122057 (*, indicates the homologous base pairs)

In order to obtain the satisfactory sequencing analysis results, it was aimed to clone 645 bp Cry1A and 415 bp Cry1Ac PCR fragments.

BLAST analysis of forward and reverse primers was also done for Cry1Ac-F/Cry1Ac-R primer set, observed data showed high specificity to Cry1Ac sequences producing an amplicon of 415 bp in length (Table 3.3).

Table 3.3; Homology search of Cry1Ac forward and reverse primers

Cry1Ac-F CAg AAA AAg Cgg AAC ggT Ag/ Cry1Ac-R gAA TCg ggg TTA CAg AAg CA			
Sequence accession no. & name	**From**	**To**	**Length**
AY122057 Bacillus thuringiensis insecticidal crystal protein Cry1A gene	1328	1740	412 bp
AY225453 Bacillus thuringiensis Cry1Ac gene	1203	1618	415 bp
AJ002514 Bacillus thuringiensis kustaki cry218 gene	1590	2005	415 bp
X54159 Bacillus thuringiensis cryIA(c) gene for insecticidal (Lepidoptera specific)	1541	1953	412 bp
AJ130970 Bacillus thuringiensis gene encoding crystal tox	1358	1773	415 bp
AF492767 Bacillus thuringiensis Cry1Ac gene	1203	1615	412 bp
AF148644 Bacillus thuringiensis insecticidal Cry1Ac partial cds	1203	1615	412 bp
U87397 Bacillus thuringiensis kurstaki crystal protein (CryIA(c)) gene	1355	1770	415 bp
U89872 Bacillus thuringiensis Cry1Ac delta-endotoxin gene	1590	2005	415 bp
U87793 Bacillus thuringiensis kurstaki insecticidal delta-endotoxin CryIA(c)gene	2178	2593	415 bp
U43606 Bacillus thuringiensis cryIA(c) gene	1203	1615	412 bp
M73249 Bacillus thuringiensis gene	1203	1618	415 bp
M73248 Bacillus thuringiensis (cryIAc3) gene	1203	1615	412 bp
M35524 B.thuringiensis delta-endotoxin gene	1441	1853	412 bp
M11068 B.thuringiensis 75 kb plasmid crystal protein gene	1590	2005	415 bp
AY525369 Synthetic construct cry1AcAT modified toxin gene	1203	1618	415 bp

3.2.4 Cloning and sequencing of fragments

645 bp Cry1A fragment from sample 24 and 415 bp Cry1Ac fragment from sample 15 were selected for cloning. Cloning of the fragments was achieved by ligation into pTZ57R/T vector and transforming into *E.coli* Dh5-α cells. PCR amplification of the selected colonies with M13 primers was performed. Eight Cry1A (with lane designation 1, 2, 3, 4, 5, 6, 7, 8) and eight Cry1Ac (with lane designation 9, 10, 11, 12, 13, 14, 16, 17, 18, 20) colonies observed to be carrying the expected size inserts (Figure 3.15).

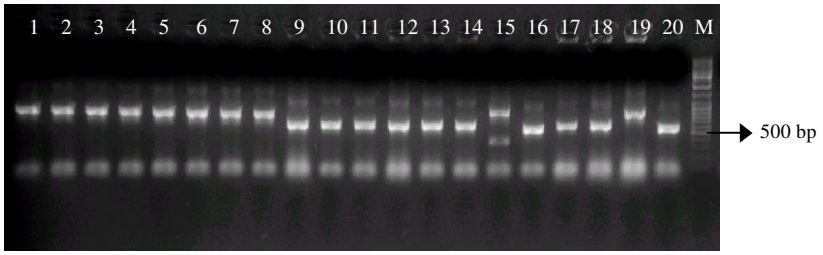


Figure 3.15; M13 PCR results with 645 bp Cry1A and 415 bp Cry1Ac clones, line 1-8; Cry1A samples, line 9-20; Cry1Ac samples and line M; 100 bp DNA Ladder

Plasmids of two selected Cry1A (with lane designation 1 and 5) and Cry1Ac (with lane designation 9 and 13) colonies were isolated and prepared for sequencing. In order to confirm the isolation of plasmids carrying the correct inserts were also electrophoresed after double digestion with EcoRI and PstI enzymes (Figure 3.16). It was understood from the double digestion results that Cry1A fragments contain an additional restriction site either for EcoRI or PstI.

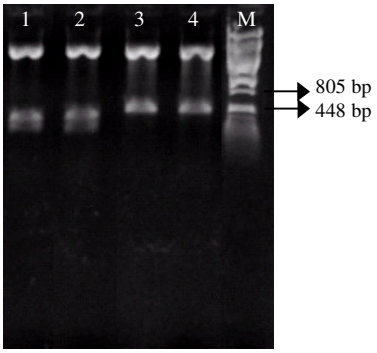


Figure 3.16; Double digestion results of selected plasmids with EcoRI and PstI, line 1-2; Cry1A samples, line 3-4; Cry1Ac samples and line M; pstI digested lambda (λ) DNA

Isolated plasmids that is expected to contain the 645 bp Cry1A fragments were automatic sequenced with T7 primers, results compared with targeted Cry1A sequence AY122057 did not show homology (Figure 3.17). High similarity was observed form the comparison of sequence analysis results of Cry1A clones with previous Cry1A sequencing results (Appendix H). Nucleotide and amino acid sequence alignments of sequenced Cry1A clones were checked with nucleotide-nucleotide and protein-protein BLAST programmes in order to identify these sequences, obtained results did not exhibit any meaningful homology with sequences found in GenBank database.

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	AY122057	720	2	1A	639	3
1	AY122057	720	3	2A	639	2
2	1A	639	3	2A	639	94

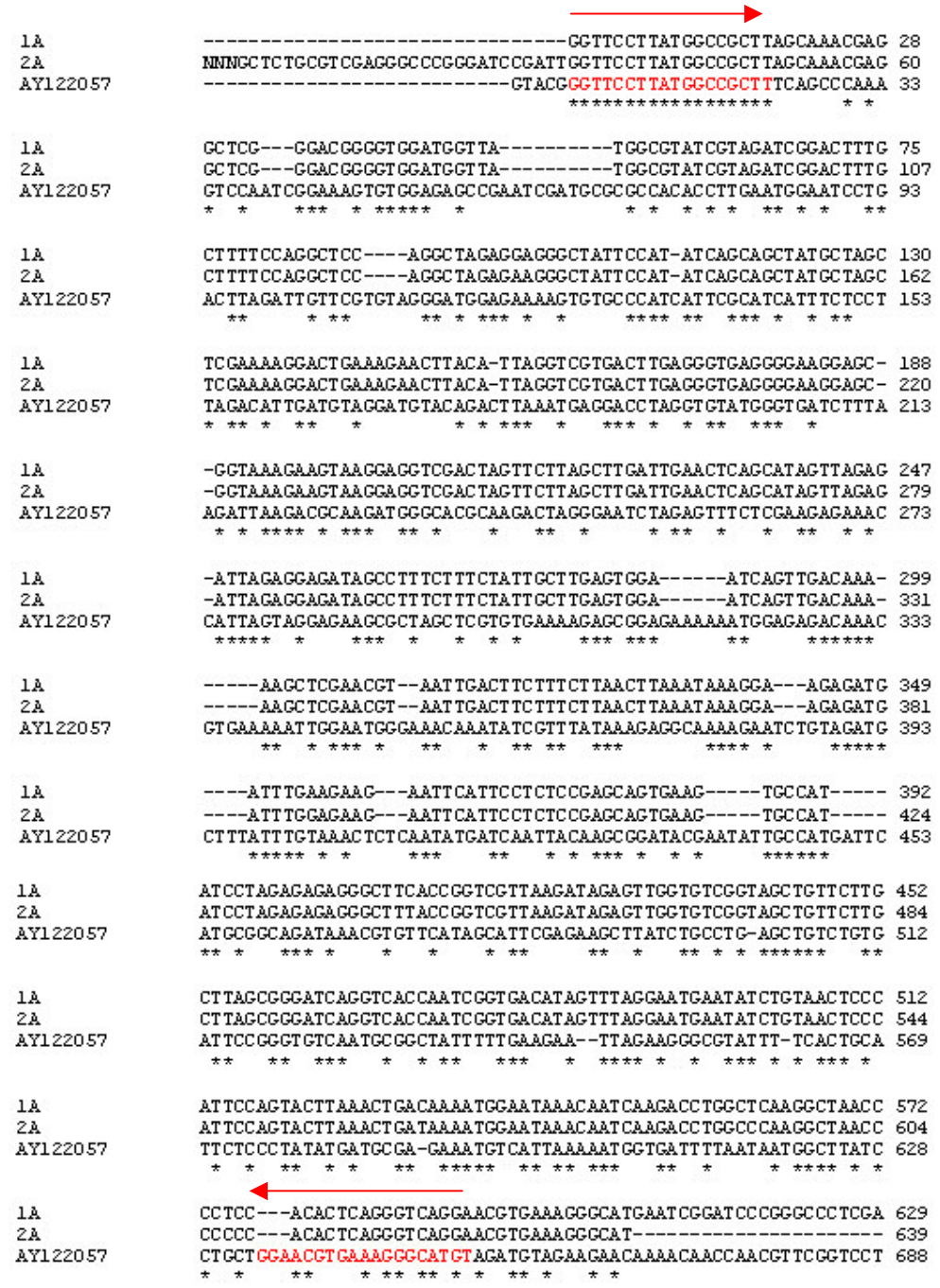


Figure 3.17; ClustalW analysis of Cry1A clone sequencing results 1A and 2A and targeted sequence AY122057 (*, indicates the homologous base pairs)

Nucleotide-nucleotide BLAST analysis results of 415 bp Cry1Ac fragments, indicate an homology with Cry genes (Appendix I). The reason for that low complementarity might arise from the synthetic construction of inserted gene. DNA sequencing results of two cloned Cry1Ac fragments exhibited 98% homology with each other, but on the other hand score obtained from comparison of sequenced fragments and targeted AY122057 sequence did not give satisfactory result (Figure 3.18).

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	AY122057	540	2	Ac1	463	4
1	AY122057	540	3	Ac2	464	4
2	Ac1	463	3	Ac2	464	98

```
Ac1             -------CAGAAAAAGCGGAACGGTAGCCTACGAAGCATGACAATGGACCAGATGTTTGA 53
Ac2             -------CAGAAAAAGCGGAACGGTAGCCTACGAAGCATGACAATGGACCAGATGTTTGA 53
AY122057        CTGTATACAGAAAAAGCGGAACGGTAGATT-CGCTGGATGAAATACCACCACAGAATAAC 59
                       ********************  * **  * **** *    **** *   *

Ac1             GAAATGGGGCGGTGGTGAGGTTCTCCGGAGGGCCGTTCGGGGGATGTTGCCCAAGAACCG 113
Ac2             GAAATGGGGCGGTGGTGAGGTTCTCCGGAGGGCCGTTCGGGGGATGTTGCCCAAGAGCCG 113
AY122057        AACGTGCCACCTAGGCAAGGATTT---AGTCATCGATTAAGCCATGTT--TCAATGTTTC 114
                 *  **   *   **  *** * *         ** *   *  *****   ***

Ac1             GCTTCGGGATAAGAGGTTGGCTCGGTTGAAGAGTGAGTCTTTGGGTCTTTGCTGTTCGAA 173
Ac2             GCTTCGGGATAAGAGGTTGGCTCGGTTGAAGAGTGAGTCTTTGGGTCTTTGCTGTTCGAA 173
AY122057        GTTCAGGCTCTAGTAGTAGTGTAAGTATAATAA-GAGCTCCTATGTTCTCTTGGATACAT 173
                * *  **    **  ** * *  *  **  ** *  ***    *  **  *    * *  *

Ac1             TGTACTACGCAAAGTGAGACTGTTGCTGACTTGAACAGCCTT---CGAGGGTCTG----- 225
Ac2             TGTACTACGCAAAGTGAGACTGTTGCTGACTTGAACAGCCTT---CGAGGGTCTG----- 225
AY122057        CGTAGTGCTGAATTTAATAATATAATTGCATCGGATAGTATTACTCAAATCCCTGCAGTG 233
                 *** * *  **  * * * * *   **  * * * **  **   * *    ***

Ac1             --GCTCATCCTTACAAGGAGAATATCGTAAAGTTCGGTAACAAG-TCGGTTATTGGCAAT 282
Ac2             --GCTCATCCTTACAAGGAGAATATCGTAAAGTTCGGTAACAAG-TCGGTTATTGGCAAT 282
AY122057        AAGGGAAACTTTCTTTTTAATGGTTCTGTAATTTCAGGACCAGGATTTACTGGTGGGGAC 293
                  *   * * **      *     **   ** *** * * ** * *    *  ***  *

Ac1             TTGCCGGAGGTGCAACAGGCCTTCAAGAAGCCT--GAGGCTGAGGCCAAGCAGTCTGCAT 340
Ac2             TTGCCGGAGGTGCAACAGGCCTTCAAGAAGCCT--GAGGCTGAGGCCAAGCAGTCTGCAT 340
AY122057        TTAGTT-AGATTAAATAGTAGTGGAAATAACATTCAGAATAGAGGGTATATTGAAGTTCC 352
                **     ** *  ** **   *  **  * * *        ****  *    *

Ac1             AAATGGTGCTTGGTCAAGGGGGAC--GAAATCGAACAAAAGTGAAAT---CATCTCTCAC 395
Ac2             AAATGGTGCTTGGTCAAGGGGGAC--GAAATCGAACAAAAGTGAAAT---CATCTCTCAC 395
AY122057        AATTCACTTCCCATCGACATCTACCAGATATCGAGTTCGTGTACGGTATGCTTCTGTAAC 412
                ** *         ** *     **  ** *****      **    *   * *** * **

Ac1             CTCAGAT-GCGACGCCATCTGCTTCTGTAACCC--CGATTCAATCGGATCCCGGGCCCTC 452
Ac2             CTCAGAT-GCGACGCCATCTGCTTCTGTAACCC--CGATTCAATCGGATCCCGGGCCCTC 452
AY122057        CCCGATTCACCTCAACGTTAATTGGGGTAATTCATCCATTTTTTCCAATACAGTACCAGC 472
                * *   *  *  *  * *    *   ****  *  * ***   **  ** * *  **  *

Ac1             GACTGCAGAGC---------------------------------------------- 463
Ac2             GACGCAAGCTNC--------------------------------------------- 464
AY122057        TACAGCTACGTCATTAGATAATCTACAATCAAGTGATTTTGGTTATTTTGAAAGTGCCAA 532
                 **
```

Figure 3.18; ClustalW analysis of Cry1Ac clone sequencing results Ac1 and Ac2 and targeted sequence AY122057 (*, indicates the homologous base pairs)

3.2.5 Interpretation of results

The control of extracted DNA samples were done by patatin gene specific PCR. Twenty-eigth out of twenty-nine amplicon produced the expected amplicon, which indicates the presence and amplification capacity of our DNA samples. The sample 21, that has a 1.222 A_{260}/ A_{280} value did not produce an amplicon and eliminated from further screening and identification studies.

In our screening tests, only NptII PCR exhibited positive signals, in Nos terminator and 35S promoter specific PCRs it was not observed any amplification signal. Samples 1, 3, 25, 26, 28 and 29 produces only an amplicon in NptII specific PCR whereas they did not produce any product in 35S promoter, Nos terminator and in Cry identification PCR systems. Among approved genetically modified tomato lines, NptII kanamycin resistance gene is present in nearly all. Therefore it gives a great opportunity to screen them by targeting this resistance gene. But in the case of 35S promoter and Nos terminator, they could not provide such a general detection, the presence of these three targets in GM tomatoes were summarized in Table 3.4.

Table 3.4; Summarization of genetic elements of approved transgenic tomato lines (Bruderer & Leitner, 2003)

Event name	35S promoter	Nos terminator	NptII gene	Approval type	Approval place
117, 1046, 1204, 1208 (vir. res.)	No map information available			Field production-import	Japan
1345-4 (del. rip.)	+	+	+	Food, Field production-food	Canada, USA
35 1 N (del. rip.)	–	+	+	Field production-food	USA
405, 707(vir. res.)	No map information available			Field production-import	Japan
5345 (ins. res.)	+	+	+	Food, Field production-food	Canada, USA
8338 (del. rip.)	+	+	+	Field production-food	USA
B, Da, F (del. rip.)	+	+	+	Food, Field production-food	Canada, USA
China tomato 1 (vir. res.)	No map information available			Field production-food, feed	China
China tomato 2 (vir. res.)	No map information available			Field production-food, feed	China
Flavr Savr (del. rip.)	+	–	+	Food, Field production-import, Field production-food feed	Canada, Japan, Mexico, USA
ICI9, ICI13 (del. rip.)	No map information available			Field production-import	Japan
Japan tomato 1 (vir. res.)	No map information available			Field production-import	Japan
N°4-7 (vir. res.)	No map information available			Field production-import	Japan

(vir. res.; virus resistant, del. rip.; delayed ripening, ins. res.; insect resistant)

The reason for our non-detection, could be the other promoter and terminator regions used in transgenic tomato lines instead of 35S promoter and Nos terminator. It was known that in recent studies, scientists begin to improve crops by using their own promoter and terminator regions. And also in genetically modified crops, different sequence mutants of P-35S fragments can be used. Therefore we could not detect those regions with our primer sets.

As mentioned in the report of Bruderer & Leitner, (2003), genetically modified tomato lines with variable improved properties were approved in different parts of the world. Virus resistant tomato with event name 117-1046-1204-1208, 405-707,

ICI9-ICI13, Japan tomato in Japan, and additionally China tomato 1 and 2 in China are the approved transgenic tomato lines with no genetic map information. In order to evaluate our NptII positive results whether they improved to provide virus resistance or not, it needs genetic information. Our kanamycin positive samples with no 35S promoter and Nos terminator could be also belong to that virus resistant genetically modified tomato group.

In our identification studies, seventeen positive amplification signals were observed with Cry1A-F/Cry1A-R primer set and ten amplification signals with Cry1Ac-F/Cry1Ac-R specific primer set. In samples 4, 10, 11, 12, 13,14 and 24, we detected 645 bp amplicon by using Cry1A-F/Cry1A-R primers that refers the presence of Cry genes, but we could not observed any 415 bp fragment with Cry1Ac-F/Cry1Ac-R primers. The observed 645 bp fragment might result from any Cry gene instead of Cry1Ac gene, indicating a non-approved transgenic line. The sample 14 which produced positive amplification signal only with Cry1A-F/Cry1A-R primers could be an unknown transgenic line.

In samples 2, 5, 6, 7, 8, 9, 15, 17, 18 and 19 positive amplification signals were observed with Cry1A-F/Cry1A-R and Cry1Ac-F/Cry1Ac-R primer sets. Detection of these 645 and 415 bp products indicates probable existance of Cry1Ac gene. In order to identify them as transgenic line 5345, it is necessary to detect 35S promoter and Nos terminator regions beside the NptII and Cry1Ac genes,. The reason for our not detection could be the non-homology of our primer pairs, at that point it is very important to have tomato line 5345 as positive control to make a comparison.

Another estimation is that, detected tomatoes could belong to a different, non approved insect resistant tomato line.

Table 3.5; Summarization of obtained results

Sample No:	Sample ID:	Pat	35S	Nos	NptII	Cry1A	Cry1Ac
1	Ankara (Eryaman)	+	-	-	+	-	-
2	Ankara (Etimesgut)	+	-	-	+	+	+
3	Ankara (Yıldız)	+	-	-	+	-	-
4	Ankara (Yıldız)	+	-	-	+	+	-
5	Ankara (Armada)	+	-	-	+	+	+
6	Ankara (Etimesgut)	+	-	-	+	+	+
7	Ankara (Çankaya)	+	-	-	+	+	+
8	Ankara (Bilkent)	+	-	-	+	+	+
9	Ankara (Yıldız)	+	-	-	+	+	+
10	Spain 1	+	-	-	+	+	-
11	Spain 2	+	-	-	+	+	-
12	Belgium 1	+	-	-	+	+	-
13	Belgium 2	+	-	-	+	+	-
14	Çanakkale	+	-	-	-	+	-
15	Ayaş	+	-	-	+	+	+
16	Eskişehir	+	-	-	-	-	-
17	Isparta	+	-	-	+	+	+
18	Antalya	+	-	-	+	+	+
19	Afyon	+	-	-	+	+	+
20	China 1	+	-	-	-	-	-
21	China 2	-	-	-	-	-	-
22	Antalya-Kalkan 1	+	-	-	-	-	-
23	Antalya-Kalkan 2	+	-	-	-	-	-
24	Antalya-Kalkan 3	+	-	-	+	+	-
25	Antalya-Kalkan 4	+	-	-	+	-	-
26	USA 1	+	-	-	+	-	-
27	USA 2	+	-	-	-	-	-
28	Mersin 1	+	-	-	+	-	-
29	Mersin 2	+	-	-	+	-	-

CHAPTER IV

CONCLUSION

Turkey is one of the most important country concerning the advances of its ecological and agricultural properties and also one of the main tomato producer in the world. It was known that including the tomato, many genetically modified crop takes place in our food markets and it is really necessary to detect and evaluate the presence of GM foods in Turkey food markets.

In this study we attempt to screen and identify tomatoes collected from Turkey food market whether they are genetically modified insect resistant tomatoes or not. The detection of presence of genetically modified crops has a special importance in the aspect of acceptance of Turkey in GM food market. By this way it could force the regulatory agencies to develop required regulations.

In this study, it was aimed to detect genetically modified insect resistant tomatoes. There is not any available construct and sequence information about genetically modified insect resistant tomato which our country imports from Israel, Holland and USA. So Monsanto's transgenic tomato line 5345 has been taken as a model, and focused on the main target genes of this genetically modified insect resistant tomato

line. This insect resistant line has an approval for food use in Canada and USA since 2000 and 1998 respectively. Even for a scientific study, in order to import Monsanto's GM tomato line 5345, a permission is needed from Ministry of Agriculture. Although our great efforts, resulting from the absence of required permission, we could not obtain the positive control 5345 tomato line and it caused difficulties in identification experiments.

In order to detect insect resistant GM tomatoes, three screening targets and an identification target gene were used, 8 primer sets that were specific to 35S promoter, Nos terminator, NptII kanamycin resistance gene, Cry1A and Cry1Ac insect resistance genes were designed.

In conclusion NptII gene was detected in most of our samples, this screening target is used both in delayed ripened and insect resistant GM tomatoes. In our seventeen samples positive amplification signals were observed indicating the presence of Cry genes and in our ten samples amplicons were obtained with Cry1Ac-F/Cry1Ac-R primers. These obtained data indicates the existance of Turkey in GM food market.

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APPENDIX A

Annealing sites of PatGc-PatGd primer set was illustrated on the Solanum tuberosum gene for patatin with the NCBI accession no. X03932 (Jaccaud *et al.,* 2003).

2641 gatcaatact attatgtgtt gtacgtgcaa tgcaggcatt attatccatt agcgttgcaa

2701 cgaaacttgc acaagtggat ccaaaatttg cttcaattaa gtcattgaat tacaagcaaa

2761 tgttgttgct ctcattaggc actggcacta cttcagagtt tgataaaaca tatacagcag

2821 aagagacagc taaatggggt actgcacgat ggatgttagt tatacagaaa atgactagtg

2881 cagcaagttc ttacatgact gattattacc tttctactgc ttttcaagct cttgattcac

2941 aaaacaatta cctaagggtt caagtaagtc ccaactatta ttatagggaa cttacataaa

3001 tctcactagt ttgcaatatt acctatttta ccaaattttg atatatctag gatacatggt

3061 caaaattagg tgtaatagtt ctaaatacat tctatccaag tggattcaca tggatctcta

3121 gaatcagtga cggattcagg atttacatta aaggggttaa aaagaaaaaa acgagtagtg

3181 cctaagattt gatcttggat ccttaaggtg agttttgaac ccctcgacca ctgagtcaac

3241 ctctaatctt acatttagga ggctcaaaat caatatatag acataaaaat tcttaaaatt

3301 gccttaaata tacaacggac gtaagttttt gttgtgaacc cccttgtcaa cctctagggc

3361 cgcccctgtc tagaatacat tagcaaatct ttctcgctct cttcaatctc gcttgccact

3421 ctcccatgta tttggtattc cagatacatg agaatcacac cagatgcata cagatacagt

3481 acgtatctag tgcgatttgc atgtatcttg gatacatacg tatctcactc acctttctca

3541 ctatttttgt gtatctggta gcaaaaatac atgtatctaa gtatattttt cttaatatat

3601 gataggaaat tcttaataag tggtaagata cgtactattt cagaaatata tatgatagtg

APPENDIX B

ClustalW analysis (ClustalW homology search programme, 2004, http://www.ebi.ac.uk/clustalw) results indicates the non-homology between commonly used 35S promoter sequence AF078810 (Jaccaud *et al.,* 2003) and 35S promoter sequence with EMBL GenBank accession no. CMV7626 that was used in primer design. Blue color is indicating the first primer set 35S-F/35S-R that was used in our study, and the pink color is indicating the second primer set, 35S-1/35S-2. In some transgenic crops, the duplicated versions of 35S promoter are used (AF078810), the sequence carries a second complementary annealing site for the 35S-1 primer (Jaccaud *et al.,* 2003).

```
SeqA Name        Len(nt)  SeqB Name        Len(nt)  Score
=========================================================
1    CMV7626     423      2    AF078810    755      8
=========================================================
```

```
                                    ----------> 35S-F
CMV7626          GGATCCTCTAGAGTCCCCCGTGTTCTCTCCAAATGAAATGAACTTCCTTATATAGAGGAA 60
AF078810         ------CCTGCAGGTCAACATGGTGGAGCACGACACACTTGTCTACTCCAAAAATATCAA 54
                       **  **  *  * ** *    *   *   * *   ** *   * * * *  **

CMV7626          GGGTCTTGCGAAGGATAGTGGGATTGTGCGTCATCCCTTACGTCAGTGGA-GATTCCAGA 119
AF078810         AGATACAGTCTCAGAAGACCAAAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGG 114
                  * *   *     **       *  *    * *  * ** *  **  **   **  * *

CMV7626          TAGGCCTAACGCTTGTCCAAG---ATCTATTCAGGATTCCAG-ATAGGCCTAACGCTTGT 175
AF078810         AAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAA 174
                  *  *    **  *  *   *   * ****     * *  *   *     **  *
                          ----------> 35S-1
CMV7626          CCAAGATCTATTCAGGATATCACATCAATCCACTTGCTTTGAAGACGTGGTTGGAACGTC 235
AF078810         GGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAA--GAT 232
                   *** *   * *   * **  ***** * *  *       * * * * **** *  *
                                    <---------- 35S-R
CMV7626          TTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCATCTTTG-GGACCACTGTCGGCAG 294
AF078810         GCCTCTGCCGACAGTGGTCC-CAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAA 291
                   ** *  * **  ** *** *   * ***    *** *   * *** **  ** *  *
```


CMV7626 AGGCATC--TTCAACGATGGCCTTTCCTTTATCGCAATGATGGCATT---TGTAGGAGC- 348
AF078810 AGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAACATGGTGGAGCA 351
CMV7626 -----CACCTTCCTTTTCCACTATCTTCACA-ATAAAGTGACAGATAGCT---GGGCAAT 399
AF078810 CGACACACTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAGGGCAAT 411
CMV7626 GGAATCC---------GAGGAGGTTTCCGGATA---------- 423
AF078810 TGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTCCATTGCCCAGCTAT 471
35S-1
CMV7626 ----------
AF078810 CTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTG 531
CMV7626 ----------
AF078810 CGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACC 591
CMV7626 ----------
AF078810 CCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGT 651
35S-2
CMV7626 ----------
AF078810 GGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCA 711

APPENDIX C

Annealing sites of Nos-1/Nos-3 primer set was illustrated on sequence with the NCBI accession no. U12540.

```
1201 tgaccgacca agcgacgccc aacctgccat cacgagattt cgattccacc gccgccttct
1261 atgaaaggtt gggcttcgga atcgttttcc gggacgccgg ctggatgatc ctccagcgcg
1321 gggatctcat gctggagttc ttcgcccacg ggatctctgc ggaacaggcg gtcgaaggtg
1381 ccgatatcat tacgacagca acggccgaca agcacaacgc cacgatcctg agcgacaata
1441 tgatcgggcc cggcgtccac atcaacggcg tcggcggcga ctgcccaggc aagaccgaga
1501 tgcaccgcga tatcttgctg cgttcggata ttttcgtgga gttcccgcca cagacccgga
1561 tgatccccga tcgttcaaac atttggcaat aaagtttctt aagattgaat cctgttgccg
1621 gtcttgcgat gattatcata taatttctgt tgaattacgt taagcatgta ataattaaca
1681 tgtaatgcat gacgttattt atgagatggg tttttatgat tagagtcccg caattataca
1741 tttaatacgc gatagaaaac aaaatatagc gcgcaaacta ggataaatta tcgcgcgcgg
1801 tgtcatctat gttactagat cgggcctcct gtcaatgctg gcggcggctc tggtggtggt
1861 tctggtggcg gctctgaggg tggtggctct gagggtggcg gttctgaggg tggcggctct
1921 gagggaggcg gttccggtgg tggctctggt tccggtgatt ttgattatga aaagatggca
```

APPENDIX D

Selected kanamycin sequences were aligned and alignments were checked. Primer annealing sites for kanamycin resistance primer sets represented as red colour.

```
274586AF        CCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACT 457
274974AF        CCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACT 479
274975AF        CCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACT 343
                ************************************************************

274586AF        GGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCG 517
274974AF        GGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCG 539
274975AF        GGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCG 403
                ************************************************************

274586AF        AGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCT 577
274974AF        AGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCT 599
274975AF        AGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCT 463
                ************************************************************

274586AF        GCCCATACGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCG 637
274974AF        GCCCATACGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCG 659
274975AF        GCCCATACGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCG 523
                ************************************************************

274586AF        GTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGT 697
274974AF        GTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGT 719
274975AF        GTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGT 583
                ************************************************************

274586AF        TCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATG 757
274974AF        TCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATG 779
274975AF        TCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATG 643
                ************************************************************

274586AF        CCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCC 817
274974AF        CCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCC 839
274975AF        CCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCC 703
                ************************************************************

274586AF        GGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAG 877
274974AF        GGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAG 899
274975AF        GGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAG 763
                ************************************************************

274586AF        AGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATT 937
274974AF        AGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATT 959
274975AF        AGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATT 823
                ************************************************************

274586AF        CGCAGCGGATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 981
274974AF        CGCAGCGGATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 1003
274975AF        CGCAGCGGATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 867
                ********************************************
```

APPENDIX E

Comparison of Cry1Ab and Cry1Ac sequences, that indicates the non-homology between U63372 and other Cry1A sequences at primer binding region. AF254640 belongs to Cry1Ab (blue color), AY122057, M11068 sequences (green color) and U63372 sequence belongs to Cry1Ac groups. Red color indicates the primer pair Cry-F/Cry-R that is expected to produce an amplicon 136 bp in length.

```
SeqA Name        Len(nt)   SeqB Name        Len(nt)   Score
=========================================================
1    AF254640    4293      2    AY122057    3659      88
1    AF254640    4293      3    M11068      3537      88
1    AF254640    4293      4    U63372      1859      69
2    AY122057    3659      3    M11068      3537      99
2    AY122057    3659      4    U63372      1859      82
3    M11068      3537      4    U63372      1859      82
```

==========

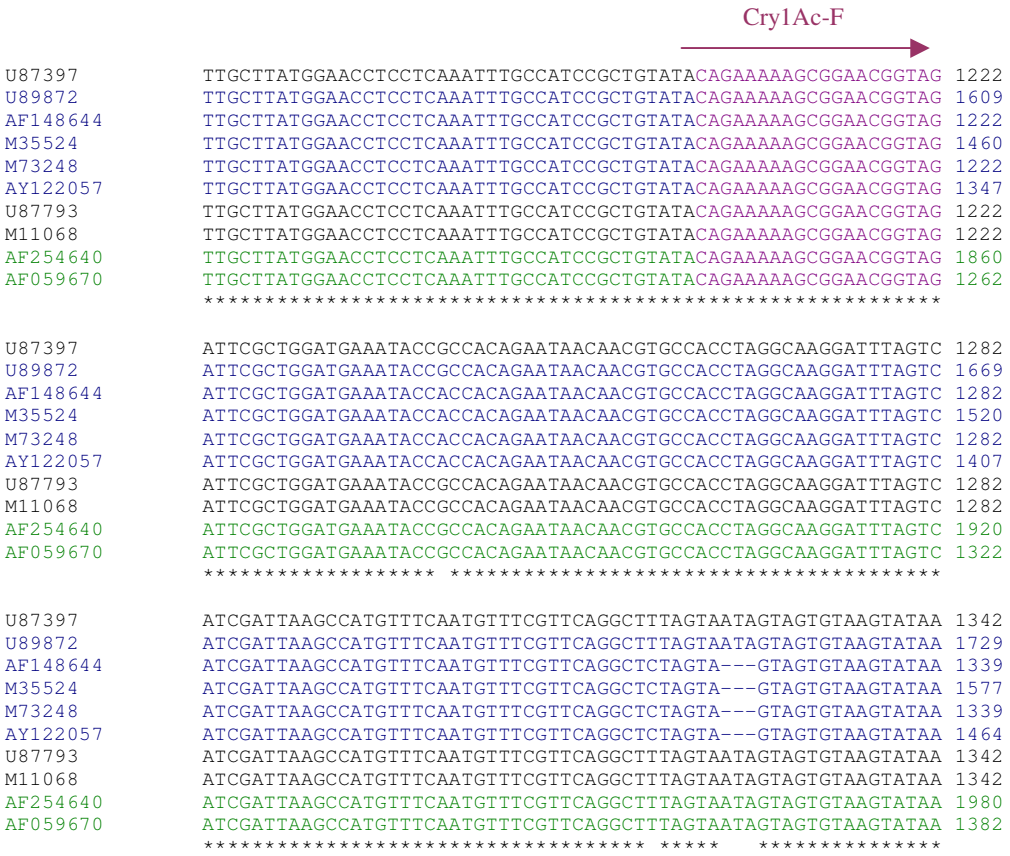
```
                                                  Cry-F
AF254640        ATGAATGCATTCCTTATAATTGTTTAAGTAACCCTGAAGTAGAAGTATTAGGTGGAGAAA 720
AY122057        ATGAATGCATTCCTTATAATTGTTTAAGTAACCCTGAAGTAGAAGTATTAGGTGGAGAAA 207
M11068          ATGAATGCATTCCTTATAATTGTTTAAGTAACCCTGAAGTAGAAGTATTAGGTGGAGAAA 82
U63372          ACGAGTGCATTCCTTACAACTGCCTGAGCAACCCTGAGGTTGAGGTGCTGGGTGGAGAAC 92
                * ** *********** ** **  * ** ******** ** ** **  * *********

AF254640        GAATAGAAACTGGTTACACCCCAATCGATATTTCCTTGTCGCTAACGCAATTTCTTTTGA 780
AY122057        GAATAGAAACTGGTTACACCCCAATCGATATTTCCTTGTCGCTAACGCAATTTCTTTTGA 267
M11068          GAATAGAAACTGGTTACACCCCAATCGATATTTCCTTGTCGCTAACGCAATTTCTTTTGA 142
U63372          GGATTGAGACTGGTTACACACCTATCGACATCTCGTTGTCACTTACCCAATTCCTTTTGT 152
                * ** ** *********** ** ***** ** ** ***** ** ** ***** ******
                         Cry-R
AF254640        GTGAATTTGTTCCCGGTGCTGGATTTGTGTTAGGACTAGTTGATATAATATGGGGAATTT 840
AY122057        GTGAATTTGTTCCCGGTGCTGGATTTGTGTTAGGACTAGTTGATATAATATGGGGAATTT 327
M11068          GTGAATTTGTTCCCGGTGCTGGATTTGTGTTAGGACTAGTTGATATAATATGGGGAATTT 202
U63372          CAGAGTTCGTGCCCGGTGCTGGATTCGTGCTTGGACTTGTCGATATCATTTGGGGAATCT 212
                  ** ** ** ************** *** * ***** ** ***** ** ******** *

AF254640        TTGGTCCCTCTCAATGGGACGCATTTCTTGTACAAATTGAACAGTTAATTAACCAAAGAA 900
AY122057        TTGGTCCCTCTCAATGGGACGCATTTCTTGTACAAATTGAACAGTTAATTAACCAAAGAA 387
M11068          TTGGTCCCTCTCAATGGGACGCATTTCTTGTACAAATTGAACAGTTAATTAACCAAAGAA 262
U63372          TTGGTCCCTCTCAATGGGACGCCTTTCTTGTACAGATAGAGCAACTTATCAACCAAAGGA 272
                ********************** ********** ** ** **  * ** ******** *
```

APPENDIX F

Comparison of Cry1A and Cry1Ac sequences by ClustalW software programme, in order to design primers, complementary to homologous regions. Black colored sequences are the B.t.k HD73 strains (Cry1Ac), blue colored ones are Cry1Ac and the green colored ones are belonging to Cry1Ab genes. Purple color indicates the region where Cry1Ac primers were designed and the red color indicates the region of forward and reverse Cry1A primers.



```
U87397          TAAGAGCTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCAT 1402
U89872          TAAGAGCTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCAT 1789
AF148644        TAAGAGCTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCAT 1399
M35524          TAAGAGCTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCAT 1637
M73248          TAAGAGCTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCAT 1399
AY122057        TAAGAGCTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCAT 1524
U87793          TAAGAGCTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCAT 1402
M11068          TAAGAGCTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCAT 1402
AF254640        TAAGAGCTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTCCTT 2040
AF059670        TAAGAGCTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTCCTT 1442
                ********************************************************* * *

U87397          CGGATAGTATTACTCAAATCCCTGCAGTGAAG---GGAAACTTTCTTTTTAATGGTTCTG 1459
U89872          CGGATAGTATTACTCAAATCCCTGCAGTGAAG---GGAAACTTTCTTTTTAATGGTTCTG 1846
AF148644        CGGATAGTATTACTCAAATCCCTGCAGTGAAG---GGAAACTTTCTTTTTAATGGTTCTG 1456
M35524          CGGATAGTATTACTCAAATCCCTGCAGTGAAG---GGAAACTTTCTTTTTAATGGTTCTG 1694
M73248          CGGATAGTATTACTCAAATCCCTGCAGTGAAG---GGAAACTTTCTTTTTAATGGTTCTG 1456
AY122057        CGGATAGTATTACTCAAATCCCTGCAGTGAAG---GGAAACTTTCTTTTTAATGGTTCTG 1581
U87793          CGGATAGTATTACTCAAATCCCTGCAGTGAAG---GGAAACTTTCTTTTTAATGGTTCTG 1459
M11068          CGGATAGTATTACTCAAATCCCTGCAGTGAAG---GGAAACTTTCTTTTTAATGGTTCTG 1459
AF254640        CATCACAAATTACACAAATACCTTTAACAAAATCTACTAATCTTGGCTCTGGAACTTCTG 2100
AF059670        CATCACAAATTACACAAATACCTTTAACAAAATCTACTAATCTTGGCTCTGGAACTTCTG 1502
                *       ***** ***** ***  *   **       **  **   * *     *****

U87397          TAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATA 1519
U89872          TAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATA 1906
AF148644        TAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATA 1516
M35524          TAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATA 1754
M73248          TAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATA 1516
AY122057        TAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATA 1641
U87793          TAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATA 1519
M11068          TAATTTCAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATA 1519
AF254640        TCGTTAAAGGACCAGGATTTACAGGAGGAGATATTCTTCGAAGAACTTCACCTGG--CCA 2158
AF059670        TCGTTAAAGGACCAGGATTTACAGGAGGAGATATTCTTCGAAGAACTTCACCTGG--CCA 1560
                *  **  *************** ** ** **  *  ** **  ** *     ***    *

U87397          ACATTCAGAATAGAGGGTATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGAT 1579
U89872          ACATTCAGAATAGAGGGTATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGAT 1966
AF148644        ACATTCAGAATAGAGGGTATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGAT 1576
M35524          ACATTCAGAATAGAGGGTATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGAT 1814
M73248          ACATTCAGAATAGAGGGTATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGAT 1576
AY122057        ACATTCAGAATAGAGGGTATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGAT 1701
U87793          ACATTCAGAATAGAGGGTATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGAT 1579
M11068          ACATTCAGAATAGAGGGTATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGAT 1579
AF254640        GATTTCAACCTTAAGAGTAAATA-----------TTACTGC--ACCATTATCACAAAGAT 2205
AF059670        GATTTCAACCTTAAGAGTAAATA-----------TTACTGC--ACCATTATCACAAAGAT 1607
                   ****   *  ** *** **           * *** *  * *   ***    ****
                          Cry1Ac-R ◄──────────

U87397          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1637
U89872          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 2024
AF148644        ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1634
M35524          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1872
M73248          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1634
AY122057        ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1759
U87793          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1637
M11068          ATCGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAA--CGTTAATTGGGG 1637
AF254640        ATCGGGTAAGAATTCGCTACGCTTCTACCACAAATTTACAATTCCATACATCAATTGACG 2265
AF059670        ATCGGGTAAGAATTCGCTACGCTTCTACCACAAATTTACAATTCCATACATCAATTGACG 1667
                **** **  *  * ** ** ******   **     * **  ** *  * * *****  *

U87397          TAATTCATCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGA-TAATCTAC 1696
U89872          TAATTCATCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGA-TAATCTAC 2083
AF148644        TAATTCATCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGA-TAATCTAC 1693
M35524          TAATTCATCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGA-TAATCTAC 1931
M73248          TAATTCATCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGA-TAATCTAC 1693
AY122057        TAATTCATCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGA-TAATCTAC 1818
U87793          TAATTCATCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGA-TAATCTAC 1696
M11068          TAATTCATCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGA-TAATCTAC 1696
AF254640        GAAG--ACCTATTAATCAGGGGAATTTTTCAGCAACTATGA-GTAGTGGGAGTAATTTAC 2322
AF059670        GAAG--ACCTATTAATCAGGGGAATTTTTCAGCAACTATGA-GTAGTGGGAGTAATTTAC 1724
                 **   * * ***  *      *   *  **** **    * **  *  ** **** ***
```

```
U87397          AATCAAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCA------TTAG 1750
U89872          AATCAAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCA------TTAG 2137
AF148644        AATCAAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCA------TTAG 1747
M35524          AATCAAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCA------TTAG 1985
M73248          AATCAAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCA------TTAG 1747
AY122057        AATCAAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCA------TTAG 1872
U87793          AATCAAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCA------TTAG 1750
M11068          AATCAAGTGATTTTGGTTATTTTGAAAGTGCCAATGCTTTTACATCTTCA------TTAG 1750
AF254640        AGTCCGGAAGCTTTAGGACTGTAGGTTTTACTACTCCGTTTAACTTTTCAAATGGATCAA 2382
AF059670        AGTCCGGAAGCTTTAGGACTGTAGGTTTTACTACTCCGTTTAACTTTTCAAATGGATCAA 1784
                * **  *    *** *   * * *    * * * * * ****  * ****      * *

U87397          GTAA---TATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATAGACAGAT 1807
U89872          GTAA---TATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATAGACAGAT 2194
AF148644        GTAA---TATAGTAGGTGTTAGAAATTTTAGTGGAACTGCAGGAGTGATAATAGACAGAT 1804
M35524          GTAA---TATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATAGACAGAT 2042
M73248          GTAA---TATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATAGACAGAT 1804
AY122057        GTAA---TATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATAGACAGAT 1929
U87793          GTAA---TATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATAGACAGAT 1807
M11068          GTAA---TATAGTAGGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATAGACAGAT 1807
AF254640        GTGTATTTACGTTAAGTGCTCATGTCTTCAATTCAGGCAATGAAGTTTATATAGATCGAA 2442
0AF59670        GTGTATTTACGTTAAGTGCTCATGTCTTCAATTCAGGCAATGAAGTTTATATAGATCGAA 1844
                **     **   ** *** *      ** * *          * ***    *****  **

U87397          TTGAATTTATTCCAGTTACTGCAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGA 1867
U89872          TTGAATTTATTCCAGTTACTGCAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGA 2254
AF148644        TTGAATTTATTCCAGTTACTGCAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGA 1864
M35524          TTGAATTTATTCCAGTTACTGCAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGA 2102
M73248          TTGAATTTATTCCAGTTACTGCAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGA 1864
AY122057        TTGAATTTATTCCAGTTACTGCAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGA 1989
U87793          TTGAATTTATTCCAGTTACTGCAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGA 1867
M11068          TTGAATTTATTCCAGTTACTGCAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGA 1867
AF254640        TTGAATTTGTTCCGGCAGAAGTAACCTTTGAGGCAGAATATGATTTAGAAAGAGCACAAA 2502
AF059670        TTGAATTTGTTCCGGCAGAAGTAACCTTTGAGGCAGAATATGATTTAGAAAGAGCACAAA 1904
                ******** **** *     * ***  * ***** ****** ** * ******** ** *

U87397          AGGCGGTGAATGCGCTGTTTACGTCTACAAACCAACTAGGGCTAAAAACAAATGTAACGG 1927
U89872          AGGCGGTGAATGCGCTGTTTACGTCTACAAACCAACTAGGGCTAAAAACAAATGTAACGG 2314
AF148644        AGGCGGTGAATGCGCTGTTTACGTCTACAAACCAACTAGGGCTAAAAACAAATGTAACGG 1924
M35524          AGGCGGTGAATGCGCTGTTTACGTCTACAAACCAACTAGGGCTAAAAACAAATGTAACGG 2162
M73248          AGGCGGTGAATGCGCTGTTTACGTCTACAAACCAACTAGGGCTAAAAACAAATGTAACGG 1924
AY122057        AGGCGGTGAATGCGCTGTTTACGTCTACAAACCAACTAGGGCTAAAAACAAATGTAACGG 2049
U87793          AGGCGGTGAATGCGCTGTTTACGTCTACAAACCAACTAGGGCTAAAAACAAATGTAACGG 1927
M11068          AGGCGGTGAATGCGCTGTTTACGTCTACAAACCAACTAGGGCTAAAAACAAATGTAACGG 1927
AF254640        AGGCGGTGAATGAGCTGTTTACTTCTTCCAATCAAATCGGGTTAAAAACAGATGTGACGG 2562
AF059670        AGGCGGTGAATGAGCTGTTTACTTCTTCCAATCAAATCGGGTTAAAAACAGATGTGACGG 1964
                ************ ********* *** * ** *** * *** ******** **** ****

U87397          ATTATCATATTGATCAAGTGTCCAATTTAGTTACGTATTTATCGGATGAATTTTGTCTGG 1987
U89872          ATTATCATATTGATCAAGTGTCCAATTTAGTTACGTATTTATCGGATGAATTTTGTCTGG 2374
AF148644        ATTATCATATTGATCAAGTGTCCAATTTAGTTACGTATTTATCGGATGAATTTTGTCTGG 1984
M35524          ATTATCATATTGATCAAGTGTCCAATTTAGTTACGTGTTTATCGGATGAATTTTGTCTGG 2222
M73248          ATTATCATATTGATCAAGTGTCCAATTTAGTTACGTATTTATCGGATGAATTTTGTCTGG 1984
AY122057        ATTATCATATTGATCAAGTGTCCAATTTAGTTACGTATTTATCGGATGAATTTTGTCTGG 2109
U87793          ATTATCATATTGATCAAGTGTCCAATTTAGTTACGTATTTATCGGATGAATTTTGTCTGG 1987
M11068          ATTATCATATTGATCAAGTGTCCAATTTAGTTACGTATTTATCGGATGAATTTTGTCTGG 1987
AF254640        ATTATCATATTGATCAAGTATCCAATTTAGTTGAGTGTTTATCTGATGAATTTTGTCTGG 2622
AF059670        ATTATCATATTGATCAAGTATCCAATTTAGTTGAGTGTTTATCTGATGAATTTTGTCTGG 2024
                ****************** ************  ** ****** ****************

U87397          ATGAAAAGCGAGAATTGTCCGAGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGCA 2047
U89872          ATGAAAAGCGAGAATTGTCCGAGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGCA 2434
AF148644        ATGAAAAGCGAGAATTGTCCGAGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGCA 2044
M35524          ATGAAAAGCGAGAATTGTCCGAGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGCA 2282
M73248          ATGAAAAGCGAGAATTGTCCGAGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGCA 2044
AY122057        ATGAAAAGCGAGAATTGTCCGAGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGCA 2169
U87793          ATGAAAAGCGAGAATTGTCCGAGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGCA 2047
M11068          ATGAAAAGCGAGAATTGTCCGAGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGCA 2047
AF254640        ATGAAAAAAAAGAATTGTCCGAGAAAGTCAAACATGCGAAGCGACTTAGTGATGAGCGGA 2682
AF059670        ATGAAAAAAAAGAATTGTCCGAGAAAGTCAAACATGCGAAGCGACTTAGTGATGAGCGGA 2084
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U87397          ATTTACTCCAAGATTCAAATTTCAAAGACATTAATAGGCAACCAGAACGTGGGTGGGGCG 2107
U89872          ATTTACTCCAAGATTCAAATTTCAAAGACATTAATAGGCAACCAGAACGTGGGTGGGGCG 2494
AF148644        ATTTACTCCAAGATTCAAATTTCAAAGACATTAATAGGCAACCAGAACGTGGGTGGGGTG 2104
M35524          ATTTACTCCAAGATTCAAATTTCAAAGACATTAATAGGCAACCAGAACGTGGGTGGGGCG 2342
M73248          ATTTACTCCAAGATTCAAATTTCAAAGACATTAATAGGCAACCAGAACGTGGGTGGGGCG 2104
AY122057        ATTTACTCCAAGATTCAAATTTCAAAGACATTAATAGGCAACCAGAACGTGGGTGGGGCG 2229
U87793          ATTTACTCCAAGATTCAAATTTCAAAGACATTAATAGGCAACCAGAACGTGGGTGGGGCG 2107
M11068          ATTTACTCCAAGATTCAAATTTCAAAGACATTAATAGGCAACCAGAACGTGGGTGGGGCG 2107
AF254640        ATTTACTTCAAGATCCAAACTTTAGAGGGATCAATAGACAACTAGACCGTGGCTGGAGAG 2742
AF059670        ATTTACTTCAAGATCCAAACTTTAGAGGGATCAATAGACAACTAGACCGTGGCTGGAGAG 2144
                ******* ****** **** ** * **  ** ***** **** *** ***** *** * *

U87397          GAAGTACAGGGATTACCATCCAAGGAGGGGATGACGTATTTAAAGAAAATTACGTCACAC 2167
U89872          GAAGTACAGGGATTACCATCCAAGGAGGGGATGACGTATTTAAAGAAAATTACGTCACAC 2554
AF148644        GAAGTACAGGGATTACCATCCAAGGAGGGGATGACGTATTTAAAGAAAATTACGTCACAC 2164
M35524          GAAGTACAGGGATTACCATCCAAGGAGGGGATGACGTATTTAAAGAAAATTACGTCACAC 2402
M73248          GAAGTACAGGGATTACCATCCAAGGAGGGGATGACGTATTTAAAGAAAATTACGTCACAC 2164
AY122057        GAAGTACAGGGATTACCATCCAAGGAGGGGATGACGTATTTAAAGAAAATTACGTCACAC 2289
U87793          GAAGTACAGGGATTACCATCCAAGGAGGGGATGACGTATTTAAAGAAAATTACGTCACAC 2167
M11068          GAAGTACAGGGATTACCATCCAAGGAGGGGATGACGTATTTAAAGAAAATTACGTCACAC 2167
AF254640        GAAGTACGGATATTACCATCCAAGGAGGCGATGACGTATTCAAAGAGAATTACGTTACGC 2802
AF059670        GAAGTACGGATATTACCATCCAAGGAGGCGATGACGTATTCAAAGAGAATTACGTTACGC 2204
                ******* *  ***************** *********** ***** ******** ** *

U87397          TATCAGGTACCTTTGATGAGTGCTATCCAACATATTTGTATCAAAAAATCGATGAATCAA 2227
U89872          TATCAGGTACCTTTGATGAGTGCTATCCAACATATTTGTATCAAAAAATCGATGAATCAA 2614
AF148644        TATCA---------------------------------------------------------- 2169
M35524          TATCAGGTACCTTTGATGAGTGCTATCCAACATATTTGTATCAAAAAATCGATGAATCAA 2462
M73248          TATCAGGTACCTTTGATGAGTGCTATCCAACATATTTGTATCAAAAAATCGATGAATCAA 2224
AY122057        TATCAGGTACCTTTGATGAGTGCTATCCAACATATTTGTATCAAAAAATCGATGAATCAA 2349
U87793          TATCAGGTACCTTTGATGAGTGCTATCCAACATATTTGTATCAAAAAATCGATGAATCAA 2227
M11068          TATCAGGTACCTTTGATGAGTGCTATCCAACATATTTGTATCAAAAAATCGATGAATCAA 2227
AF254640        TATTGGGTACCTTTGATGAGTGCTATCCAACGTATTTATATCAAAAAATAGATGAGTCGA 2862
AF059670        TATTGGGTACCTTTGATGAGTGCTATCCAACGTATTTATATCAAAAAATAGATGAGTCGA 2264
                ***

U87397          AATTAAAAGCCTTTACCCGTTATCAATTAAGAGGGTATATCGAAGATAGTCAAGACTTAG 2287
U89872          AATTAAAAGCCTTTACCCGTTATCAATTAAGAGGGTATATCGAAGATAGTCAAGACTTAG 2674
AF148644        ------------------------------------------------------------
M35524          AATTAAAAGCCTTTACCCGTTATCAATTAAGAGGGTATATCGAAGATAGTCAAGACTTAG 2522
M73248          AATTAAAAGCCTTTACCCGTTATCAATTAAGAGGGTATATCGAAGATAGTCAAGACTTAG 2284
AY122057        AATTAAAAGCCTTTACCCGTTATCAATTAAGAGGGTATATCGAAGATAGTCAAGACTTAG 2409
U87793          AATTAAAAGCCTTTACCCGTTATCAATTAAGAGGGTATATCGAAGATAGTCAAGACTTAG 2287
M11068          AATTAAAAGCCTTTACCCGTTATCAATTAAGAGGGTATATCGAAGATAGTCAAGACTTAG 2287
AF254640        AATTAAAAGCCTATACCCGTTACCAATTAAGAGGGTATATCGAAGATAGTCAAGACTTAG 2922
AF059670        AATTAAAAGCCTATACCCGTTACCAATTAAGAGGGTATATCGAAGATAGTCAAGACTTAG 2324

U87397          AAATCTATTTAATTCGCTACAATGCAAAACATGAAACAGTAAATGTGCCAGGTACGGGTT 2347
U89872          AAATCTATTTAATTCGCTACAATGCAAAACATGAAACAGTAAATGTGCCAGGTACGGGTT 2734
AF148644        ------------------------------------------------------------
M35524          AAATCTATTTAATTCGCTACAATGCAAAACATGAAACAGTAAATGTGCCAGGTACGGGTT 2582
M73248          AAATCTATTTAATTCGCTACAATGCAAAACATGAAACAGTAAATGTGCCAGGTACGGGTT 2344
AY122057        AAATCTATTTAATTCGCTACAATGCAAAACATGAAACAGTAAATGTGCCAGGTACGGGTT 2469
U87793          AAATCTATTTAATTCGCTACAATGCAAAACATGAAACAGTAAATGTGCCAGGTACGGGTT 2347
M11068          AAATCTATTTAATTCGCTACAATGCAAAACATGAAACAGTAAATGTGCCAGGTACGGGTT 2347
AF254640        AAATCTATTTAATTCGCTACAATGCCAAACACGAAACAGTAAATGTGCCAGGTACGGGTT 2982
AF059670        AAATCTATTTAATTCGCTACAATGCCAAACACGAAACAGTAAATGTGCCAGGTACGGGTT 2384
                                  Cry1A-F

U87397          CCTTATGGCCGCTTTCAGCCCAAAGTCCAATCGGAAAGTGTGGAGAGCCGAATCGATGCG 2407
U89872          CCTTATGGCCGCTTTCAGCCCAAAGTCCAATCGGAAAGTGTGGAGAGCCGAATCGATGCG 2794
AF148644        ------------------------------------------------------------
M35524          CCTTATGGCCGCTTTCAGCCCAAAGTCCAATCGGAAAGTGTGGAGAGCCGAATCGATTCG 2642
M73248          CCTTATGGCCGCTTTCAGCCCAAAGTCCAATCGGAAAGTGTGGAGAGCCGAATCGATGCG 2404
AY122057        CCTTATGGCCGCTTTCAGCCCAAAGTCCAATCGGAAAGTGTGGAGAGCCGAATCGATGCG 2529
U87793          CCTTATGGCCGCTTTCAGCCCAAAGTCCAATCGGAAAGTGTGGAGAGCCGAATCGATGCG 2407
M11068          CCTTATGGCCGCTTTCAGCCCAAAGTCCAATCGGAAAGTGTGGAGAGCCGAATCGATGCG 2407
AF254640        CCTTATGGCCGCTTTCAGCCCCAAGTCCAATCGGAAAATGTG------------------- 3024
AF059670        CCTTATGGCCGCTTTCAGCCCCAAGTCCAATCGGAAAATGTG------------------- 2426
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U87397          CGCCACACCTTGAATGGAATCCTGACTTAGATTGTTCGTGTAGGGATGGAGAAAAGTGTG 2467
U89872          CGCCACACCTTGAATGGAATCCTGACTTAGATTGTTCGTGTAGGGATGGAGAAAAGTGTG 2854
AF148644        ------------------------------------------------------------
M35524          CGCCACACCTTGAATGGAATCCTGACTTAGATTGTTCGTGTAGGGATGGAGAAAAGTGTG 2702
M73248          CGCCACACCTTGAATGGAATCCTGACTTAGATTGTTCGTGTAGGGATGGAGAAAAGTGTG 2464
AY122057        CGCCACACCTTGAATGGAATCCTGACTTAGATTGTTCGTGTAGGGATGGAGAAAAGTGTG 2589
U87793          CGCCACACCTTGAATGGAATCCTGACTTAGATTGTTCGTGTAGGGATGGAGAAAAGTGTG 2467
M11068          CGCCACACCTTGAATGGAATCCTGACTTAGATTGTTCGTGTAGGGATGGAGAAA-GTGTG 2466
AF254640        ------------------------------------------------------------
AF059670        ------------------------------------------------------------

U87397          CCCATCATTCGCATCATTTCTCCTTAGACATTGATGTAGGATGTACAGACTTAAATGAGG 2527
U89872          CCCATCATTCGCATCATTTCTCCTTAGACATTGATGTAGGATGTACAGACTTAAATGAGG 2914
AF148644        ------------------------------------------------------------
M35524          CCCATCATTCGCATCATTTCTCCTTAGACATTGATGTAGGATGTACAGACTTAAATGAGG 2762
M73248          CCCATCATTCGCATCATTTCTCCTTAGACATTGATGTAGGATGTACAGACTTAAATGAGG 2524
AY122057        CCCATCATTCGCATCATTTCTCCTTAGACATTGATGTAGGATGTACAGACTTAAATGAGG 2649
U87793          CCCATCATTCGCATCATTTCTCCTTAGACATTGATGTAGGATGTACAGACTTAAATGAGG 2527
M11068          CCCATCATTCGCATCATTTCTCCTTAGACATTGATGTAGGATGTACAGACTTAAATGAGG 2526
AF254640        CCCATCATTCCCATCATTTCTCCTTGGACATTGATGTTGGATGTACAGACTTAAATGAGG 3084
AF059670        CCCATCATTCCCATCATTTCTCCTTGGACATTGATGTTGGATGTACAGACTTAAATGAGG 2486


U87397          ACCTAGGTGTATGGGTGATCTTTAAGATTAAGACGCAAGATGGGCACGCAAGACTAGGGA 2587
U89872          ACCTAGGTGTATGGGTGATCTTTAAGATTAAGACGCAAGATGGGCACGCAAGACTAGGGA 2974
AF148644        ------------------------------------------------------------
M35524          ACCTAGGTGTATGGGTGATCTTTAAGATTAAGACGCAAGATGGGCACGCAAGACTAGGGA 2822
M73248          ACCTAGGTGTATGGGTGATCTTTAAGATTAAGACGCAAGATGGGCACGCAAGACTAGGGA 2584
AY122057        ACCTAGGTGTATGGGTGATCTTTAAGATTAAGACGCAAGATGGGCACGCAAGACTAGGGA 2709
U87793          ACCTAGGTGTATGGGTGATCTTTAAGATTAAGACGCAAGATGGGCACGCAAGACTAGGGA 2587
M11068          ACCTAGGTGTATGGGTGATCTTTAAGATTAAGACGCAAGATGGGCACGCAAGACTAGGGA 2586
AF254640        ACTTAGGTGTATGGGTGATATTCAAGATTAAGACGCAAGATGGCCATGCAAGACTAGGAA 3144
AF059670        ACTTAGGTGTATGGGTGATATTCAAGATTAAGACGCAAGATGGCCATGCAAGACTAGGAA 2546


U87397          ATCTAGAGTTTCTCGAAGAGAAACCATTAGTAGGAGAAGCGCTAGCTCGTGTGAAAAGAG 2647
U89872          ATCTAGAGTTTCTCGAAGAGAAACCATTAGTAGGAGAAGCGCTAGCTCGTGTGAAAAGAG 3034
AF148644        ------------------------------------------------------------
M35524          ATCTAGAGTTTCTCGAAGAGAAACCATTAGTAGGAGAAGCGCTAGCTCGTGTGAAAAGAG 2882
M73248          ATCTAGAGTTTCTCGAAGAGAAACCATTAGTAGGAGAAGCGCTAGCTCGTGTGAAAAGAG 2644
AY122057        ATCTAGAGTTTCTCGAAGAGAAACCATTAGTAGGAGAAGCGCTAGCTCGTGTGAAAAGAG 2769
U87793          ATCTAGAGTTTCTCGAAGAGAAACCATTAGTAGGAGAAGCGCTAGCTCGTGTGAAAAGAG 2647
M11068          ATCTAGAGTTTCTCGAAGAGAAACCATTAGTAGGAGAAGCGCTAGCTCGTGTGAAAAGAG 2646
AF254640        ATCTAGAATTTCTCGAAGAGAAACCATTAGTAGGAGAAGCACTAGCTCGTGTGAAAAGAG 3204
AF059670        ATCTAGAATTTCTCGAAGAGAAACCATTAGTAGGAGAAGCACTAGCTCGTGTGAAAAGAG 2606


U87397          CGGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATCGTTTATA 2707
U89872          CGGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATCGTTTATA 3094
AF148644        ------------------------------------------------------------
M35524          CGGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATCGTTTATA 2942
M73248          CGGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATCGTTTATA 2704
AY122057        CGGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATCGTTTATA 2829
U87793          CGGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATCGTTTATA 2707
M11068          CGGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATCGTTTATA 2706
AF254640        CGGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATTGTTTATA 3264
AF059670        CGGAGAAAAAATGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATTGTTTATA 2666

U87397          AAGAGGCAAAAGAATCTGTAGATGCTTTATTTGTAAACTCTCAATATGATCAATTACAAG 2767
U89872          AAGAGGCAAAAGAATCTGTAGATGCTTTATTTGTAAACTCTCAATATGATCAATTACAAG 3154
AF148644        ------------------------------------------------------------
M35524          AAGAGGCAAAAGAATCTGTAGATGCTTTATTTGTAAACTCTCAATATGATCAATTACAAG 3002
M73248          AAGAGGCAAAAGAATCTGTAGATGCTTTATTTGTAAACTCTCAATATGATCAATTACAAG 2764
AY122057        AAGAGGCAAAAGAATCTGTAGATGCTTTATTTGTAAACTCTCAATATGATCAATTACAAG 2889
U87793          AAGAGGCAAAAGAATCTGTAGATGCTTTATTTGTAAACTCTCAATATGATCAATTACAAG 2767
M11068          AAGAGGCAAAAGAATCTGTAGATGCTTTATTTGTAAACTCTCAATATGATCAATTACAAG 2766
AF254640        AAGAGGCAAAAGAATCTGTAGATGCTTTATTTGTAAACTCTCAATATGATAGATTACAAG 3324
AF059670        AAGAGGCAAAAGAATCTGTAGATGCTTTATTTGTAAACTCTCAATATGATAGATTACAAG 2726
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U87397          CGGATACGAATATTGCCATGATTCATGCGGCAGATAAACGTGTTCATAGCATTCGAGAAG 2827
U89872          CGGATACGAATATTGCCATGATTCATGCGGCAGATAAACGTGTTCATAGCATTCGAGAAG 3214
AF148644        ------------------------------------------------------------
M35524          CGGATACGAATATTGCCATGATTCATGCGGCAGATAAACGTGTTCATAGCATTCGAGAAG 3062
M73248          CGGATACGAATATTGCCATGATTCATGCGGCAGATAAACGTGTTCATAGCATTCGAGAAG 2824
AY122057        CGGATACGAATATTGCCATGATTCATGCGGCAGATAAACGTGTTCATAGCATTCGAGAAG 2949
U87793          CGGATACGAATATTGCCATGATTCATGCGGCAGATAAACGTGTTCATAGCATTCGAGAAG 2827
M11068          CGGATACGAATATTGCCATGATTCATGCGGCAGATAAACGTGTTCATAGCATTCGAGAAG 2826
AF254640        CGGATACCAACATCGCGATGATTCATGCGGCAGATAAACGCGTTCATAGCATTCGAGAAG 3384
AF059670        CGGATACCAACATCGCGATGATTCATGCGGCAGATAAACGCGTTCATAGCATTCGAGAAG 2786

U87397          CTTATCTGCCTGAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAG 2887
U89872          CTTATCTGCCTGAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAG 3274
AF148644        ------------------------------------------------------------
M35524          CTTATCTGCCTGAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAG 3122
M73248          CTTATCTGCCTGAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAG 2884
AY122057        CTTATCTGCCTGAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAG 3009
U87793          CTTATCTGCCTGAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAG 2887
M11068          CTTATCTGCCTGAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAG 2886
AF254640        CTTATCTGCCTGAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAG 3444
AF059670        CTTATCTGCCTGAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAG 2846

U87397          AAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTG 2947
U89872          AAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTG 3334
AF148644        ------------------------------------------------------------
M35524          AAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTG 3182
M73248          AAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTG 2944
AY122057        AAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTG 3069
U87793          AAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTG 2947
M11068          AAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTG 2946
AF254640        AAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTG 3504
AF059670        AAGGGCGTATTTTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTG 2906
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Cry1A-R ◄——

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U87397          ATTTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAA 3007
U89872          ATTTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAA 3394
AF148644        ------------------------------------------------------------
M35524          ATTTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAA 3242
M73248          ATTTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAA 3004
AY122057        ATTTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAA 3129
U87793          ATTTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAA 3007
M11068          ATTTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAA 3006
AF254640        ATTTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAA 3564
AF059670        ATTTTAATAATGGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAA 2966
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APPENDIX G

Comparison of sequence analysis results of Cry1A PCR.

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	92-15-F	611	2	14-F	640	20
1	92-15-F	611	3	14-fromR	865	23
1	92-15-F	611	4	211-17-F	607	22
1	92-15-F	611	5	211-17-fromR	636	30
2	14-F	640	3	14-fromR	865	5
2	14-F	640	4	211-17-F	607	1
2	14-F	640	5	211-17-fromR	636	1
3	14-fromR	865	4	211-17-F	607	81
3	14-fromR	865	5	211-17-fromR	636	84
4	211-17-F	607	5	211-17-fromR	636	78

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14-fromR          NNNNNNNNNNNNNNNNNNNNNCNNNNNNNNCNNNNNNCNANNNNNNNNNNNNNNNNTTGGN 240
211-17-F          ------------------------------------------------------------
211-17-fromR      ------------------------------------------------------------NN 2
92-15-F           ------------------------------------------------------------
14-F              ----------------------------------------------------NGCGCGCA 8


14-fromR          NNNNNNTNNGNGNANTTGNNCNNNNCN-AANANNNNNNNCNNNNNNTGGTTCCTTATGGC 299
211-17-F          ------------------------------------------------------------
211-17-fromR      NNNNNNNNNNNNNNNNNNNNNNNNNNN-NNNNNNNNNNNNNNNNNTTTNGNTNCCCCNNNN 61
92-15-F           --GGTCTGCATNGTGNATNCCNTTTTG-TNCCGGTNATACTGGAATGGGAGTNACAGATA 57
14-F              NGGACATCCATTATCCGGAAGAAACAAATAACGATTTTACCACCAAGTTTTCTGTCAACA 68


14-fromR          CGCTTAGCAAACGAGGCTCGGGACGGGGTGGATGGTTATGGCGTATCGTAGATCGGACTT 359
211-17-F          -GCCGCGTTGGTGTCGTTTATGG-GCCNTCTGTGGTATCGTTATNTCGCGACTTG----- 53
211-17-fromR      NGGGCTACAAACGAGGCTCGGGACGGGGTGGATGGTTATGGCGTATCGTAGATCGGACTT 121
92-15-F           TGATG-GCTAANNTATGTCACGATTGGTGACCTGATCCCGCTA-ANCAAGAACAGCACCG 115
14-F              TCTTCGGCGATAAAAGAGATCCATTATTCAAATGAGATAATTAATGGTTATAACTTATAA 128
                                                 **


14-fromR          -TGCTTTTCCAGGCTCCAGGCTAGAGAAGGGCTAT-TCCATATCA-GCAGCTATGCTAGC 416
211-17-F          -TGCTTTTCCAGGCTCCTGGCTAGAGAAGGGCTAT-TCCNTATCA-GCAGCTATGCTAGC 110
211-17-fromR      -TGCTTTTCCAGGCTCCAGGCTAGAGAAGGGCTAT-TCCATATCA-GCAGCTATGCTAGC 178
92-15-F           ACACNNTNTCTATCTTAACNATCGGTAAAGCCTCTCTCTACGATATGGCACNTCACTGGT 175
14-F              CTCCAGTGTTCAGTCTTGTTCGCCGGAAAGAATGGAGTTTCGCTA-TGAATCTCCTTACC 187
                     *  *                  ** *  *          *          *


14-fromR          TCGAA-AAGGACTGAAAGAACTTACATTAGGTCGTGACTTGAGGGTGAGGGGAAGGAGCG 475
211-17-F          TCGAA-AAGGACTGAAAGAACTTACATTAGGTCGTGACTTGAGGGTGAGGGGAAGGAGCG 169
211-17-fromR      TCGAA-AAGGACTGAAAGAACTTACATTAGGTCGTGACTTGAGGGTGAGGGGAAGGAGCG 237
92-15-F           NCNGACGAGGANTGAATCCNCTC-CTTTTCACCNCNNCTTTCATNNCANGNTNAGGANN- 233
14-F              TTTTAATTGGTGGGGATGGATTT-GGTTAGGTGGACATTTATGTAGCATTATAATATACA 246
                      *   **   * *     *    **          **       *     *
```

```
14-fromR          GTAAAGAAGTAAGGAGGTCGACTAGTTCTTAGCTTGATTGAACTCAGCATAGTTAGANAT 535
211-17-F          GTAAAGAAGTAAGGAGGTCGACTAGTTCTTAGCTTGATTGAACTCAGCATAGTTAGAGAT 229
211-17-fromR      GTAAAGAAGTAAGGAGGTCGACTAGTTCTTAGCTTGATTGAACTCAGCATAGTTAGAGAT 297
92-15-F           -----GAAGTCNAANTNNGTTCCANCTNTTTGANCACNTGNATNCNACNNCAANNCNATT 288
14-F              -CATTGAATGCGACACCTCATTTTGTTAATAGGAAGGGTAGAAGTGA-TTCAATAGTAAG 304
                       ***                 *  * *      *  *

14-fromR          TAGAGGAGATAGCCTTTCTTTCTATTGCTTGAGTGGAATCAGTTGACAAAAAGCTCGAAC 595
211-17-F          TAGAGGAGATAGCCTTTCTTTCTATTGCTTGAGTGGAATCAGTTGACAAAAAGCTCGAAC 289
211-17-fromR      TAGAGGAGATAGCCTTTCTTTCTATTGCTTGAGTGGAATCAGTTGACAAAAAGCTCGAAC 357
92-15-F           NNAANANAANCGGTGNTCNNCCTNNAACTNNNCT--AACNGANTGNNTGAG-GTNCAANC 345
14-F              AGGTAAAGGATAATTTTCAGCTAAAACATAATGAAGAATATATTTGCTCTA--TTTTAAA 362
                               **          *       **     *             *

14-fromR          GTAATTGACTTCTTTCTTAACTTAAATAAAGGAAGAGATGATTTTNAGAAGAATTCNTTC 655
211-17-F          GTAATTGACTTCTTTCTTAACTTAAATAAAGGAAGAGATGATTTGGAGAAGAATTCATTC 349
211-17-fromR      GTAATTGACTTCTTTCTTAACTTAAATAAAGGAAGAGATGATTTGGAGAAGAATTCATTC 417
92-15-F           TGCGNCNNCAACNCNGNCAACC--GNCCNTNANCNCNCTTACCCGCAN-------CTTCN 396
14-F              TGTTTTGGGGGAAAAGGGATATGGGTTTTGGGTCGGATATGGGTAGGATAAGTTATTTAA 422
                                    *                                     *

14-fromR          CTCTCCGNGGCAGTGAAGTGCCNTATCCTAGAGAGAGGGCTTTCCCGNTCGTTAAGATAG 715
211-17-F          CTCTCCGAG-CAGTGAAGTGCCATATCCTAGAGAGAGGGCTTTACCGGTCGTTAAGATAG 408
211-17-fromR      CTCTCCGAG-CAGTGAAGTGCCATATCCTAGAGAGAGGGCTTTACCGGTCGTTAAGATAG 476
92-15-F           CNCCCNCANCNNNTTCANNNNCNCNNCCNNANTCGAACNANNCNTTACAAGTTCGAGTNN 456
14-F              AAGAGGGNTATAATTGAACCATAAAATATGGATAATAAAAGGGTTTAATGGGTCGGATTT 482
                             *  *                                  * *    *

14-fromR          AGNTTGGTGTCGGT-AGCTGTTCTTGCTTAGCGGGATCAGGTCGCCACATCCGGTGAGCA 774
211-17-F          AGTT-GGTGTCGGT-AGCTGTTCTTGCTTAGCGGGATCAGGTCACCAA--TCGGTGA-CA 463
211-17-fromR      AGTT-GGTGTCGGT-AGCTGTTCTTGCTTAGCGGGATCAGGTCACCAA--TCGGTGA-CA 531
92-15-F           CTGTCCCNNCNCAN-ANNCNCNCGCNCATNNNCNACNNGNNNCNTCGN--TCNNCNCTCG 513
14-F              TTAGGGGCATATTTGACCCTTTCTCTTTTGTAATATAAATGCCACGATGTAATATTAAAA 542
                             *      *     *             *

14-fromR          TAGTTTAGGAATGAATNTCCGTANCTCCCATNCCAGTACTTAANGCTGACAAAATGGAAT 834
211-17-F          TAGTTTAGGAATGAATATCTGTAACTCCCATTCCAGTACTTAAA-CTGACAAAATGGAAT 522
211-17-fromR      TAGTNTAGGAATNAATATNNGNNACTCCCNATCCGNNNNTNAAANGGCATNANGCGATNG 591
92-15-F           CCACNCCGN--TNNAATNTNGCNNNTGNCATGACCGCNNCNNTCACNNCCGNTGNCTCCN 571
14-F              TGACGTGGAAAATCCATTTGGGCAATTAAAGAAAAGTAAAATGCGCTG--GATATTTTGA 600
                         *           *    *

14-fromR          AAACAATCAAGACCTGNTCCAGG--CANCCTCC---------------------------- 865
211-17-F          AAACAATCAAGACCTGGCTCAAGGCTAACCCCTCCACACTCAGGGTCAGCGGGNAAAANG 582
211-17-fromR      GAACNGNNAAGGNCATGNGNACANGGGAACGNAAANGGCANGGCG---------------- 636
92-15-F           CNNNNCTCNTN-CCNTCNCCNCATCNNGNNANCCATNNCTC-------------------- 611
14-F              CGTGGCCAACTAACG-CTNAANGGGAANCCAAANNNNNNNNN-------------------- 640
                             *

14-fromR          -------------------------
211-17-F          GGGGGNNGGGNANNNNNNNNNNNNN 607
211-17-fromR      -------------------------
92-15-F           -------------------------
14-F              -------------------------
```

APPENDIX H

Comparison of automatic and clone sequence analysis results of Cry1A PCR.

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	2A	639	2	1A	639	94
1	2A	639	3	14-fromR	865	87
1	2A	639	4	211-17-F	607	88
1	2A	639	5	211-17-fromR	636	81
2	1A	639	3	14-fromR	865	86
2	1A	639	4	211-17-F	607	89
2	1A	639	5	211-17-fromR	636	81
3	14-fromR	865	4	211-17-F	607	81
3	14-fromR	865	5	211-17-fromR	636	84
4	211-17-F	607	5	211-17-fromR	636	78

```
2A                --------------NNNGCTCTGCGTCGAGGGCCCGGGATCCGATTGGTTCCTTATGGCC 46
1A                ---------------------------------------------GGTTCCTTATGGCC 14
211-17-F          ------------------------------------------------------------
14-fromR          NNNNNNTNNGNGNANTTGNNCNNNNCNAANANNNNNNNCNNNNNNTGGTTCCTTATGGCC 300
211-17-fromR      NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTTTNGNTNCCCCNNNNN 62


2A                GCTTAGCAAACGAGGCTCGGGACGGGGTGGATGGTTATGGCGTATCGTAGATCGGACTTT 106
1A                GCTTAGCAAACGAGGCTCGGGACGGGGTGGATGGTTATGGCGTATCGTAGATCGGACTTT 74
211-17-F          GCCGCGTTGGTGTCGTTTATGG-GCCNTCTGTGGTATCGTTATNTCG-----CGACTTGT 54
14-fromR          GCTTAGCAAACGAGGCTCGGGACGGGGTGGATGGTTATGGCGTATCGTAGATCGGACTTT 360
211-17-fromR      GGGCTACAAACGAGGCTCGGGACGGGGTGGATGGTTATGGCGTATCGTAGATCGGACTTT 122
                  *          *  * *   *  *   *   ****   *   * ***     **   * *

2A                GCTTTTCCAGGCTCCAGGCTAGAGAAGGGCTATTCCATATCAGCAGCTATGCTAGCTCGA 166
1A                GCTTTTCCAGGCTCCAGGCTAGAGGAGGGCTATTCCATATCAGCAGCTATGCTAGCTCGA 134
211-17-F          GCTTTTCCAGGCTCCTGGCTAGAGAAGGGCTATTCCNTATCAGCAGCTATGCTAGCTCGA 114
14-fromR          GCTTTTCCAGGCTCCAGGCTAGAGAAGGGCTATTCCATATCAGCAGCTATGCTAGCTCGA 420
211-17-fromR      GCTTTTCCAGGCTCCAGGCTAGAGAAGGGCTATTCCATATCAGCAGCTATGCTAGCTCGA 182
                  *************** ******** *********** ***********************

2A                AAAGGACTGAAAGAACTTACATTAGGTCGTGACTTGAGGGTGAGGGGAAGGAGCGGTAAA 226
1A                AAAGGACTGAAAGAACTTACATTAGGTCGTGACTTGAGGGTGAGGGGAAGGAGCGGTAAA 194
211-17-F          AAAGGACTGAAAGAACTTACATTAGGTCGTGACTTGAGGGTGAGGGGAAGGAGCGGTAAA 174
14-fromR          AAAGGACTGAAAGAACTTACATTAGGTCGTGACTTGAGGGTGAGGGGAAGGAGCGGTAAA 480
211-17-fromR      AAAGGACTGAAAGAACTTACATTAGGTCGTGACTTGAGGGTGAGGGGAAGGAGCGGTAAA 242
                  ************************************************************

2A                GAAGTAAGGAGGTCGACTAGTTCTTAGCTTGATTGAACTCAGCATAGTTAGAGATTAGAG 286
1A                GAAGTAAGGAGGTCGACTAGTTCTTAGCTTGATTGAACTCAGCATAGTTAGAGATTAGAG 254
211-17-F          GAAGTAAGGAGGTCGACTAGTTCTTAGCTTGATTGAACTCAGCATAGTTAGAGATTAGAG 234
14-fromR          GAAGTAAGGAGGTCGACTAGTTCTTAGCTTGATTGAACTCAGCATAGTTAGANATTAGAG 540
211-17-fromR      GAAGTAAGGAGGTCGACTAGTTCTTAGCTTGATTGAACTCAGCATAGTTAGAGATTAGAG 302
                  ************************************************* *******
```

```
2A               GAGATAGCCTTTCTTTCTATTGCTTGAGTGGAATCAGTTGACAAAAAGCTCGAACGTAAT 346
1A               GAGATAGCCTTTCTTTCTATTGCTTGAGTGGAATCAGTTGACAAAAAGCTCGAACGTAAT 314
211-17-F         GAGATAGCCTTTCTTTCTATTGCTTGAGTGGAATCAGTTGACAAAAAGCTCGAACGTAAT 294
14-fromR         GAGATAGCCTTTCTTTCTATTGCTTGAGTGGAATCAGTTGACAAAAAGCTCGAACGTAAT 600
211-17-fromR     GAGATAGCCTTTCTTTCTATTGCTTGAGTGGAATCAGTTGACAAAAAGCTCGAACGTAAT 362
                 ************************************************************

2A               TGACTTCTTTCTTAACTTAAATAAAGGAAGAGATGATTTGGAGAAGAATTCATTCCTCTC 406
1A               TGACTTCTTTCTTAACTTAAATAAAGGAAGAGATGATTTGAAGAAGAATTCATTCCTCTC 374
211-17-F         TGACTTCTTTCTTAACTTAAATAAAGGAAGAGATGATTTGGAGAAGAATTCATTCCTCTC 354
14-fromR         TGACTTCTTTCTTAACTTAAATAAAGGAAGAGATGATTTTNAGAAGAATTCNTTCCTCTC 660
211-17-fromR     TGACTTCTTTCTTAACTTAAATAAAGGAAGAGATGATTTGGAGAAGAATTCATTCCTCTC 422
                 ************************************  ********** ********

2A               CGAG-CAGTGAAGTGCCATATCCTAGAGAGAGGGCTTTACCGGTCGTTAAGATAGAGTT- 464
1A               CGAG-CAGTGAAGTGCCATATCCTAGAGAGAGGGCTTCACCGGTCGTTAAGATAGAGTT- 432
211-17-F         CGAG-CAGTGAAGTGCCATATCCTAGAGAGAGGGCTTTACCGGTCGTTAAGATAGAGTT- 412
14-fromR         CGNGGCAGTGAAGTGCCNTATCCTAGAGAGAGGGCTTTCCCGNTCGTTAAGATAGAGNTT 720
211-17-fromR     CGAG-CAGTGAAGTGCCATATCCTAGAGAGAGGGCTTTACCGGTCGTTAAGATAGAGTT- 480
                 ** * ************ ******************  *** ************** *

2A               GGTGTCGGTAGCTGTTCTTGCTTAGCGGGATCAGGTCACCAA--TCGGTGA-CATAGTTT 521
1A               GGTGTCGGTAGCTGTTCTTGCTTAGCGGGATCAGGTCACCAA--TCGGTGA-CATAGTTT 489
211-17-F         GGTGTCGGTAGCTGTTCTTGCTTAGCGGGATCAGGTCACCAA--TCGGTGA-CATAGTTT 469
14-fromR         GGTGTCGGTAGCTGTTCTTGCTTAGCGGGATCAGGTCGCCACATCCGGTGAGCATAGTTT 780
211-17-fromR     GGTGTCGGTAGCTGTTCTTGCTTAGCGGGATCAGGTCACCAA--TCGGTGA-CATAGTNT 537
                 ************************************ ***    ****** ****** *

2A               AGGAATGAATATCTGTAACTCCCATTCCAGTACTTAAA-CTGATAAAATGGAATAAACAA 580
1A               AGGAATGAATATCTGTAACTCCCATTCCAGTACTTAAA-CTGACAAAATGGAATAAACAA 548
211-17-F         AGGAATGAATATCTGTAACTCCCATTCCAGTACTTAAA-CTGACAAAATGGAATAAACAA 528
14-fromR         AGGAATGAATNTCCGTANCTCCCATNCCAGTACTTAANGCTGACAAAATGGAATAAACAA 840
211-17-fromR     AGGAATNAATATNNGNNACTCCCNATCCGNNNNTNAAANGGCATNANGCGATNGGAACNG 597
                 ****** *** *  *   *****   **     * **     *  *   *     ***

2A               TCAAGACCTGGCCCAAGGCTAACCCCCCCACACTCAGGGTCAGGAACGTGAAAGGGCAT- 639
1A               TCAAGACCTGGCTCAAGGCTAACCCCTCCACACTCAGGGTCAGGAACGTGAAAGGGCATG 608
211-17-F         TCAAGACCTGGCTCAAGGCTAACCCCTCCACACTCAGGGTCAGCGG-GNAAAANGGGGGG 587
14-fromR         TCAAGACCTGNTCCAGG--CANCCTCC---------------------------------- 865
211-17-fromR     NNAAGGNCATGNGNACANGGGAACGNAAANGGCANGGCG---------------------- 636
                   ***  *      *        *

2A               --------------------------------
1A               AATCGGATCCCGGGCCCTCGACGCGAGCTNC 639
211-17-F         NNGGGNANNNNNNNNNNNNN----------- 607
14-fromR         --------------------------------
211-17-fromR     --------------------------------
```

APPENDIX I

Nucleotide-nucleotide BLAST analysis results of sequenced 415 bp Cry1Ac fragment.

Sequences producing significant alignments: (bits) Value

gi|23344759|gb|AY122057.1|	Bacillus thuringiensis insectici...	42	1.1
gi|29293656|gb|AY225453.1|	Bacillus thuringiensis Cry1Ac (c...	42	1.1
gi|2584728|emb|AJ002514.1|BTAJ2514	Bacillus thuringiensis k...	42	1.1
gi|40274|emb|X54159.1|BTCRYIAC	Bacillus thuringiensis cryIA...	42	1.1
gi|3979716|emb|AJ130970.1|BTH130970	Bacillus thuringiensis ...	42	1.1
gi|46409856|gb|AY570733.1|	Bacillus thuringiensis serovar k...	42	1.1
gi|33321715|gb|AF492767.1|	Bacillus thuringiensis Cry1Ac ge...	42	1.1
gi|5052773|gb|AF148644.1|AF148644	Bacillus thuringiensis in...	42	1.1
gi|1842094|gb|U87397.1|BTU87397	Bacillus thuringiensis kurs...	42	1.1
gi|42717975|gb|AY525369.1|	Synthetic construct cry1AcAT mod...	42	1.1
gi|1888558|gb|U89872.1|BTU89872	Bacillus thuringiensis Cry1...	42	1.1
gi|1839245|gb|U87793.1|BTU87793	Bacillus thuringiensis kurs...	42	1.1
gi|1171234|gb|U43606.1|BTU43606	Bacillus thuringiensis cryI...	42	1.1
gi|143125|gb|M73249.1|BACKURS	Bacillus thuringiensis gene, ...	42	1.1
gi|142741|gb|M73248.1|BACCRYIACC	Bacillus thuringiensis (cr...	42	1.1
gi|142739|gb|M35524.1|BACCRYIA	B.thuringiensis delta-endoto...	42	1.1
gi|142721|gb|M11068.1|BACCRSG	B.thuringiensis 75 kb plasmid...	42	1.1

Alignments

>gi|23344759|gb|AY122057.1| Bacillus thuringiensis insecticidal crystal protein Cry1Ac (cry1Ac)
gene, complete cds
Length = 3659

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

```
Query: 30   tgaatcggggttacagaagca 50
             ||||||||||||||||||||
Sbjct: 1741 tgaatcggggttacagaagca 1721
```

>gi|29293656|gb|AY225453.1| Bacillus thuringiensis Cry1Ac (cry1Ac) gene, complete cds
Length = 3537

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

```
Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 1619 tgaatcggggttacagaagca 1599
```

>gi|2584728|emb|AJ002514.1|BTAJ2514 Bacillus thuringiensis kurstaki cry218 gene
Length = 4190

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

```
Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 2006 tgaatcggggttacagaagca 1986
```

>gi|40274|emb|X54159.1|BTCRYIAC Bacillus thuringiensis cryIA(c) gene for insecticidal crystal protein
(Lepidoptera specific) (partial)
Length = 2192

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

```
Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 1954 tgaatcggggttacagaagca 1934
```

>gi|3979716|emb|AJ130970.1|BTH130970 Bacillus thuringiensis gene encoding crystal toxin protein
Length = 3692

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

```
Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 1774 tgaatcggggttacagaagca 1754
```

>gi|46409856|gb|AY570733.1| Bacillus thuringiensis serovar kurstaki clone 44 cry1A type crystal
protein gene, partial cds
Length = 2381

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

```
Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 1595 tgaatcggggttacagaagca 1575
```

>gi|33321715|gb|AF492767.1| Bacillus thuringiensis Cry1Ac gene, complete cds
Length = 3534

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

```
Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 1616 tgaatcggggttacagaagca 1596
```

>gi|5052773|gb|AF148644.1|AF148644 Bacillus thuringiensis insecticidal protein Cry1Ac (cry1Ac) gene,
partial cds
Length = 2169

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

Query: 30 tgaatcggggttacagaagca 50
||||||||||||||||||||
Sbjct: 1616 tgaatcggggttacagaagca 1596

>gi|1842094|gb|U87397.1|BTU87397 Bacillus thuringiensis kurstaki crystal protein (CryIA(c)) gene,
complete cds
Length = 3693

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

Query: 30 tgaatcggggttacagaagca 50
||||||||||||||||||||
Sbjct: 1771 tgaatcggggttacagaagca 1751

>gi|42717975|gb|AY525369.1| Synthetic construct cry1AcAT modified toxin gene, complete cds
Length = 2028

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

Query: 30 tgaatcggggttacagaagca 50
||||||||||||||||||||
Sbjct: 1619 tgaatcggggttacagaagca 1599

>gi|1888558|gb|U89872.1|BTU89872 Bacillus thuringiensis Cry1Ac delta-endotoxin gene, complete cds
Length = 4178

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

Query: 30 tgaatcggggttacagaagca 50
||||||||||||||||||||
Sbjct: 2006 tgaatcggggttacagaagca 1986

>gi|1839245|gb|U87793.1|BTU87793 Bacillus thuringiensis kurstaki insecticidal delta-endotoxin CryIA(c)
(cryIA(c)) gene, complete cds
Length = 5319

Score = 42.1 bits (21), Expect = 1.1
Identities = 21/21 (100%)
Strand = Plus / Minus

Query: 30 tgaatcggggttacagaagca 50
||||||||||||||||||||
Sbjct: 2594 tgaatcggggttacagaagca 2574

>gi|1171234|gb|U43606.1|BTU43606 Bacillus thuringiensis cryIA(c) gene, partial cds
          Length = 1821
 Score = 42.1 bits (21), Expect = 1.1
 Identities = 21/21 (100%)
 Strand = Plus / Minus

Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 1616 tgaatcggggttacagaagca 1596

>gi|143125|gb|M73249.1|BACKURS Bacillus thuringiensis gene, complete CDS
          Length = 3537

 Score = 42.1 bits (21), Expect = 1.1
 Identities = 21/21 (100%)
 Strand = Plus / Minus

Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 1619 tgaatcggggttacagaagca 1599

>gi|142741|gb|M73248.1|BACCRYIACC Bacillus thuringiensis (cryIA(c)3) gene, complete
CDS
          Length = 3534

 Score = 42.1 bits (21), Expect = 1.1
 Identities = 21/21 (100%)
 Strand = Plus / Minus

Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 1616 tgaatcggggttacagaagca 1596

>gi|142739|gb|M35524.1|BACCRYIA B.thuringiensis delta-endotoxin gene, complete cds
          Length = 4320

 Score = 42.1 bits (21), Expect = 1.1
 Identities = 21/21 (100%)
 Strand = Plus / Minus

Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 1854 tgaatcggggttacagaagca 1834

>gi|142721|gb|M11068.1|BACCRSG B.thuringiensis 75 kb plasmid crystal protein gene
and flanks
          Length = 4300

 Score = 42.1 bits (21), Expect = 1.1
 Identities = 21/21 (100%)
 Strand = Plus / Minus

Query: 30   tgaatcggggttacagaagca 50
            ||||||||||||||||||||
Sbjct: 2006 tgaatcggggttacagaagca 1986