# DEVELOPMENT OF ANALYSIS METHODS FOR *CRY1AC* AND *SAM-K* GENE LINES IN TOMATO USING PCR AND REAL-TIME PCR

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

SAHRA UYGUN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

MAY 2010

Approval of the thesis:

**DEVELOPMENT OF ANALYSIS METHODS FOR *CRY1AC* AND *SAM-K* GENE LINES IN TOMATO USING PCR AND REAL-TIME PCR**

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**I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.**

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# ABSTRACT

DEVELOPMENT OF ANALYSIS METHODS FOR *CRY1AC* AND *SAM-K* GENE LINES IN TOMATO USING PCR AND REAL-TIME PCR

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May 2010, 174 pages

Genetically modified organisms are entering the food chain in all over the world. In order to have transparency in the foods that are being consumed, there is a need to trace the genetically modified organisms (GMOs) in the market and consequently, this brings the necessity of analytical methods that are capable of detecting, identifying and quantifying the transgenic events. These analytical methods also form the basis of the labeling regulations that are tried to be formed regarding GMOs.

The main aim of this study is to develop and apply the detection methods for the two of the tomato events, delayed ripening and insect resistant. Currently the only validated detection methods are mainly for the corn, soybean, and cotton. There is no validated detection method for tomato. Tomato is one of the most consumed food products in Turkey and it is also among the controversial organisms in terms of genetic modification and labeling, therefore the analysis of the genetic modifications in tomato is crucial.

In this study, DNA-based detection is performed, with polymerase chain reaction (PCR) being the chosen method of study. In order to detect the GMO-derived DNA, the method of analysis includes the following studies: species-specific, screening, gene-specific, construct-specific and inverse PCR. In addition, the quantification method is developed

using the real-time PCR. In order to develop the procedure of identification method, the reference samples are used and the unknown varieties that are to be analyzed using this method are expected to have similarities with the authorized transgenic events.

**Keywords:** GMOs, detection methods, tomato, delayed ripening, insect resistant

# ÖZ

DOMATESTEKİ *CRY1AC* VE *SAM-K* GEN HATLARI İÇİN PZR VE EŞ-ZAMANLI PZR KULLANILARAK ANALİZ METODLARININ GELİŞTİRİLMESİ

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Genetiği değiştirilmiş gıdalar dünyanın bir çok yerinde beslenme zincirinde yerini almıştır. Tüketilen gıdaların besin içeriği açısından şeffaflığını sağlamak için piyasadaki genetiği değiştirilmiş organizmaları (GDO) belirlemek ve onların izlenebilirliğini sağlamak gerekmektedir. Bu da, transgenik içeriğin saptanması, tanımlanması ve miktarının belirlenmesi için analitik yöntemlere ihtiyaç olduğunu göstermektedir. Ayrıca, bu analitik metotlar GDOların etiketlenmesi ile ilgili olan düzenlemelerin de temelini oluşturmaktadır.

Bu çalışmanın başlıca amacı domateste olgunlaşması geciktirilmiş ve böcek direnci olan ürünlerin tanımlanması için metot oluşturmak ve uygulamaktır. Yapılan çalışmalarda mısır, soya ve pamuk için transgenik içeriği saptama yöntemlerinin geçerliliği kanıtlanmıştır. Buna karşın, domateste transgenik içeriği saptamak amaçlı geliştirilen yöntemler resmi bir otorite tarafından onaylanmamıştır. Domates, Türkiyede en çok tüketilen gıda ürünleri arasındadır ve ayrıca genetik modifikasyonlar açısından da tartışmaya yol açan bir üründür, bu yüzden domatesteki genetik modifikasyonların araştırılması önemlidir.

Bu çalışmada DNA üzerinden belirleme çalışması yapılmıştır ve polimeraz zincir reaksiyonu (PZR) çalışma metodu olarak şeçilmiştir. GDO'dan elde edilmiş DNA'nın belirlenmesi için uygulanan analiz metotları; tür-spesifik, tarama, gen-spesifik, konstrakt-spesifik ve invers PZR yöntemlerini kapsamaktadır. Ayrıca, miktar belirleme metodu da eş-zamanlı PZR kullanılarak geliştirilmiştir. Tanımlama metodundaki prosedürün gelistirilmesi için referans örnekler kullanılmıştır ve bu metot kullanılarak analiz edilen bilinmeyen örneklerin izin verilen transgenik ürünlerle benzerlik göstermesi beklenmiştir.

**Anahtar Kelimerler:** GDO, transgenik ürünlerin belirlenmesi, domates, olgunlaşması geciktirilmiş, böcek direnci

To My Parents; Ayşegül, Öner

# ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Assoc. Prof. Dr. G Candan Gürakan, for her guidance, support and patience throughout this study. I am very grateful for her invaluable advices.

I would also like to express my gratitude to my co-supervisor, Dr. Remziye Yılmaz, for her endless support during this study. I am very grateful for her guidance and insight in every aspect.

I also wish to express my appreciation to Prof. Dr. Meral Yücel, for kindly letting me conduct some of my experiments in METU Central Laboratory. Her encouragement made things easier in this study. I would like to extend my appreciation to Dr. Can Özen for his suggestions. His scientific approach always inspired me.

My thanks are extended to Betül Söyler, Sümeyra Gürkok and Cengiz Baloğlu for their help and suggestions and to my friends Bengü Öztürk, Leyla Nesrin Kahyaoğlu and Çağrı Taşar for their love and friendship.

I also want to thank the Scientific and Technological Research Council of Turkey, TUBITAK and the Unit of this Council, BIDEB for the financial support during this study.

Finally I have to say that I completed this study with the support and love from my family. I also would like to thank them for their patience and positive approach. I dedicate this study to my parents who are always there for me.

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A260: Absorbance value at the 260nm

*aad*: 3"(9)-O-aminoglycoside adenylyltransferase

APHIS: The Animal and Plant Health Inspection Service

ARDB:Antibiotic Resistance Genes Database

BCH: Biosafety Clearing House

BGSC: Bacillus Genetic Stock Center

BMELV: German Federal Ministry of Food, Agriculture and Consumer Protection

bp: Base pair

Bt: *Bacillus thruringiensis*

BgVV: German Institute for Consumer Health Protection and Veterinary Medicine

CaMV: Cauliflower Mosaic Virus

cDNA: complementary DNA

CFIA: Canadian Food Inspection Agency

CIBIOGEM: Mexican Comision Intersecretarial de Bioseguridad de los Organismos Gene´ticamente Modificados

CONABIA: Comision Nacional Asesora de Biotecnologia Agropecuaria

CORESTA: Cooperative Centre for the Scientific Research Relative to Tobacco

Cp: Crossing point

CRM: Certified reference material

Ct: Threshold cycle

CTAB: Cetyltrimethylammonium bromide

CTNBio: National Biosafety Technical Commission

Dir. for Nat.: Norwegian Directorate for Nature Management

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide

DR: Delayed ripening

EC: European Commission

EDTA: Ethylenediaminetetraacetic acid

EFSA: European Food Safety Authority

ELISA: Enzyme-linked immunosorbant assay

ENGL: European Network of GMO laboratories

EPA: Environmental Protection Agency

EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase

ERMA: Environmental Risk Management Authority New Zealand

EtBr: Ethidium Bromide

EU: European Union

FAO: Food and Agriculture Organization

FDA: Food and Drug Administration

FFDCA: Federal Food, Drug and Cosmetic Act

FIFRA: Federal Insecticide, Fungicide and Rodenticide Act

FSANZ: Food Standards Australia New Zealand

GBE: Green Biotechnology Europe

GHCL: Guanidine hydrochloride

GMDD: GMO Detection Method Database

GMOs: Genetically Modified Organisms

GMODL-SJTU: GMO Detection Laboratory in Shanghai Jiao Tong University

GM: Genetically modified

HGE: Haploid genome equivalent

HPLC: High performance liquid chromatography

IARI: Indian Agricultural Research Institute

IPCR: Inverse PCR

IR: Insect resistant

IRMM: Institute for Reference Materials and Measurements

ISAAA: International Service for the Acquisition of agri-biotech applications

ISO: International Organization for Standardization

JRC: Joint Research Centre

KKGM: Koruma ve Kontrol Genel Müdürlüğü

LB: Lysogeny broth

LC: LightCycler Roche Real-Time Systems

LFS: Lateral flow strips

µg: micro gram

µl: micro liter

mM: milimolar

MALDI-TOF: Matrix-assisted laser desorption/ionization- time-of-flight mass spectrometer

NASBA: Nucleic Acid Sequence-Based Amplification

NCBI: National Center for Biotechnology Information

NCBP: National Committee on Biosafety of the Philippines

NIRS: Near Infrared Spectroscopy

NMR: Nuclear Magnetic Resonance

*nptII*: neomycin-phosphotransferase II

OECD: Organization for Economic Co-operation and Development

OGTR: Office of the Gene Technology Regulator

PCR: Polymerase Chain Reaction

PPA: Plant Protection Act

PVP: Polyvinylpyrrolidone

RA: Risk Assessment

RDA: Recommended daily allowance

RIKILT: Institute of Food Safety, Wageningen University and Research Center

RNA: Ribonucleic acid

*sam-k*: S-Adenosyl methionine

SDS: Sodium dodecyl sulfate

SECB: Swiss Expert Committee for Biosafety

SGN: SOL Genomics Network

SOL: International *Solanaceae* Genomics Project

TAE: Tris-acetate-EDTA

TAGEM: Tarımsal Araştırmalar Genel Müdürlüğü

TBE: Tris-borate-EDTA

Ti: Tumor inducing

ToLCV: Tomato leaf curl virus

TUBITAK: Türkiye Bilimsel ve Teknolojik Araştırma Kurumu

TUGEM: Tarımsal Üretim ve Geliştirme Genel Müdürlüğü

TSCA: Toxic Substances Control Act

USDA: Unites States Deparment of Agriculture

qPCR: Quantitative Polymerase Chain Reaction

WTO: World Trade Organization

# CHAPTER 1

# INTRODUCTION

## 1.1 Genetic Modifications

Genetically modified organisms (GMOs) are the organisms whose genetic material has been altered using the recombinant DNA technology. This change in genetic material can be an addition of a novel gene or a deletion of the existing gene (Ovesna et al, 2008). In some cases the expression of a gene can be altered. New qualities could be added or existing characteristics could be changed according to the modification. In most cases of GMOs, there is a gene construct that is inserted into the genome of the recipient organism and this method is called transformation (Holst-Jensen, 2001).

The gene construct or the gene cassette that is inserted into the genome of the organisms consists of basic elements like the promoter for the control of the gene expression, the gene for conferring a specific feature and the terminator which ends the expression of the gene. Apart from these basic elements, there are also other elements whose function is to stabilize the gene construct and also to show the presence of the construct in the genome. These elements can be broadly classified as enhancers and marker gene consecutively (Holst-Jensen, 2001).

In addition to biotechnological methods, conventional breeding of the plants also forms different varieties and hybrids that have desired qualities. However, biotechnological methods enable one to add qualities from unrelated organisms like in the case of addition of a bacterial toxin gene to confer insect resistance in a crop plant. These new qualities can be related to minimizing the overall cost and also the environmental pollution (Adugna and Mesfin, 2008).

Apart from the technology behind these organisms, the debate related to the genetic modifications in the food chain is continuing. The reason for the ongoing arguments about these organisms involves health issues, consumer rights and also transparency in the food chain. These issues reflect the need for the detection and identification of individual transgenic events.

### 1.2 Transgenic Events

The genetic lines that are commercialized worldwide can be categorized into groups as the first, second and third generation GMOs. First generation GMOs have the attributes of herbicide tolerance, insect and viral resistance which are mostly profitable to the grower of these organisms (Burton et al, 2002). These traits could be added to the organisms without any change of other characteristics related to the taste or appearance of the final organism. It can be said that they are producer-oriented (Celec et al, 2005). Second generation GMOs have enhanced qualities which propose to provide direct benefits to the consumers. These organisms have improved output traits rather than having improved input traits as in the case of first generation GMOs. The improved output traits can be related to the altered ripening of the product or modified color of the end-product. Similar to the second generation of GMOs, the third generation consists of consumer-oriented products like the enhanced nutritional values of organisms or the use of GMOs for vaccination. Improvement of organisms for producing functional end-products is the main aim of the third generation GMOs (Celec et al, 2005).

While in early forms of GMOs, there is only single trait changes, in recently developed forms of GMOs, multiple agronomic traits are combined through gene stacking or pyramiding. Instead of possessing single agronomic trait genes, the future GMOs may be transformed with gene clusters like encoding partial or complete synthetic pathways. Artificial, engineered chromosomes can be formed and accordingly combining the desired genes on stable inheritable chromosomes (Holst-Jensen, 2009).

After the development of the transgenic organisms, there have been debates related to the benefits and the risks of these organisms in various aspects. It is not enough to comment on GMOs as completely safe or take them as purely a risk element. It is important to consider that the GMOs do not represent a single technology. Each novel organism that is developed should be considered differently considering the potential risks and benefits

related to health and environment. Also the opinions of the scientists, private companies and regulators should be taken into consideration (Pretty and Hine, 2001).

The potential risks of the GMOs should be assessed taking the foreseen hazards into consideration. Some of the risks that are considered are as follows: Horizontal gene flow (gene exchange), new forms of resistance caused by the antibiotic genes inserted, effects of novel toxin products, changes in farm practice leading to loss of biodiversity, allergic immune reactions (Pretty and Hine, 2001).

The potential benefits of the GMOs differ according to the categories/generations that were outlined in the beginning of this section. Some of the characteristics that are added to the GMOs can be related to the altered amino acid or fatty acid composition, fertility restoration, herbicide tolerance, insect resistance, male sterility, altered color, virus resistance (AGBIOS, http://www.agbios.com).

These potentials risks and benefits could give a general idea about the characteristics of the transgenic events; however, each individual event of GMO should be assessed on its own before its commercialization and placement on the market.

### 1.3 Detection and Identification of Transgenic Events

The ability to detect the presence of genetic modifications in a sample is essential to ensure the food safety and also to give the choice to the consumer about their diets. The identification methods are formed in order to ensure the full traceability of the GMOs at all stages, from consumer choices to post-market studies for the GMO products (Zel et al, 2008).

In the gene cassette that is inserted, there are some common elements of promoters, terminators and marker genes. In addition, the mostly added attributes like insect resistance and herbicide tolerance have also common gene insertions. The most commonly used elements and genes have the following sources: The Cauliflower mosaic virus (CaMV) is the source of 35S promoter and 35S terminator, the soil bacterium *Agrobacterium tumefaciens* is the source of the 3'-nos terminator, the plant *Petunia hybrida* is the source of EPSPS gene encoding tolerance to the herbicide Roundup event, and the soil bacterium *Bacillus thuringiensis* is the source of cry-genes encoding insecticidal toxins (Holst-Jensen, 2007). These elements are important for the detection of

genetically modified organisms and are among the first elements that are tried to be assessed to judge on the existence of any genetic modification in the samples; screening for these common elements is essential for GMO analysis.

Screening is an important initial step for the analysis of GMOs. However it is also important to mention that the common elements of GMOs like the promoter 35S and the terminator nos exist naturally in CaMV and in the plant virus Ti plasmid respectively (Wen-Tao et al, 2009) and this increases the possibility of a contamination in the organisms that are being studied rather than any transgene that have been inserted in their genome. It is also known that the common marker genes (*nptII*: kanamycin/neomycin resistance and *aadA*: streptomycin/spectinomycin resistance) that are used in the transgenic events are also widespread in nature which also increases the possibility of a false-positive result in the analysis (Wen-Tao et al, 2009), (Statement of EFSA, 2009).

While screening of the samples for the genetic modifications gives ideas about the presence of an inserted gene cassette, it is more accurate to perform additional analysis for the presence of the gene construct. This is required for the identification and further confirmation of the detection of the GMOs.

Qualitative and also quantitative detection and identification of GMOs are required because there is a need i) to discriminate between approved and unapproved material, ii) to identify safe or potentially unsafe material, iii) to certificate the purity of identity of the material. In addition, quantitative analysis of GMOs is required to control for the legal standards of labeling thresholds. These analyses also play a role in the safety assessment and risk management of GMOs by enabling the tracing and also the drawback of the material, in a wide time frame: from the date of the characterization of the GMO, to their placement on the market (Holst-Jensen, 2009).

### 1.3.1 Reference Materials

Reference materials are essential for any detection study because the detection must be performed with comparison to known identities.

Reference materials are needed both for qualitative and quantitative detection. For qualitative detection they are used for the visual comparison of the results. For quantitative detection they are used for the formation of calibration curves. In this case, certified

reference materials (CRMs) or plasmids harboring the required sequence can be used. CRMs are the standards that are used as reference materials for GMOs. Starting from 1997, certified reference materials for various GM events were produced at Institute for Reference Materials and Measurements (IRMM) in European Union. CRMs are only available for certain crop species like maize, soybean, rapeseed, cotton, potato and sugar beet (Marmiroli et al, 2008) and they are supplied in powder form. GMO certified reference material powders that are available, are produced in concentrations from 0 % to 5 or 10 % GMO according to the event (European Commission Joint Research Centre, http://ec.europa.eu/dgs/jrc/index.cfm).

With the CRMs being available only for a limited number of species, there are various studies that are conducted with plasmid DNA in order to perform quantification of the GMOs (Taverniers et al, 2004), (Mattarucchi et al, 2005) (Taverniers et al, 2005), (Zhang et al, 2008). Indeed in these studies, it was confirmed that the plasmid DNA is commutable for using it as standards of detection and quantification.

### 1.3.2 DNA Based Detection Methods

The GMOs are the result of genetic modifications and consequently the most direct method to detect them are the ones that aim the genetic modification itself. In this case it is the modified DNA (Miraglia et al, 2004). In this respect, the major advantages of DNA based detection methods of GMOs are specificity and sensitivity.

There are also some limitations of the DNA based detection methods. The cost of the method and the competence that is required to conduct the analysis are some drawbacks. In addition, sequence information of the genetic modifications is mostly kept confidential by the companies that produce the events (Holst-Jensen et al, 2003). This fact makes the detection difficult while trying to identify the inserted construct.

Polymerase chain reaction is the dominating DNA based detection method of GMO analysis and this method will be explained in detail, in the following sections. There are also other methods which are isothermal amplification, direct detection of genomic DNA by electrochemical sensors, cDNA analysis by microarray and direct hybridization of genomic DNA to microarrays. These methods are also proposed for the detection of GMOs (Holst-Jensen, 2009).

### 1.3.2.1 DNA Isolation

DNA isolation from the plant genome fundamentally depends on the following steps: i) removal of envelops (cell walls/membranes) around the DNA, ii) separation of DNA from the cell components (cell wall debris and metabolic substances), iii) maintenance of integrity of DNA during the procedure. The last step covers the protection of the resulting DNA from nucleases and mechanical shearing (Varma et al, 2007). CTAB (cetyltrimethylammonium bromide) method is a widely used DNA isolation method applied to plants (Somma, JRC, Session 4, http://ec.europa.eu/dgs/jrc/).

The following table (Table 1.1) consists of the DNA isolation methods as well as commercial kits that can be employed in GMO analysis. It is possible to see the chemicals that are used in each method.

**Table 1.1 Methods that can be used for DNA isolation during GMO analysis (Marmiroli et al, 2008)**

| Short name | Manufactured by | Main steps of the method | | Sample | | Cost |
|---|---|---|---|---|---|---|
| | | Lysis | Purification | Fresh weight (mg) | Dry weight (mg) | €/sample |
| DNeasy | Qiagen | Detergents | Salts, silica membrane | 100 | 20 | 2.82 |
| Nucleon | Amersham | SDS | Resin, chloroform, isopropanol | 100 | 20 | 2.38 |
| NucleoSpin | Macherey Nagel | CTAB | Silica membrane | 100 | 100 | 2.33 |
| Roche | Roche | Not known | Isopropanol | 200 | 40 | 5.34 |
| PrepMan | Applied Biosystems | Not known | Not known | 100 | 20 | 1.40 |
| WizardGen | Promega | Detergents | Salts, isopropanol | 40 | 40 | 1.11 |
| WizardPlus | Promega | G-HCl, SDS, proteinase K | Resin | 300 | 100 | 2.65 |
| Wizard Magnetic | Promega | G-HCl, SDS, proteinase K | Magnetic particles | 300 | 100 | |
| CTAB | Pietsch et al. [20] | CTAB | Chloroform, isopropanol | 300 | 100 | 0.07 |
| PVP | Kim et al. [21] | SDS | PVP, isopropanol, chloroform/isoamyl alcohol | 100 | 20 | 0.27 |
| QIAamp DNA Stool | Qiagen | Not known | Silica membrane | 300 | 100 | ND |
| Nucleospin Food | Macherey Nagel | Not known | Silica resin | 300 | 100 | ND |
| NucleoSpin Plant | Macherey Nagel | Not known | Silica resin and detergents | 300 | 100 | ND |
| GeneClean kit | Q-Biogene | Detergents and particles | Microcolumn membrane | 300 | 100 | ND |
| GenElute Plant | Sigma Aldrich | Detergents | Microcolumn membrane | 300 | 100 | ND |

Consequently, the extraction of DNA from the samples to be analyzed in a GMO study is a crucial step in order to ensure further analysis (Marmiroli et al, 2008). It is also needed to assess the quality of the isolated DNA in order to meet the standards for the purity and integrity.

Different methods are available to assess the quality of the DNA which can be given as gel electrophoresis, spectrometric analysis, restriction digestion, PCR amplification, and chromatographic techniques (Varma et al, 2007). Among these techniques, spectrometric analysis is the one that is frequently used. The ratio of absorbance at 260/280 nm is recorded to comment on the purity of the DNA that is isolated. A ratio of 1.8 is required for a pure DNA sample and a decrease indicates contamination by proteins, while an increase in the ratio indicates RNA contamination (above 2.0) (Varma et al, 2007).

Apart from the contamination with RNA or protein, plant sources suffer from the impurities of polysaccharides and polyphenols (especially fresh tissues) and it is possible to comment on this by the spectrophotometric measurements at 230 nm and 270 nm. The ratio A260/A230 must be greater than 1.8 and ratio 260/270 should lie between 1.2 and 1.3 in DNA preparations that are free from polysaccharides and polyphenols, respectively (Varma et al, 2007).

#### 1.3.2.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) uses the principle of DNA replication that takes place in the living cells. With PCR, it is possible to amplify a region of a gene, any DNA sequence using a thermo-stable DNA polymerase that is isolated from the bacteria *Thermus aquaticus*, two primers surrounding the target sequence, nucleotides and other reagents like PCR buffer and $MgCl_2$ solution.

PCR is a cyclic process and involves three main steps: i) the separation of the two strands of the DNA molecule, ii) the binding of the primers to the complementary strands, iii) the assembly of two copies of the original double-stranded DNA with the addition of nucleotides (Ovesna et al, 2008). These steps can be observed in the following figure (Fig 1.1).

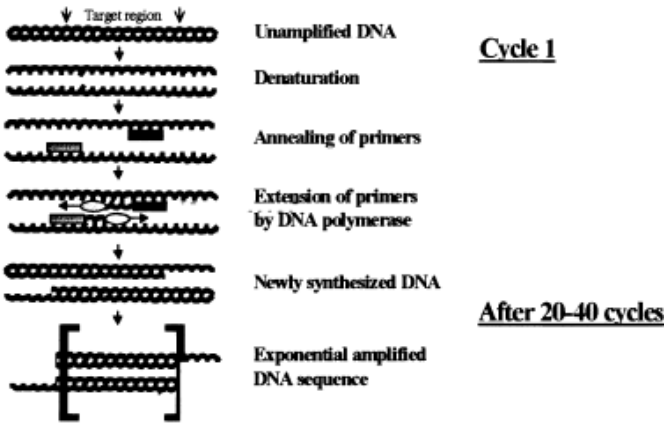



**Figure 1.1 DNA Amplification by PCR (Meyer, 1999)**

PCR based detection methods of the GMOs can be divided into mainly four groups: screening, gene-specific, construct-specific and event specific amplification (Figure 1.2) (Holst-Jensen et al, 2003). Additionally, there is also a control PCR that must be performed before these detections. This control PCR involves a species-specific gene amplification.


**Figure 1.2 The Types of PCR Assays (Holst-Jensen et al, 2003)**

#### 1.3.2.2.1 Species-Specific Detection

Species-specific amplification serves as a check point for the detection analysis of GMOs. In this PCR, a gene that is found in the sample under study is chosen so that only samples from this species enter the amplification reaction. Within this respect, after the DNA isolation, species-specific PCR should be performed in order to check the amplificability of sample DNA for further analysis.

The examples of species-specific genes can be given as: *zein* gene in corn, *lectin* gene in soybean, *SPS* gene in rice, *Sad1* gene in cotton (Randhawa et al, 2009). For tomato, different species-specific genes were employed in the studies. For example in the study of Yang et al, *LAT52* gene was taken as the species-specific gene while in the study of Jaccaud et al, *patatin* gene was used for the genus *Solanum*.

#### 1.3.2.2.2 Screening for Common Genetic Elements

Screening involves the amplifications in the commonly inserted elements of the gene cassette that is transformed into the organism. Most of the transformation events that are authorized have been transformed with CaMV 35S promoter (P-35S) and also with the nopaline synthase terminator (T-Nos) from *Agrobacterium tumefaciens* (Holst-Jensen et al, 2003). The presence of these screening elements is not enough to conclude that the organism under study is a GMO and these elements cannot be used for the identification studies. Contamination with CaMV or *A.tumefaciens* can be the source of the presence of these elements. Therefore additional analysis should be performed along with further studies for the elimination of the idea of false positive results. For example in the study of Wolf et al,2000 an additional PCR system is proposed with the amplification of CaMV-specific genes in order to eliminate the possibility of false positive P-35S amplifications.

For screening, the widely used marker genes are also used in amplification studies. For example most vectors used in transformation studies contain the gene that codes for the resistance to kanamycin (*nptII*) antibiotics, which is also used in the screening studies (Holst-Jensen et al, 2003).

#### 1.3.2.2.3 Gene-Specific Detection

Gene-specific amplification is an essential step for the identification of the GMOs. The gene that is included in the gene cassette for the transformation can be found in its natural

form or it may have undergone some modifications. These modifications do not alter the protein product that is expressed, rather they improve the expression in the transformed organism with the help of truncation or altered codon usage (Holst-Jensen et al, 2003). The specificity of detection increases in gene-specific PCR compared to screening methods.

#### 1.3.2.2.4 Construct-Specific Detection

As it is reflected in the name of the method, the amplification in construct-specific detection is aimed at the junctions between the gene of interest and the common regulatory elements like promoter or terminator. It can also be named as modification-specific detection because the modification itself is tried to be amplified.

For this method, a positive signal namely the expected amplicon, is obtained in the presence of a genetic modification. Yet, the same cassette can be inserted into more than one organism which should also be taken into account while examining the unique transgenic event (Holst-Jensen et al, 2003).

The importance of this method lays in the fact that identification specificity being higher than both gene-specific and screening methods. Additionally and more importantly, with the construct analysis, it is possible to detect the eliminated and unapproved lines that can escape during the field trials of the GMOs. At this point, it is important to give an example from the incident of Bt10 corn.

Detection is not aimed at those that have not undergone safety assessment for approval, since these events are not intended to be commercialized. The event Bt10 is a transformation product of the same recombinant DNA sequence that was used to transform its ‘sister’ event Bt11 corn (which is an approved GMO) (Watanabe et al, 2007). It means that amplification of the construct sequence that is used in the transformation is necessary. This fact emphasizes the importance of construct-specific detection, rather than solely event-detection of GM events (Bluth und Bahrdt, 2009).

#### 1.3.2.2.5 Event-Specific Detection

A GMO is actually defined by a unique transformation event (Holst-Jensen et al, 2003). Therefore for the identification of a GMO, event-specific detection is necessary. This system amplifies the junction between the recipient organism and the gene-construct that is inserted and it is also called line specific detection (Bluth und Bahrdt, 2009).

Although event-specific detection is required for the true identification of an event, it has its own limitations. First of all, the DNA sequence is mostly unknown for the junction sequences of the construct. Although it is possible to find the DNA sequence of some of the approved GMOs from the databases, they are mostly kept by the regulatory organizations and the developers. The sequence of the transgene and its integration site are required in order to direct the amplifications (Marmiroli et al, 2008)

Another limitation is related to the gene stacking. When two events are crossed, the resulting plant may contain the genetic modifications of each event and it would be indistinguishable from the parent plants in a PCR test that aims a single event (Holst-Jensen et al, 2003).

#### 1.3.2.2.6 Inverse PCR

Conventional PCR studies yield a fragment, a part of the gene of interest. In these studies it is necessary to know the sequence that is being studied. However in the detection of transgenic events, it is often difficult to know the DNA sequence of the event and even the construct when both the authorized and unauthorized events are considered. As more transgenic events are being commercialized in the world, the problem of "asynchronus authorization" arises, which results in the unintentional release of the unknown transgenic organisms in the food chain to be detected for genetic modifications (Ruttink et al, 2010). Inverse PCR (IPCR) technique has been used in GMO detection for obtaining the sequence information of the events. Zimmermann et al (2000) has used IPCR in order to obtain the flanking sequences of Bt11 corn and apply event specific analysis.

Inverse PCR study allows amplifying both the upstream and downstream of the region whose sequence is known (Garces et al, 2004). In other words, with inverse PCR it is possible to characterize the unknown flanking regions that surround either the gene or the construct that is inserted. The main steps of this method include restriction enzyme digestion, ligation of the fragments and finally polymerase chain reaction with inverse primers. These steps are illustrated in Figure 1.3. The primers used in inverse PCR have their 3' ends oriented outward.

Restriction enzyme digestion is a crucial step in the IPCR because the entire method depends on the cleavage of the genomic DNA which is going to be further analyzed (The University of Oklahoma, http://www.genome.ou.edu/ protocol_book/protocol_ partI.html).

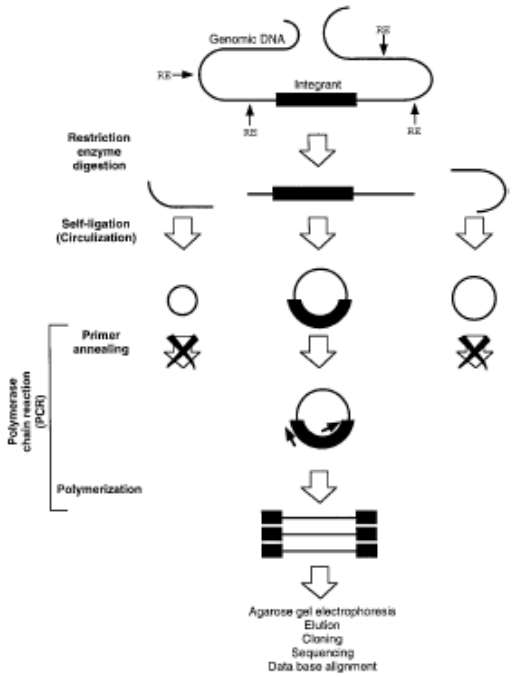



**Figure 1.3 The Principles of IPCR (Hui et al, 1998)**

Ligation of DNA fragments using DNA ligase is also an essential step of the IPCR. It is essential of restricted fragments of genomic DNA to circularize rather than the linear concatamers to form. Diluting the digested DNA into a 100-µL reaction ensures that the concentration does not exceed 3µg/mL. Experimentally, it has been found that at DNA concentrations below 3µg/mL circles rather than the formation of linear concatamers (Triglia, 2000).

The major problems that are faced in inverse PCR studies results from obtaining either no product or some undesired amplication products or smears. These results might be due to the intrinsic complexity of ligation sample or due to low copy numbers of circularized DNA that is being used as template in PCR analysis (Wo et al, 2006).

#### 1.3.2.2.7 Quantification in GMO Analysis

Conventional PCR can easily detect the presence of a genetic modification; however quantification of these modifications is also required to ensure the thresholds in the labeling scheme. Real-time PCR, for gene amplification, is mostly used for the quantification in order to assess the copy numbers of the transgenes. Real-time polymerase chain reaction is currently the method of choice to test labeling with EU regulation 1829/2003 (Gaudron et al, 2009).

Different chemistries can be used in the real-time PCR analysis and some of these can be given as: TaqMan® (Applied Biosystems, Foster City, CA, USA), Molecular Beacons, Scorpions® and SYBR® Green (Molecular Probes). All of these chemistries allow detection of PCR products with a generation of fluorescent signal (Dharmaraj, http://www.ambion.com/techlib/basics/rtpcr/index.html). In the following table (Table 1.2) it is possible to see these chemistries and also other chemistries used in real-time PCR. Additionally, the instruments for real-time PCR can also be seen in this table. Mostly in GMO studies TaqMan® and SYBR® Green chemistries are used.

In TaqMan probes, there are two types of fluorophores, which are the fluorescent parts of reporter proteins. These probes are in fact oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher in the 3' end of it (Figure 1.4) The probe is designed in a way that it attaches to a part of the target DNA that is an internal part of a PCR product (amplified with a pair of primers). When the probe is not bound, the quencher prevents the fluorescent signal. During amplification, the polymerase enzyme replicates the template on which the probe is bound and consequently the 5'- nuclease activity of the polymerase cleaves the probe. With this cleavage, fluorescence is coupled and quenching dyes and FRET are no longer in action. As a result fluorescence signal increases through each cycle, related to the amount of probe cleavage (Dharmaraj, http://www.ambion.com/techlib/basics/rtpcr/index.html).

**Table 1.2 Chemistry and Instruments for Real-time PCR (Marmiroli et al, 2008)**

| | Chemistry | Instrument |
|---|---|---|
| **Non-Specific** | SYBR green I<br>LC Green (Idaho Technology) | ABI 7700 (Applied Biosystems)<br>HR-1 (high resolution melting instrument (Idaho Technology) |
| **Specific** | TaqMan probe<br>MGB TaqMan probe<br>FRET<br>Molecular beacons<br>Plexor technology<br>Scorpion<br>LNA (locked nucleic acid) probe<br>LUX (light upon extension) primers<br>Amplifluor<br>FDSP (fluorophore double stranded probe)<br>CPT (cycling probe technology) | ABI 7700, ABI 7900 HT (Applied Biosystems)<br>LightCycler (Roche Molecular Biosystems)<br>Smart Cycler (Cepheid)<br>iCycler (Bio-Rad)<br>Mx4000 (Stratagene) |

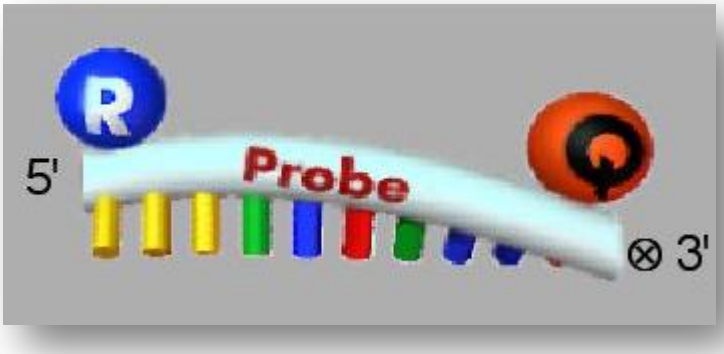



**Figure 1.4 The illustration of the TaqMan probe (University of Otago, http://cgr.otago.ac.nz/SLIDES/TAQMAN/SLD004.HTM)**

SYBR Green is the simple and economical chemistry for detection and quantification of PCR products in real-time reactions. It binds to double-stranded DNA, and in its excitation emits light. Therefore, as a PCR product accumulates, fluorescence increases. SYBR Green dye binds to double-stranded DNA, for this reason; there is no need to design a probe for any particular sequence that is being analyzed. Yet, in the detection using SYBR Green optimization stage is required in order to eliminate undesired amplifications (primer-dimers, unspecific amplifications ) (Dharmaraj, http://www.ambion.com/techlib/ basics /rtpcr/index.html).

The standard curve and comparative methods are the two main approaches used in real-time PCR studies of GMOs in order to estimate the target molecule concentration from the fluorescence intensities recorded from samples (Applied Biosystems, 2001). These two methods can be categorized as absolute quantification and relative quantification.

While forming a standard curve, a reference standard for extrapolating quantitative results is used. This standard can be genomic or plasmid DNA according to the analysis that is being performed and also according to the organism (availability of standards). The other approach involves comparison of the Ct values. Ct value (threshold cycle) represents the cycle number where amplification begins with respect to threshold that is set (Bluth und Bahrdt, 2009). Ct values of both the calibrator and the samples of interest are normalized to an endogenous housekeeping gene according to the organism that is being analyzed (Dharmaraj, http://www.ambion.com/techlib/basics/rtpcr/index.html).

The units of measurement for quantification can be given as i) mass (the content of GM material expressed as w/w ratio, not DNA amount), ii) % GM DNA (the relative ratio compared to a corresponding reference material and it is expressed as % GMO), iii) % HGE (Haploid genome equivalents which is the number of genetic modifications compared to total genome copies and it is expressed as % GMO HGE). The latter unit of measurement is the official unit of measurement that is employed in European Union (Bluth und Bahrdt, 2009).

An example of real-time PCR amplification can be observed in the Figure 1.5. It is possible to see a typical amplification curve showing important feature of the system (threshold, baseline region, Ct and the sample and no template control).

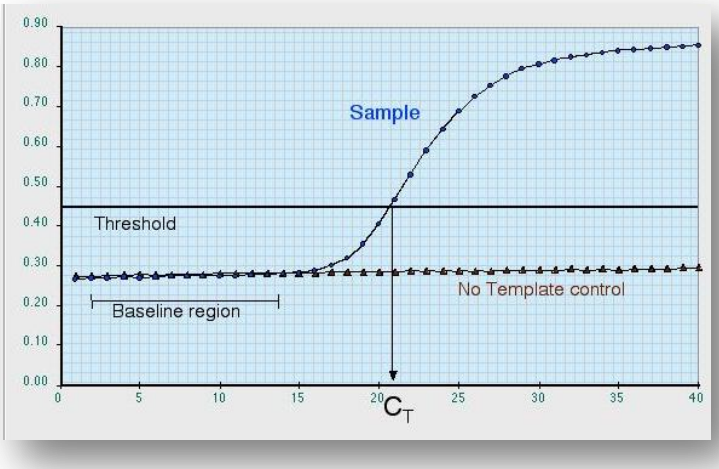



**Figure 1.5 Amplification Plot of Real-Time PCR (Normalized Reported Fluorescence vs PCR cycle number) (University of Otago, http://cgr.otago.ac.nz/SLIDES/TAQMAN/SLD004.HTM)**

#### 1.3.2.2.8 Agarose Gel Electrophoresis

Gel electrophoresis is the method for the separation of macromolecules regarding size, electric charge and other physical properties. Using agarose in gel electrophoresis while separating DNA molecules is a standard method that is widely employed (Somma and Querci, JRC, Session 5, http://ec.europa.eu/dgs/jrc/). Accordingly, in order to evaluate the PCR products, agarose gel electrophoresis is commonly used as the post-PCR analysis.

The concentration of agarose should be adjusted according to the expected band size of the PCR amplification (Table 1.3).

During electrophoresis a voltage is applied in the gel and DNA molecules are forced to move through the pores of agarose. The rate of migration of the molecules depends on i) strength of the field, ii) size of the DNA molecule, iii) ionic strength and temperature of the buffer (Somma and Querci, JRC, Session 5, http://ec.europa.eu/dgs/jrc/).

Other alternatives to conventional gel electrophoresis for the analysis of PCR amplified products can be given as capillary gel electrophoresis, hybridization to labeled and colored beads, flow cytometry, array hybridization and electrochemical sensors (Holst-Jensen, 2009).

**Table 1.3 Recommended Agarose Gel Concentrations for the Separation of DNA (Somma and Querci, JRC, Session 5, http://ec.europa.eu/dgs/jrc/)**

| % Agarose | DNA size range(bp) |
|---|---|
| **0.75** | 10.000-15.000 |
| **1.00** | 500-10.000 |
| **1.25** | 300-5000 |
| **1.50** | 200-4000 |
| **2.00** | 100-2500 |
| **2.50** | 50-1000 |

### 1.3.3 Other Methods of GMO Detection

Mainly GMO detection can be performed using various methods ranging from bioassays, protein or RNA based assays, analysis of metabolites and also DNA based assays which were previously discussed. The table 1.4 summarizes some of these methods and also gives the examples, matrices that these assays can be performed on.

**Table 1.4 The Overview of the Detection Methods of GMOs (Ovesna et al, 2008)**

| Target | Method | Matrix (example) |
|---|---|---|
| DNA | PCR, qPCRm DNA/DNA hybridisation (e.g. Southern blot, DNA arrays), MALDI-TOF | Processed/unprocessed food__ Meat products as sausages, paté, spring sausages, salami |
| RNA | DNA/RNA hybridisation e.g. Northern blot), qRT-PCR | Living GMOs |
| Protein | ELISA (Enzyme Linked Immonoabsorbent Assay) | Unprocessed food |
| Metabolite | NMR, HPLC, NIRS | Containing lipids and fatty acid |

Bioassays involve the analysis of GMOs according to the qualities that are expected from them. For example exposure of the samples to the herbicide would discriminate the ones with herbicide tolerance from the others. Evaluation of the survivors and comparison with the number of affected plants would give the relative GMO content in a lot. There are some advantages of bioassays and these are low cost of the assay, few requirements for the analysis and the ability of the assays to confirm the desired biological properties of the GMOs. The major disadvantages of bioassays are that they can only be applied to certain biological properties and they usually require longer time compared with other GMO detection methods (Holst-Jensen, 2009).

RNA can be another target for GMO analysis. With the isolation of mRNA from the samples that are being analyzed, it is possible to perform separation and hybridization with the help of reverse transcription, in order to detect the genetic modifications. Some of the techniques that can be used employing this principle are NASBA and Real-time PCR (Taverniers, 2008). NASBA is nucleic acid sequence-based amplification. The main goal of NASBA is to develop multiplexing of GMO analysis and integration with microarray. It also enables high-throughput identification and quantification of GMOs (Morisset, 2008). This method relies on specific binding between the RNA molecule and a synthetic RNA or DNA molecule, the primer. The primer must be complementary to the nucleotide sequence at the start of the RNA molecule. Accordingly, the result is a double stranded molecule which is similar to DNA. The binding between the RNA molecule and the primer is

continued with reverse transcription. At the end, the cDNA can be amplified with PCR or it can be translated into copies of the original RNA molecule and when the procedure is repeated by using each copy as a template it leads to the technique: NASBA (Holst-Jensen, 2001). However since RNA is not a stable molecule compared to DNA, in most studies of GMO analysis, DNA is preferred.

Proteins from the GMOs can be detected with immunological and physicochemical techniques. The most common protein based assays are immunoassays where the target proteins are detected by specific antibodies coupled to a colorimetric detection system (Holst-Jensen, 2009). One major drawback of protein based methods is that they are not able to detect a genetic modification if the modified gene is inactive in the cells from which the sample is derived (Miraglia et al, 2004). There is also the high cost of the production of antibodies for specific proteins that are to be detected.

When the composition of GMO is the trait of modification, like in the case of change in fatty acids, conventional chemical methods such as chromatography can be used to detect differences in the chemical profile. Metabolites are in this case the products of the transgenic protein activity, can easily be detected by physical methods, like NMR (Nuclear Magnetic Resonance), HPLC (High Performance Liquid Chromatography) or NIRS (Near Infrared Spectroscopy) (Ovesna et al, 2008).

### 1.3.4 Novel Detection Techniques

High throughput detection methods are required when there is a time limit and a great number of samples are to be tested. In such cases rapid and simultaneous results are required. These high throughput methods are either based on combinations of one or more oligoplex PCRs followed by multiplex identification of the amplified DNA. Automation is the key element in these methods (Holst-Jensen, 2009). Large-scale screening enables a large number of samples to be tested and is required for risk assessment of the commercialized products (Gu et al, 2009).

These novel GMO methods also include biosensors and also the microarray technology.

Biosensors are the sensors which carry an element that is derived from a biological recognition system for the analyte, and this is enabled for converting and transmitting a signal. This element can be either a complex molecule or an isolated single molecule like

DNA. DNA-based sensors are related to sequence complementarities, like a hybridization assay. It is also required that the sensor DNA remains immobilized on the surface of the transducer element (Marmiroli et al, 2008).

The microarray has the potential to combine detection, identification, and quantification of many number of GM events in a single experiment. Microarray enables the identification of several GMOs in one step and it would decrease the number of analyses that are required. The principle of microarray can be given as: labeled nucleic acid molecules are subjected to other nucleic acid molecules attached to a solid support. Considering GMO detection, the pattern of hybridization would show if the sample represents a GMO, and also which GM events are present in the samples (Marmiroli et al, 2008). The first validated microarray for use in GMO detection is from the Eppendorf Biochip Systems. They have developed DualChip GMO and this method was validated in the EU for use in qualitative GMO screening. The microarray carries the detection sequences, capture probes, for the amplicons. In cases where there is an amplicon in the mixture that is complementary to the capture probe, hybridization occurs, and the biotinylated sequence is linked to the microarray (Euro|BioTech|News, 2007).

As large-scale detection systems are crucial in the future of GMO analysis, so are the detection of masked transgenic events and also the unknown event identification. The events that are tried to be detected became more complex than the earlier forms of the GMOs. Two transgenic lines can be combined to obtain better qualities or multiple traits are transformed in the organism making the detection tougher. Additionally, as it was mentioned in Section 1.3.2.2.6, unknown events are the crucial elements in the future of GMO detection. Amplification of the flanking sequences and the characterization of the events lead to the unknown event identification (Ruttink et al, 2010).

In the following figure (Figure 1.6), it is possible to observe the evolution of GMO detection methods through time and these include DNA based, protein based or metabolite based methods as well as reference materials used in these studies.

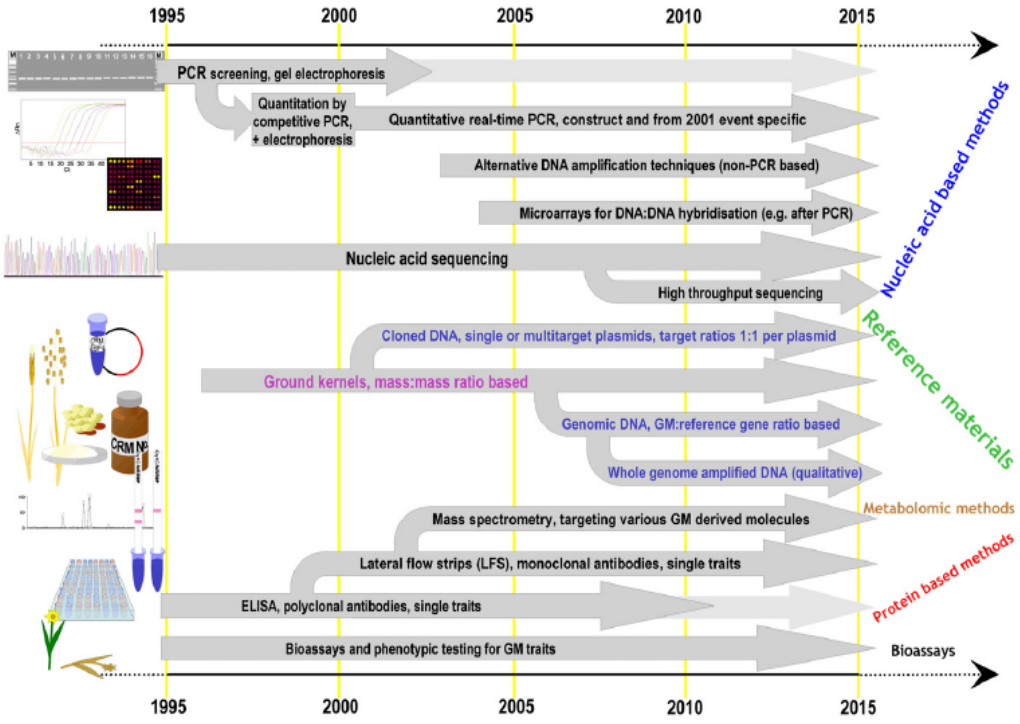



**Figure 1.6 The Evolution of GMO Detection Methods (Holst-Jensen, 2009)**

## 1.4 Procedures and Testing to be followed Considering GMOs

In the development of detection techniques of GMOs, it is important to know the procedures that these transgenic events go through. After the development of the transgenic line by the companies, these events should follow some evaluation procedures before their release into the environment and consequently to feed or food chain. These procedures vary with country as well as the regulations regarding the usage of these products. Some countries lack an evaluation system for the handling of GMOs or try to establish the set of rules. Developing countries try to form their regulatory frameworks based on the models of developed nations (Ramessar et al, 2008).

### 1.4.1 Authorization and Approval of Transgenic Events

In all countries where there is a GMO legislation, the basic requirement is that, any transformation event should go through an authorization procedure before it can be grown

or used. This is in place because only safe products are tried to be placed on the market for the consumption (Holst-Jensen et al, 2006).

In the authorization step of the transgenic events, the risk assessment (RA) plays an important role. RA is to identify and evaluate potential adverse effects, risks of a GMO before this event is commercialized and placed on the market. After the RA has been performed, the results can then be used by the relevant authority to make a decision about whether or not to give approval for transgenic event (for the import or cultivation) (Craig et al, 2008). The regulatory bodies of different countries that are involved in this process can be observed in the table 1.5. Accordingly, each country has its own approval procedure and risk evaluation of the transgenic lines.

**Table 1.5 Regulatory Authorities of Countries Related to GMOs (Craig et al, 2008)**

| Country/Region | Authority |
|---|---|
| **Argentina** | CONABIA |
| **Australia** | OGTR |
| **Brazil** | CTNBio |
| **Cambodia** | BCH |
| **Canada** | CFIA |
| **China** | BCH |
| **European Union** | JRC, EFSA |
| **India** | BCH |
| **Mexico** | CIBIOGEM |
| **Japan** | BCH |
| **Switzerland** | SECB |
| **USA** | Unified |

The acronyms: Comision Nacional Asesora de Biotecnologia Agropecuaria (CONABIA); Office of the Gene Technology Regulator (OGTR); National Biosafety Technical Commission (CTNBio); Canadian Food Inspection Agency (CFIA); Joint Research Council Biotechnology & GMOs Information Website (JRC); European Food Safety Authority (EFSA); Mexican Comision Intersecretarial Bioseguridad Organismos Geneticamente Modificados (CIBIOGEM); Swiss Expert Committee for Biosafety (SECB); US Regulatory Agencies Unified Biotechnology Website (unified)

Apart from the regulatory bodies of different countries, there are also international organs which deal with the transgenic organisms and the regulation of them. These organizations are: World Trade Organization (WTO), Organization for Economic Co-operation and Development (OECD), Food and Agriculture Organization of the United Nations (FAO), International Organization for Standardization (ISO) (Ovesna et al, 2008).

In addition to these organizations, the Cartagena Protocol on Biosafety, which is an international agreement, tries to protect biological diversity from the potential risks of the genetically modified organisms. This protection is tried to be accomplished with the guidelines for safe transfer, handling and use of these organisms. The rules that were set in the protocol try to control the trade and transfer of GMOs across international borders covering the labeling of shipments of GM products. Additionally, this protocol allows governments to prevent the import of GMOs when there is concern about the safety (Cosbey and Burgiel, 2000) (Ahmed, 2002).

Although there are intensive evaluations of approval stage regarding some countries, there have been some cases that unintentional releases of unauthorized GMOs occurred. Some of these can be given as Starlink corn, Bt10 corn, Shanyou63 rice, LLRice601. These incidents indicate the necessity of acquiring an efficient detection system that not only detects and identifies the currently approved GMOs but also search for other possible gene inserts (Cankar et al, 2008). The incidents of mixing during storage, transport, and processing have the possibility to take place. For example, the Starlink corn in the US is strictly limited to the use for feed, however it was found in cereals and other grains (Vergragt and Brown, 2008)

The major drawback in the analysis of the unapproved GMOs is that the sequence and the insert information is lacking. At this point, there is no strategy that has been described and internationally accepted for the detection of unapproved GMOs (Cankar et al, 2008).

#### 1.4.1.1 Authorization in USA

The regulations in the US regarding GMO products are under the principle of "substantial equivalence". This term refers to the equity that is applied to all food products (genetically modified or not). GMOs are compared with their traditional counterparts. This comparison is based on an examination of the risk factors (toxins, potential allergens, weediness, pest potential, etc.) (AGBIOS, http://www.agbios.com/dbase.php).

Considering this fact, the release and authorization of any GMO is considered under the existing legislation (Marmiroli et al, 2008). These legislations are related to the following acts: the Plant Protection Act (PPA), the Federal Food, Drug and Cosmetic Act (FFDCA), the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Toxic Substances Control Act (TSCA).

US Department of Agriculture with its branch Animal and Plant Health Inspection Service (APHIS) regulates the release of the GMOs to the environment and also to consumers and these regulations include cultivation, importation, interstate movements and also field trials. The US Food and Drug Administration (FDA) ensures the safety of the food products and all developed GM foods should enter the consultation stage of the FDA. In this stage, the information of GMOs (the genetic stability, compositional analyses, nutritional assessments, potential allergenicity and toxicology of substances present) that are developed should be provided by the developer. In addition to these steps, the Environmental Protection Agency (EPA) regulates the pesticidal properties of the newly developed organism (Table 1.6) (Michelini et al, 2008).

**Table 1.6 The US Regulatory System Flow Scheme for GMOs (AGBIOS, http://www.agbios.com/dbase.php)**

| | |
|---|---|
| **Research & Development** | - Compliance with NIH Guidelines for work with GMOs is mandatory for all scientists receiving federal funding or working for federal agencies. |
| **Field Trials** | - Following a letter of notification, developers must receive APHIS approval for field trials and submit summary reports.<br>- Trials may be inspected by APHIS and/or state department of agriculture officials.<br>- Developers must comply with APHIS performance standards developed to minimize "outcrossing" and inadvertent environmental release.<br>- APHIS also oversees transport of seed to and from trial site.<br>- For trials of pesticidal plants >10 acres, need an Experimental Use Permit from EPA. Public notification and comment is required here, but not for field trials generally. |
| **General Environmental Release** | - Developers must apply to APHIS for a determination of non-regulated status. Public notification and comments solicited.<br>- APHIS review (>=10 months) considers range of risk factors including environmental effects, wildlife effects, and potential to become a plant pest.<br>- For pesticidal plants (e.g., Bt corn), the plant-pesticide substance [e.g., Cry1A(b)] must be subject to risk assessment (>= 18 months) and registration by EPA. Public notification and comment is invited through publication in the *Federal Register.* |
| **Use as Food** | - Through its voluntary consultation process, FDA works with the producer from an early stage in product development to ensure that all food safety issues have been addressed.<br>- Based on a favourable review of summary data and a presentation to FDA scientists, FDA issues a letter stating they have no further questions. |
| **Post Commercialization** | - All three regulatory agencies have the legal power to demand immediate removal from the marketplace of any product should new, valid data indicate a question of safety for consumers or the environment. |

In USA, the applications approved between 1987 and early 2005 included GMOs with following traits herbicide tolerance (3,587), insect resistance (3,141), improved product quality (flavor, appearance, or nutrition) (2,314), virus resistance (1,239), and agronomic properties like drought resistance (1,043) and fungal resistance (647) (USDA Economic Research Service, http://www.ers.usda.gov/).

The list for the completed consultation on GMO lines for approval in USA can be found at the US FDA webpage: www.cfsan.fda.gov/~lrd/biocon.html.

#### 1.4.1.2 Authorization in EU

In European Union, the regulations are based on the "precautionary principle", which is different from the regulations of USA. The transgenic events are taken as novel products and handled strictly to provide the safeguards against contamination of GMOs to other products (Michelini et al, 2008). In the EU legislations, it is required for all GMOs and their derived products to be evaluated by European Food Safety Authority (EFSA) before they are approved and used. For any GMO or its derived food to be authorized in EU, the developer company must submit an application regarding the European legislation. The European Commission forwards the application to EFSA and requests a scientific risk assessment (EFSA, http://www.efsa.europa.eu). The elements that are included in the preauthorization safety assessments of EFSA are: availability of validated detection methods, reference materials, thresholds for labeling, postmarket monitoring, and postmarketing traceability requirements (Holst-Jensen et al, 2006)

Regarding the authorization of GMOs, it is important to mention the acts/laws of EU: i) 1829/2003/EC (regulation/approval of GM food and feed), ii) 1830/2003/EC (traceability and labeling of GM products), iii) 2001/18/EC (deliberate release into the environment and marketing of GMOs including seeds), iv) 1946/2003/EC (trans-boundary movements of GMOs outside EU-notification of the Cartegena Protocol), v) 65/2004/EC (for establishing a system for the development and assignment of unique identifiers for genetically modified organisms) (Ovesna et al, 2008) (Bluth and Bahrdt, 2009).

The approved transgenic lines for use in food and feed in EU can be found at the website of GMO Compass Database: http://www.gmo-compass.org/eng/gmo/db/ and also at the Community Reference Laboratory website: http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

### 1.4.2 Regulations and Labeling of GMOs

Like in the case of approval procedures of GMOs, there are also differences in the regulation of these organisms among countries. The following table (Table 1.7) is from Nature Biotechnology, outlining the biosafety regulations of different countries. This table shows the heterogeneity in the labeling requirements of various countries. There are countries (like USA, Hong Kong) with voluntary labeling guidelines and as opposed to them; there are mandatory labeling requirements of other countries (like Australia, EU, Japan). The difference continues in the mandatory labeling policies, whether the rules aim the presence of a genetic modification in the finished product (like Australia, New Zealand, Japan) or the use of the GM technology throughout the production (like EU, Brazil) (Michelini et al, 2008).

**Table 1.7 Current GM Bio-safety Regulations for Selected Countries (Ramessar et al, 2008)**

| Country | Regulation | Product/Process | Transparency | Labeling | Tolerance |
|---|---|---|---|---|---|
| **Argentina** | Law 18284 on Argentina Food Codex | Product | Yes | Voluntary | n.d. |
| **Australia** | Gene Technology Act 2000 | Process | Yes | Mandatory | 1% |
| **Brazil** | Biosafety Law 11.105 | Process | n.d. | Mandatory | 1% |
| **Canada** | Consumer Packaging &Labeling Act, Food and Drugs Act | Product | Yes | Voluntary | 5% |
| **China** | Under Disc. | Process | n.d. | Mandatory | 1% |
| **European Union** | E.U. Directive and Regulations | Process | Yes | Mandatory | 0.9% 0.5% |

**Table 1.7 continued**

| Country | Regulation | Product/Process | Transparency | Labeling | Tolerance |
|---|---|---|---|---|---|
| **India** | EPA 1986&1989 Rules | Process | Yes | Proposed legislation for mandatory labeling | n.d. |
| **Mexico** | Biosafety Law of Genetically Modified Organisms | Process | Yes | Mandatory | n.d. |
| **Japan** | Law concerning the conservation and Sustainable use of Biological Diversity | Process | Yes | Mandatory for selected products | 5% |
| **USA** | Federal Plant Protection Act, Federal Food Drug and Cosmetic Act | Product | Yes | Voluntary | 5% |

### 1.4.2.1 Regulations in USA

In the 1992 Policy, U.S Food and Drug Administration (FDA) asserts that: "There is no reason for concluding that GM foods differ from conventional foods in any way and foods developed through the technology of genetic engineering do not present any different or greater safety concerns than foods developed using traditional plant breeding methods." Therefore, GMOs with conventional foods are subject to the same existing labeling regulations (AGBIOS, http://www.agbios.com/dbase.php). However, the mandatory

labeling scheme changes and labeling is actually required if safety concerns (allergens, change in nutritional composition) exist in the final product (Ramessar et al, 2008).

#### 1.4.2.2 Regulations in EU

The EU act 1829/2003 requires genetically modified food and feed to be labeled with regard to applications in genetic engineering. These products include i) food which is a GMO or which consists of GMOs (like GM cotton, GM soya, GM maize), ii) food, ingredients or additives, which are produced from GMOs (starch from GM corn, cornflakes from GM corn, lecithin from GM soy beans), iii) ingredients and additives which contain genetically modified organisms (like yeast extract from GM yeast, salami with GM lactobacilli). Note that up to now, no GM yeast, GM bacteria or GM fungi are approved in the EU for use in food (GMO-Compass, http://www.gmo-compass.org).

EU requires any food containing 0.9% GM content to be labeled as such. During the production, transportation and also processing of agricultural products, an amount of mixing between different fields, shipments and lots is unavoidable, therefore rather than a 0% threshold, EU allows up to 0.9% for authorized GMOs and 0.5% for nonauthorized GMOs (Heinemann et al, 2004).

One of the differences of the systems of USA and EU is that, in USA the hybrid GMO (the plant that is the result of a cross between two GMOs) is not regulated if both parents are authorized. However in EU this hybrid GMO is treated as a new event and accordingly assessed (Holst-Jensen et al, 2003).

#### 1.4.2.3 Regulations in Turkey

The policy and the main aims of Turkey regarding genetically modified organisms is outlined in the article of Baran and Yilmaz, 2008 and it is stated that : “The policy of Turkey is to protect its biological diversity, as well as human and animal health, against the possible adverse effects of products developed by using modern biotechnology; however, it is also important to benefit from the current and future advantages of modern biotechnology applications, as long as this is done safely in accordance with national requirements.”

In Turkey there are studies and projects regarding the detection and quantification of genetically modified organisms. The Ministry of Agriculture and Rural Affairs has a

branch called TAGEM which stands for General Directorate of Agricultural Research. TAGEM has projects like "Detection and quantification of Genetically Modified Soybean (Round Up Ready) in Soybean Originated Food and Processed Foods" It is stated by TAGEM that "Turkish Government is in the process of adopting legislation that requires traceability of GM components and labeling of foods that contain genetically modified organisms above a certain level. Turkish Agricultural Market is vulnerable to GMO products due to lack of efficient research and country survey project." As is it mentioned the studies related to the detection of genetically modified organisms are of great importance and in Turkey the related institutions for these studies are universities, TUBITAK, TAGEM, TUGEM, KKGM (List of Abbreviations).

As it is stated by TAGEM about the adoption of a legislation system, there has been a recent legislation in Turkey that was published on October 26$^{th}$ 2009, concerning genetically modified organisms but it was withdrawn for further additions. The recent legislation had the following important points:

- It tried to form the risk analysis commissions for the assessment procedure of the approval of GMOs.
- It imposed requirements to the ones that import these products and also to the firms that try to sell these products.
- It was also prohibited to have the label of GMO free, because additional requirements were set related to labeling.
- The labeling was tried to be established by setting the threshold level of 0.9% for the approved GMOs which is the equivalent of the regulations in EU. Below this threshold the presence of GM material was considered to be accidental or technically unavoidable.
- The rules did not include the seeds.

### 1.4.3 Development of Detection Methods and Method Validation

Validated detection methods are necessary considering the increase in the development of transgenic events. These method developments are mainly considered in EU. Some key elements related to a validated study include: the method, test material and performance of the participants (Holst-Jensen et al, 2003). Collaborative studies are required in order to accomplish this task of forming a detection method.

In the development of the detection methods for transgenic events, it is necessary to identify the unique event that the sample harbors. For the establishment of the accurate traceability in the food chain, this identification is required. It is important for food exporters, importers, retailers, and for the authorities that are responsible for food safety, to know the extent and the nature of GMO ingredients in the products that they evaluate (Nenov and Vassilev, 2006). The reference materials are also crucial while developing the identification method, since the positive and negative controls are the basis for the method development in GMO analysis.

For European Union, European Commission's Joint Research Centre (JRC) is responsible for testing and validation of the method, submitted by the researchers for authorization to detect and identify the GMO (Zel et al, 2008). A detection method is considered ready to be in the validation step, after it acts according to the acceptance criteria. These criteria have been set by the European Network of GMO laboratories (ENGL). According to these set of rules, the method should have the following properties: It should be i) able to detect and quantify the specific GM event in a product, ii) event-specific, iii) applicable to the notified material along with other food samples (Michelini et al, 2008).

Additionally, now that more and more transgenic events are being developed and commercialized, the detection and identification methods of GMOs should cover a broad range of modifications and even cover the unknown/unauthorized events (Querci et al, 2010). In this respect, matrices are being developed for the current authorized transgenic events; so that the inserted genetic elements are summarized and this would help in the detection of the unknown and uncharacterized events (the modifications in the unknown cases are expected to have similarities with the known events).

### 1.4.4 Databases

Databases are essential tools for obtaining information about the gene cassettes that are inserted in GMOs, approval status of the transgenic events and also detection methods that have been studied considering these events. The following databases are the mostly used databases in the GMO research.

- **GMO Detection Method Database (GMDD)** (http://gmdd.shgmo.org/): This database was formed by GMO Detection Laboratory in Shanghai Jiao Tong University (GMODL-SJTU) in collaboration with RIKILT, Shanghai Academy of

Agricultural Sciences, Shanghai Food and Drug Administration, Shanghai Entry-Exit Inspection and Quarantine Bureau, and Shanghai University Bio-information Center. The aim is to provide information about GMO detection methods and gene inserts.

- **AGBIOS** (http://www.agbios.com/dbase.php): This database was formed by a Canadian company and its aim is to provide information for public policy, regulatory, and risk assessment expertise for products of biotechnology.
- **GMO-Compass** (http://www.gmo-compass.org/eng/gmo/db/): This database was formed with the support of European Union within the European Commission's Sixth Framework Program. The aim is to provide information about every genetically modified plant that has been approved or is awaiting approval in the EU.
- **GMO Methods Database (JRC)** (http://mbg.jrc.ec.europa.eu/home /ict/methodsdatabase.htm): This database was formed by the Joint Research Centre of the European Commission. The aim of this database is to provide access to information on methods of GMO analysis. It contains more than 300 different DNA-based (PCR) and protein-based (ELISA) assays.
- **GMO Register Database** (http://gmoinfo.jrc.ec.europa.eu/): This database was formed by the Joint Research Centre of the European Commission on behalf of the Directorate General for the Environment. The aim of this website is to publish information and to receive comments related to notifications about the field trials of GMOs and placement of them on the market.

### 1.5 Transgenic Organisms Grown Worldwide

The first field trials of genetically modified plants were accomplished in 1987 according to the report of Economic Research Service of Unites States Department of Agriculture. These first GM plants were insect resistant tomatoes (USDA Economic Research Service, http://www.ers.usda.gov/). In addition to field trials, the first commercial genetically modified plant that was authorized for marketing was also a tomato event (FlavrSavr tomato) and it was in the year of 1994 (Food and Drug Administration, 1994). Now, in the AGBIOS database there are 144 records/events of GMOs that are approved in various countries to be used as food and feed (May 19$^{th}$ 2010). Most of the approved GMOs

worldwide are the events of soybean, corn, cotton and canola (AGBIOS, http://www.agbios.com/dbase.php).

According to "GMO Compass", there are no genetically modified fruit or vegetables are on the market of EU (http://www.gmo-compass.org/eng/gmo/db/). Genetically modified tomatoes are not approved in Europe and relatedly, applications that were submitted have been withdrawn (Note that GMO Compass is a website related to the genetically modified organisms and it is centered in EU. This organization received financial support from the European Commission, from the Green Biotechnology Europe Unit of EuropaBio, the EU association for bioindustries, and from the German Federal Ministry of Food, Agriculture and Consumer Protection).

There are also some "Discontinued Transgenic Products", meaning that once they were distributed in the market but then withdrawn with different reasons. Among them two tomato events can be seen. The first commercialized tomato (FlavrSavr) that was genetically engineered by Calgene in 1994 had the quality of delayed ripening and it was withdrawn after being available sporadically for several years (Colorado State University, 2004). This was the first fresh genetically modified fruit or vegetable to be sold.

The other genetically modified tomato product was produced by the British firm Zeneca Seeds (later part of Syngenta). In 1996, some supermarkets in the United Kingdom started to sell this tomato puree. The product was named as "Zeneca Tomato Puree". This was the first time that a product made from a genetically modified organism has been sold in Europe (University of Reading, http://www.ncbe.reading.ac.uk/NCBE/GMFOOD/tomato.html).

It is also asserted by GMO-Compass that no genetically modified tomatoes are being grown commercially in North America or in Europe. However there are approved transgenic tomato lines in USA, allowing the usage of these events (Table 1.11). The current situation of the GM crops that are grown worldwide is summarized by the 2008 Report on Global Status of Biotech/GM Crops by International Service for the Acquisition of Agri-biotech Applications (ISAAA) and these data are given in the following figure (Figure 1.7).

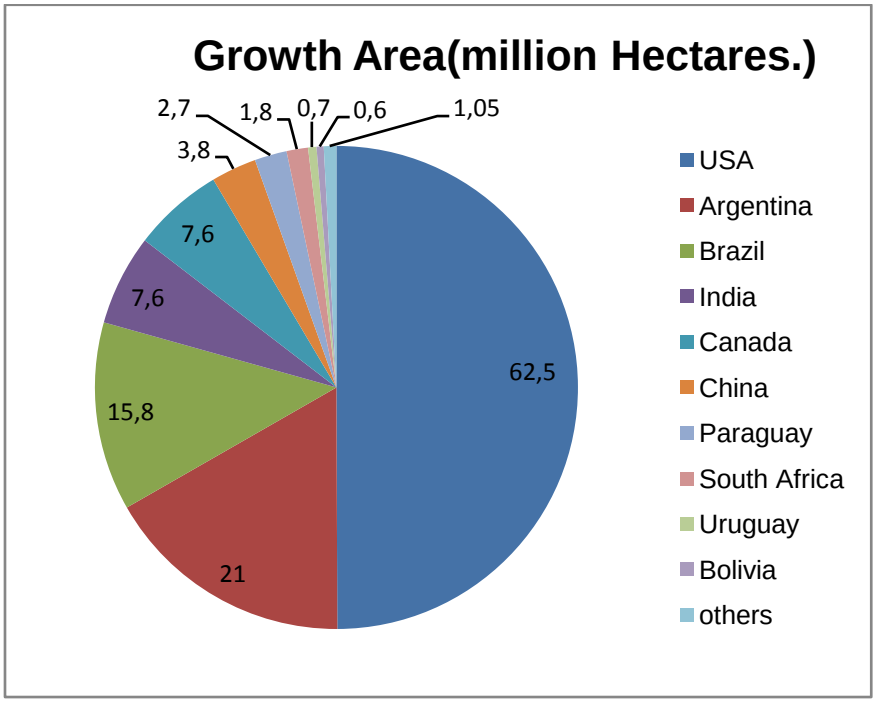



**Figure 1.7 The Growth Area of the GM Crops that are produced in Different Countries (ISAAA, 2008)**

### 1.6 Tomato

The tomato plant belongs to the family *Solanaceae*, genus *Lycopersicon*. The *Solanaceae* family also includes other important vegetable crops like potato (*Solanum tuberosum*) and tobacco (*Nicotiana tabacum*). The cultivated tomato *Solanum lycopersicum* L., formerly *Lycopersicon esculentum* Mill, is in its present status after the long domestication period that has been spent. The improvement started with the selection of preferred genotypes. Because tomato is predominantly inbreeding, namely a self fertilizing species, the genetic variation tends to decrease, even without selection. Therefore cultivated tomato has an extremely limited genetic variation (Heuvelink, 2005). Yet still, there are different tomato cultivars with different sizes, colors and other qualities (Figure 1.8).

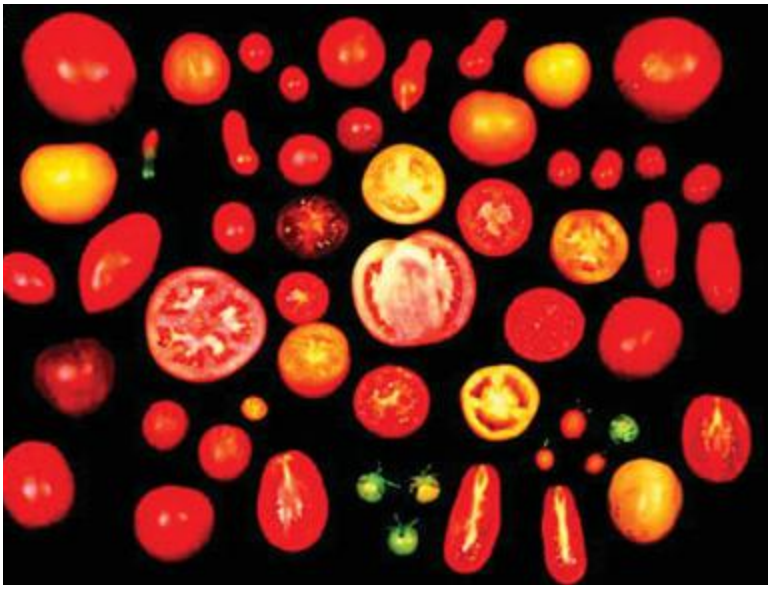


**Figure 1.8 Shape Variations in Different Tomatoes (Bai, and Lindhout, 2007)**

The leading producers of tomato are China, United States and Turkey according to the data from The Food and Agriculture Organization of the United Nations (FAO) (http://www.fao.org/) (Table 1.8). Additionally, the examples of the cultivars of tomato from various countries can be observed in table 1.9. While tomato is now grown in many places around the world, the wild-type of the tomato plant is native to South America, specifically to Peru (Fatima et al, 2008).

**Table 1.8 List of Countries by Tomato Production in 2006 based on FAO accessed in July 2008 (http://faostat.fao.org)**

| Rank | Country | Tomato Production (tonnes) |
|---|---|---|
| **1** | China | 32,540,040 |
| **2** | USA | 11,250,000 |
| **3** | Turkey | 9,854,877 |
| **4** | India | 8,637,700 |
| **5** | Egypt | 7,600,000 |
| **6** | Italy | 6,351,202 |
| **7** | Iran | 4,781,018 |
| **8** | Spain | 3,679,300 |
| **9** | Brazil | 3,272,927 |
| **10** | Mexico | 2,878,222 |

**Table 1.9 Examples of Tomato Cultivars and their Origins (Slimestad and Verheul, 2009)**

| Type/Cultivar | Origin |
|---|---|
| **Aromata** | England |
| **Assun** | Spain |
| **Biga** | Canakkale |
| **Boludo** | Spain |
| **Bornova** | Izmir Organic |
| **Burbank** | California |
| **Rio Grande** | Bursa Karacabey |
| **Campari** | New Zealand |
| **Canario** | Spain |
| **Cayambe** | Italy |
| **Dometica** | Norway |
| **Dunkan** | Spain |
| **Elanto** | Norway |
| **Extase** | Germany |
| **Felicia** | France |
| **Flavourine** | New Zealand |
| **Havanera** | Spain |
| **Haubners** | Germany |
| **Izabella** | France |

**Table 1.9 continued**.

| | |
|---|---|
| **Liso** | Spain |
| **Marglobe** | Spain |
| **Momor** | Italy |
| **Paola** | France |
| **Ponderosa** | Ohio,USA |
| **Rambo** | Spain |
| **Ramillete** | Spain |
| **Ropreco** | California |
| **Rutgers** | Ohio,USA |
| **Senior** | Spain |
| **Spectra** | England |
| **Tradiro** | New Zealand |

Tomatoes are one of the most widely eaten vegetables in the world. The processed products made from tomato can be given as: (i) tomato preserves (tomato juice, tomato purée, tomato paste, pickled tomatoes); (ii) dried tomatoes (tomato powder, dried tomato fruits); and (iii) tomato-based foods (tomato soup, tomato sauces, ketchup) (Heuvelink, 2005).

Another reason for the common usage of the tomatoes is the result of their vitamin and mineral composition. It is a vital source of Vitamin C and A; one medium-sized tomato provides 57% of the recommended daily allowance (RDA) of Vitamin C and 25% RDA of Vitamin A and 8% RDA of iron with only 35 calories (Fatima et al, 2008). Tomatoes also harbor an antioxidant called lycopene that is essential for the protection of cells against oxidative damage. There have been studies related to the linkage of lycopene to risk reduction of many cancer types (Fatima et al, 2008). In addition to lycopene, the tomato contains a number of flavonoids and phenolic acids that is required for a healthy diet (Slimestad and Verheul, 2009).

Another important point about tomato is that, its seed production is easy regarding the fact that large quantities of seeds can be easily obtained from a single plant. Each fruit can carry more than 100 seeds (Heuvelink, 2005). The main parts of the tomato fruit can be seen in the figure 1.9.

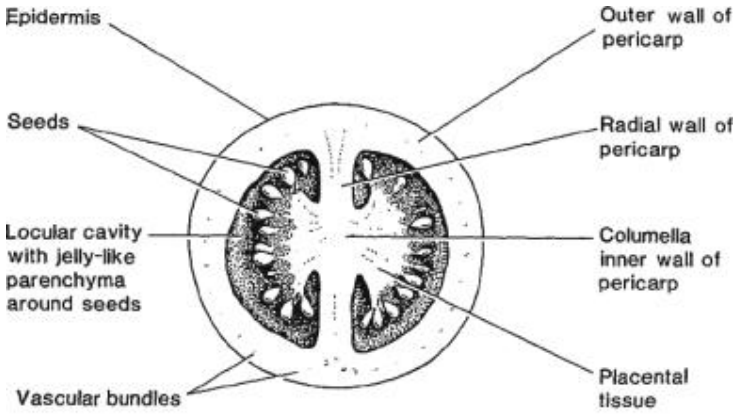



**Figure 1.9 Transverse Section of Mature Tomato fruit (Slimestad and Verheul, 2009).**

Tomato is also one of the model plants that is used in the studies because of its enriched cytogenetic, genetic and physical maps generated among different research (Gupta et al, 2009). Tomato is diploid (2n= 24), dicot, has relatively short generation time and genome size (C=0.7pg) and also genes can be transformed to it using *Agrobacterium tumefaciens* (Zamir and Tanksley, 1988).

### 1.6.1 Cultivars in Turkey

The regions in Turkey that are used for processing tomatoes are in Marmara, mostly in Balikesir and Canakkale, and in Aegean, mostly in Bergama and Turgutlu and Central Anatolia (Tokat) (Heuvelink, 2005). Seedlings are normally started in the middle of March and are transplanted after mid to late April. Harvest is between late July and early October (Sirtioglu, 2003). Tomatoes are the main greenhouse vegetables. Greenhouse cultivation of them takes place during winter and early spring (Sirtioglu, 2003), (Heuvelink, 2005).

As it is mentioned in the previous section, Turkey is one of the leading countries in tomato production and according to the report of Assoc. Prof. Dr. Ali Ergül ("Plant Genetic Resources in our Country and to What Extent do we need Transgenic Plants?" First National Genetically Modified Organisms Symposium, METU), there are 14 cultivars of tomato that are used in the agricultural production in Turkey.

The following table (Table 1.10) summarizes the number of the cultivars of some of the important vegetables that are produced in Turkey. In the same report it is possible to find information regarding the genetic resources considering the vegetable plants. It is reported that there has been studies related to the genetic resources of Turkey since 1964 under TAGEM where the analysis of genetically modified organisms are also conducted.

**Table 1.10 The Number of Cultivars that are used in Agricultural Production in Turkey (Ergul, 2008)**

| Vegetable | Number of Cultivars |
|---|---|
| **Tomato (*Solanum Lycopersicum*)** | 14 |
| **Pepper (*Capsicum Anuum L.*)** | 21 |
| **Cauliflower (*Brassica oleraceae L. Var botrytis L*)** | 2 |
| **Eggplant (*Solanum melongona*)** | 6 |

Some of the cultivars of tomato that are grown in Turkey can be given as: Bornova, Biga, Rio Grande, Astona, Dürinta, F-144, Gokce 191, Ikram, Jabot, Malika, Newton, Paskal, Selin (Slimestad and Verheul, 2009) (Akay and Kara, 2006).

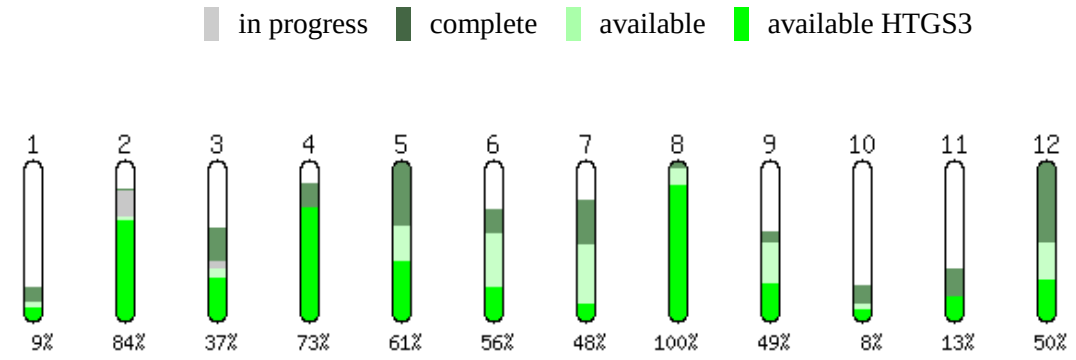
Nearly 75 percent of Turkey's total tomato production is consumed as fresh and the rest is processed. Of the 25 percent of the tomato that is processed, about 85 percent is used to produce tomato paste, 10 percent is used for canned tomatoes, and the remainder is used for ketchup, tomato juice, dried tomatoes and other products (Sirtioglu, 2003)

### 1.6.2 Genome

The cultivated tomato is a perennial diploid dicotyledon and it has 12 chromosomes (2n=24). The genome size of tomato is 950mb. The sequencing of the tomato genome is being carried out by The International *Solanaceae* Genomics Project (SOL) which was started in 2003. The genome of tomato is being sequenced by an international consortium of ten countries and forming tomato gene databases, the gene expression database, the

tomato metabolite database, genome annotations, etc, are maintained in the SOL Genomics Network (SGN; http://sgn.cornell.edu) (Bai, and Lindhout, 2007). The current situation in the sequencing can be observed from the website of the International Tomato Sequencing project (Figure 1.10) (http://solgenomics.net/).

With knowledge of tomato genome, it will be possible to learn about the evolution of the genes and also gene functions which would lead to the improvements in breeding and also in GMO studies. The reference euchromatic tomato sequence is expected to be completed during 2010 (The Tomato Genome Sequencing Consortium, 2009).




chromosome 1: USA, chromosome 2: Korea, chromosome 3: China, chromosome 4: UK, chromosome 5: India, chromosome 6: Netherlands, chromosome 7: France, chromosome 8: Japan, chromosome 9: Spain, chromosome 10: USA, chromosome 11: China, chromosome 12: Italy, HTGS: High Throughput Genomic Sequences

**Figure 1.10 Pre-release of Tomato Genome Shotgun Sequence and the Countries involved in the Sequencing of Tomato retrieved in December 2009 (http://solgenomics.net/)**

### 1.6.3 Transgenic Events of Tomato

Transgenic tomatoes are also being developed around the world like in the case of other crop plants. In order to speed up the process of plant breeding, crosses and backcrosses and transgenic technology are applied to achieve desired qualities in foods. In order to form these methods for obtaining a successful commercial transgenic event, it is necessary to (i) develop a system for the recovery of large number of whole plants from explant tissues, (ii) optimize the process of inserting a foreign gene to the plant or silencing a gene within the plant genome (Fatima et al, 2008).

In the period of 1992-2003, there had been 75 applications for field trials of GM tomatoes in EU; this application number is 635 during 1985-2008 for USA and other countries like Mexico, Japan, Canada and so on. There had not been any approval for the cultivation of GM tomatoes in EU. However in USA, in 1998 nearly 200.000 hectares were under cultivation with GM tomatoes (GMO-Compass, http://www.gmo-compass.org). Currently, there are 6 main transgenic events that are recognized by some countries (Table 1.11). These are 1345-4 (approved by Canada, Mexico, USA), 35 1 N (approved by USA), 5345 (approved by Canada and USA), 8338 (approved by USA), B, Da, F (approved by Canada, Mexico, USA), FlavrSavr (approved by Canada, Japan, Mexico, USA).

In addition to the current recognized tomatoes, there are also studies of development of transgenic tomatoes. For example, at the international level the field trials of the tomato event AtNHX1, is being carried out, which is developed by Arcadia Bioscience (USA) against salinity conditions. Also in India, in Indian Agricultural Research Institute (IARI), New Delhi, transgenic tomatoes with resistance to Leaf Curl Virus are tried to be developed using Replicase (ToLCV) and RNAi constructs (ToLCV) (Bansal 2009). The common traits that are tried to be obtained in the newly developed tomato lines mostly have the following characteristics (GMO-Compass, http://www.gmo-compass.org):

- **Product traits** such as modified composition of components (pectin, starch, fructose), enrichment of vitamins or secondary plant materials (carotenoids, lycopene, folic acid, flavonoids), reduction in allergens.
- **Agronomic traits** such as resistance to pests, insect resistance, virus and fungal resistance, adaptation to climate and location factors, drought, salinity and cold resistance.

- **Plant development characteristics** such as delayed maturity, parthenocarpy.
- **Renewable primary products** in molecular pharming (the utilization of GM tomatoes to produce pharmaceuticals).

**Table 1.11 Transgenic Tomato Events (AGBIOS Database, http://www.agbios.com/dbase.php)**

| Event | Company | Description |
|---|---|---|
| **1345-4** | DNA Plant Technology Corporation | Delayed ripening tomatoes produced by inserting a copy of a truncated gene encoding 1-aminocyclopropane-1-carboxyllic acid (ACC) synthase, which resulted in downregulation of the endogenous ACC synthase and reduced ethylene accumulation. |
| **35 1 N** | Agritope Inc. | Introduction of a gene sequence encoding the enzyme S-adenosylmethionine hydrolase that metabolizes the precursor of ethylene |
| **5345** | Monsanto Company | Resistance to lepidopteran pests, introduction of the *cry1Ac* gene from *Bacillus thuringiensis* subsp. Kurstaki. |
| **8338** | Monsanto Company | Introduction of a gene sequence encoding the enzyme 1-amino-cyclopropane-1-carboxylic acid deaminase (ACCd) that metabolizes the precursor of ethylene. |
| **B, Da, F** | Zeneca Seeds | Delayed softening tomatoes produced by inserting a truncated version of the polygalacturonase (*PG*) encoding gene in the sense or anti-sense orientation |
| **FLAVR SAVR** | Calgene Inc. | Delayed softening tomatoes produced by inserting an additional copy of the polygalacturonase (*PG*) encoding gene in the anti-sense orientation in order to reduce expression of the endogenous *PG* gene and reduce pectin degradation |

#### 1.6.3.1 Detection of Transgenic Tomatoes

Many transgenic products are being produced and commercialized; accordingly the studies related to the detection of these events gain more importance. Considering the transgenic events of tomato, there have been studies related to the adoption of the methods of identification.

There are studies related to the detection of transgenic tomato lines, for example in India, Bansal et al, 2009 developed transgenic tomatoes overexpressing *osmotin* gene, which were evaluated for drought and salt stress and the selected tolerant lines were tested in field trials. For this tomato event a study is conducted for the development of its detection methods by Randhawa et al, 2009 and multiplex PCR methods for the simultaneous detection of multiple sequences were tried to be formed for the *osmotin* gene, P-35S and *LAT 52* gene (endogenous reference gene).

Another example of a detection study can be given from China. In the study of Xie et al, conducted in 2007, the detection method of the transgenic tomato was tried to be formed using visible/near-infrared spectroscopy. Delayed ripening tomatoes with antisense *LeETR2* (ethylene receptor gene) were used as the samples of the study. They have tried to develop the principal component, discriminant and partial least-squares discriminant analysis for the detection of the differences between transgenic and non-transgenic tomatoes.

Mostly, the studies use DNA based detection methods for detection of genetic modifications in tomatoes, mainly PCR method (Boyce, 1999), (Yang et al, 2005), (Sonmezalp, 2004), (Turkoglu, 2007), (Uckun, 2007).

In table 1.12, it is possible to find the examples of detection techniques of the transgenic tomato events, which are given in the GMO databases (Section 1.4.4).

**Table 1.12 Examples of Detection of Transgenic Tomato Events**

| Event | Type of Assay | Type of Detection | Description | Database |
|---|---|---|---|---|
| **5345** | Gene | Quantitative | *cry1ac* | GMDD |
| **B, Da, F** | Gene | Qualitative | *PG* | GMDD |
| | Construct | Qualitative | *PG*-tnos | |
| | Event | Quantitative | genome-pJR16A | |
| **FlavrSavr** | Construct | Qualitative | *PG*-P35S | GMDD |
| **FlavrSavr** | Construct | Qualitative | | JRC |
| **Huafan No 1** | Construct | Qualitative | antiEFE-P35S | GMDD |
| | | Quantitative | antiEFE-P35S | |
| **Nema 282F** | Construct | Qualitative | *PG*-tnos | JRC |

### 1.6.3.2 Delayed Ripening Tomato

Ripening of fruits involve different biochemical reactions. Tomato fruit is a climacteric fruit and the ripening process is achieved by the increase of both respiration and ethylene production. The phytohormone ethylene has an important role in initiation of the early biochemical reactions initiating ripening and also the subsequent changes that occur (Fatima et al, 2008). Ethylene biosynthesis in plants has a reaction cascade that involves the reactions that are depicted in Figure 1.11.

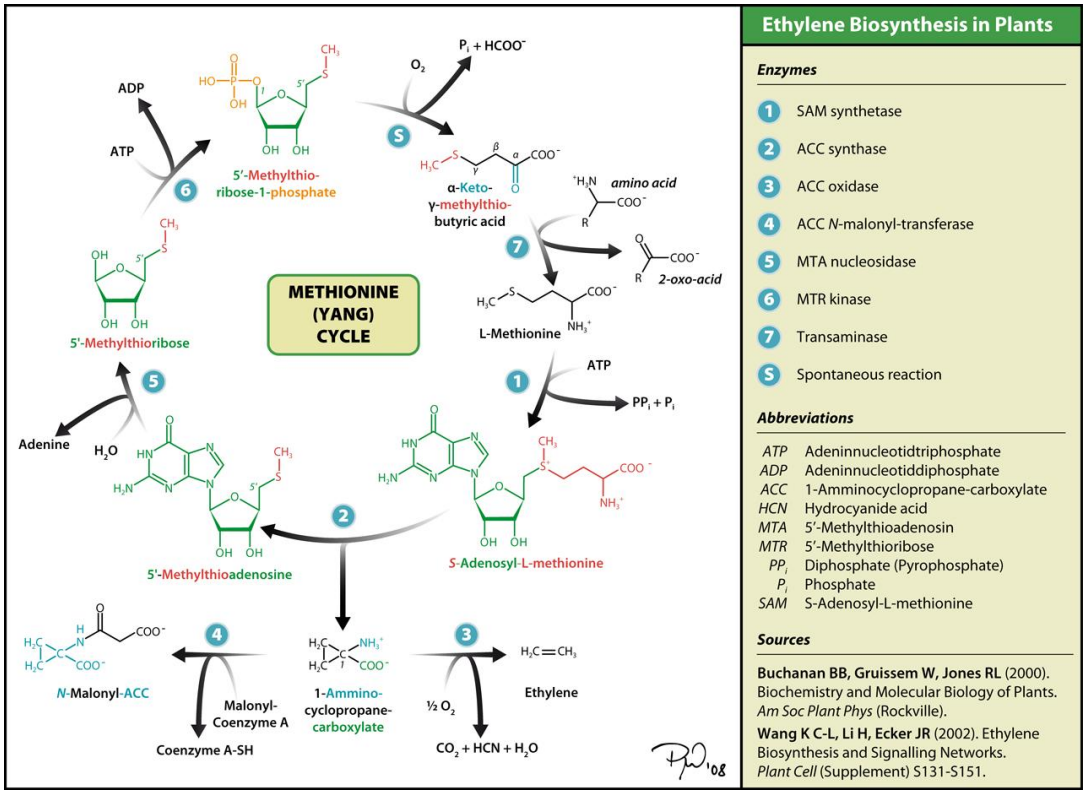


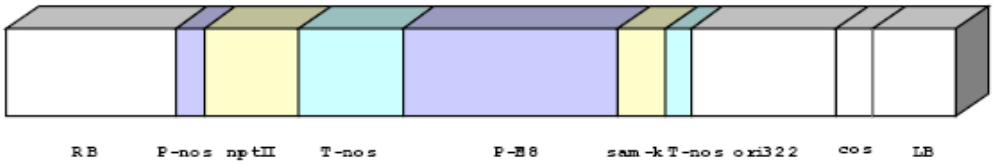

**Figure 1.11 Ethylene Biosynthesis in Plants (http://en.wikipedia.org/wiki/File:Yang-cycle.png)**

Among the 6 transgenic tomato events that are recognized (Table 1.11), most of them have the delayed ripening characteristics. For example the tomato event 1345-4 contains a truncated version of the tomato 1-aminocyclopropane-1-carboxyllic acid (ACC) synthase gene (enzyme 2 in the fig. 1.11). ACC synthase is responsible for the conversion of s-adenosylmethionine (SAM) to ACC, which is the precursor of ethylene. With the help of the truncated version of the gene, the suppression of the gene expression of the native ACC synthase is possible. Another example is the 8338 tomato event which harbors an additional gene from *Pseudomonas chlororaphis* and this gene is responsible for the enzyme that catalyzes ACC (AGBIOS, http://www.agbios.com/dbase.php).

In this study, mainly the event 35 1 N is taken into consideration. In this event SAMase encoding gene, derived from *E.coli* bacteriophage T3, is inserted into the genome. With this insertion, the level of the SAM (the product between the 1$^{st}$ and 2$^{nd}$ enzymes in Fig.

1.11) is expected to decrease, which is the substrate for the conversion to ACC. In this case ripening is delayed, and when these tomatoes are exposed to exogenous ethylene, they ripen normally (Kramer et al, 1995).

The delayed ripening event of 35 1 N (Agritope Inc.) was approved in USA only and this was in the year 1996 (AGBIOS, http://www.agbios.com/dbase.php). The gene cassette of this event that has been inserted into the tomato genome can be observed in figure 1.12.




| Abbreviation | Element-Name | Size [KB] |
|---|---|---|
| RB | Right Border | 1.8 |
| P-nos | P-nos | 0.3 |
| nptII | neomycin phosphotransferase | 1.02 |
| T-nos | T-nos | 1.1 |
| P-E8 | P-E8 | 2.3 |
| sam-k | S-adenosylmethionine hydrolase | 0.51 |
| T-nos | T-nos | 0.27 |
| ori322 | ori322 | 1.54 |

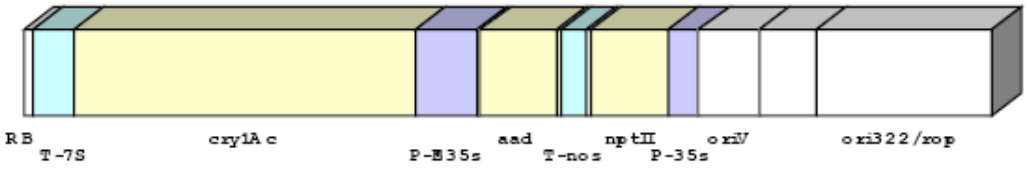
**Figure 1.12: Linear Map of DNA Construct used in the Transformation of 35 1N Tomato (Bruderer and Leitner 2003)**

### 1.6.3.3 Insect Resistant Tomato

The attack of the insects to the crops is a serious agricultural problem and it leads to yield losses and reduced product quality. Insects can cause damage both in the field and during storage of the agricultural crops (GMO-Compass, http://www.gmo-compass.org).

The insect resistant tomato (5345) harbors the insecticidal protein, Cry1Ac, encoded by the *cry1Ac* gene from the soil bacterium *Bacillus thuringiensis* subsp. kurstaki (B.t.k) strain HD73. The toxin is insecticidal to Lepidoptera larvae; these larvae are pests impacting tomato production (Gustafson et al, 1997).

The insect resistant event of 5345 was approved in USA (1998) and in Canada (2000) (AGBIOS, http://www.agbios.com/dbase.php). This event was developed by the firm Monsanto. The gene cassette of this event that has been inserted into the tomato genome can be observed in figure 1.13. This gene cassette is identical to the one that is inserted in the transgenic cotton line 531 which is also commercially known as Bollgard Cotton.




| Abbreviation | Element-Name | Size [KB] |
|---|---|---|
| RB | Right Border | 0.09 |
| T-7S | T-7S | 0.43 |
| | cry1Ac delta-endotoxin | 3.5 |
| P-E35s | P-E35s | 0.62 |
| aad | 3"(9)-O-aminoglycoside adenylyltransferase | 0.79 |
| T-nos | T-nos | 0.26 |
| nptII | neomycin phosphotransferase | 0.79 |
| P-35s | P-35s | 0.32 |
| oriV | oriV | 0.62 |
| Space | Space | - |
| ori322/rop | ori322/rop | 1.8 |

**Figure 1.13: Linear Map of DNA Construct used in the Transformation of 5345 Tomato (Bruderer and Leitner 2003)**

## 1.7 Aim of the Study

There is a need for establishing an efficient identification system for the genetic modifications, as more transgenic events are being developed and commercialized in the world. In order to ensure the traceability of the GMOs, the detection, identification and the quantification of the transgenic events are required. This study tries to form the basic procedures for the establishment of such a system.

The aim of this study was to demonstrate the steps for obtaining detection and identification methods for the two of the transgenic tomato events: delayed ripening and insect resistant using conventional and real-time amplification of significant fragments. In addition, for the quantification of the samples harboring an insect resistant toxin gene (*cry1ac*), real-time PCR conditions were tried to be optimized and standard curves were generated using two real-time PCR systems. Also, inverse PCR technique was performed with the aim of learning the DNA sequences of the flanking regions of the known gene fragment that is inserted.

The screening and gene-specific detection was formed using the positive control tomato seed material. The tomato seeds that were collected in previous studies were the sample material to be analyzed in this study. The amplifications of the samples were either compared with the positive control or were sent to sequencing. With the sequencing of the amplifications, it was possible to compare them with the expected results and the databases.

The transgenic events 35 1 N (delayed ripening, Agritope) and 5345 (insect resistant, Monsanto) were the model events in this study and accordingly the promoter, terminator, marker gene, the expressed gene and the constructs were the elements that are considered in the PCR detection.

In the amplifications, different DNA concentrations were used in order to be able to apply the detection methods to a wide range of concentration that would be achieved in the DNA extraction from food samples.

# CHAPTER 2

## MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Seed Samples

In this study, in order to develop the identification methods for the two of the model transgenic tomatoes (delayed ripening and insect resistant) previously screened tomato seeds were used as the samples to be analyzed. A total of 6 tomato samples were studied. These seeds were screened for the common transgenic elements as well as the genes that were expected to be inserted in the tomato genome.

#### 2.1.2 Reference Materials

As reference materials, both positive and negative controls were used. The tomato seed sample harboring 35S promoter, nos terminator and *nptII* resistance gene was used as the positive control for screening studies. This sample had been kindly provided by Chris Bowler, Laboratory of Molecular Plant Biology Stazione Zoologica, Italy to use as a reference material in the studies concerning tomato samples. For the positive control of the *cry1ac* gene, a plasmid that was provided by the *Bacillus* Genetic Stock Center was used. *E. coli* strain containing recombinant plasmid vector pKK223-3 was kindly provided by Daniel R. Zeigler, Ohio State University, USA. pKK223-3 plasmid contains *cry1Ac* gene and it is 8.4kbp in length (Appendix C). The untransformed plasmid is 4584bp in length.

### 2.2 Methods

#### 2.2.1 DNA Isolation

In DNA isolation CTAB (Cetyltrimethylammonium bromide) extraction method was used as it is outlined in the report of European Commission Joint Research Center (Somma,

JRC, Session 4, http://ec.europa.eu/dgs/jrc/). This is an important step to obtain the DNA from the seeds for further analysis and it directly affects the quality of the polymerase chain reaction.

#### 2.2.1.1 Surface Sterilization

Surface sterilization step was performed according to the method that is defined in Koc et al. 2007. This step is crucial for eliminating contamination in DNA isolation which could interfere with the reactions following DNA isolation. The seed samples were surface-sterilized for 1 min with 70% ethanol, and then soaked for 10 min in sodium hypochlorite solution. The seeds were then washed four times with distilled water in order to remove any remains.

#### 2.2.1.2 DNA Isolation from Seeds

For the DNA isolation, 150 mg of tomato seeds were weighed from the lot that was surface-sterilized and were ground with liquid nitrogen. The ground seeds were then transferred to 2 ml micro-centrifuge tube which is followed with the addition of 300 µl sterile water and 500 µl CTAB buffer (Applichem). The contents of the tube were mixed using a loop. 20 µl of Proteinase K (20mg/ml, Fermentas) was added, mixed and the tube was placed in 65$^{o}$C for 90 min. Following this incubation, 20 µl of RNase A (10mg/ml, Fermentas) was added, mixed and the tube was again placed in 65$^{o}$C for 10 min. After the incubation, the sample was placed in the micro-centrifuge (Hettich Zentrifugen Mikro 12-24) and was centrifuged at a speed of 16,000xg for 10 min. The supernatant that is produced after centrifugation was transferred into a new micro-centrifuge tube and 500 µl of chloroform was added and the tube was shaken for 30 sec. The tube was again centrifuged at 16,000xg and the supernatant part was transferred. This chloroform extraction step was repeated three times. Then the supernatant was transferred into a new micro-centrifuge tube and 2 volumes of CTAB precipitation solution (5 g/l CTAB (Applichem), 0.04M NaCl (Merck)) was added and mixed by pipetting which is followed by incubation at room temperature for an hour. The incubation is preceded with centrifugation at 16,000xg for 5 min and the supernatant was discarded. The precipitate was then dissolved with 350 µl NaCl (1.2M (Merck)) and 350 µl of chloroform was added to this tube. The contents were centrifuged for 10 min at 16,000xg and the upper layer was transferred into a new tube and 0.6 volumes of isopropanol (Applichem) was added and

mixed. Again this step was followed with a centrifugation of 10 min at 16,000xg. The supernatant was then discarded and a final was with ethanol was performed. 500 µl of ethanol was added, mixed and then centrifuged at 16,000xg for 10min. After this final centrifugation the supernatant was discarded and the pellets inside the tube were dried at room temperature. Then distilled water (100-150 µl) was added in order to re-dissolve DNA.

### 2.2.2 Concentration Determination

The concentration of the DNA, isolated from seeds was determined spectrophotometrically using AlphaSpec™ Spectrophotometer (Alpha Innotech, USA). The purity of the isolated DNA was also checked regarding the absorbance ratios at 260 nm and 280 nm. The quality and the purity of the DNA are critical factors for further analysis (Somma, 2002). CTAB protocol for extraction was chosen specifically for plant samples especially in order to eliminate polysaccharides and polyphenolic compounds which directly affect DNA purity and quality. Approximately a ratio of 1.8 is expected from a pure DNA sample. As the ratio goes up to 2.0, RNA contamination can be estimated.

The quality of the isolated DNA was also checked with agarose gel electrophoresis after the DNA isolation step. Intact bands were expected from the properly isolated DNA. Smears that were seen in the agarose gel can imply either contamination or the improper handling of the sample during DNA isolation.

### 2.2.3 Plasmid Isolation

As a reference material for the *cry1ac* gene, pKK223-3 plasmid was used which was transformed into *E.coli*. In order to obtain the plasmid, alkaline lysis method was employed. Firstly the colonies were grown on LB plates containing ampicillin because the *E.coli* strain that was obtained is resistant to ampicillin. The plates were incubated overnight at 37$^{o}$C. Prior to the extraction, the single colonies were inoculated to 5 ml LB broth containing falcon tubes together with 5 µl ampicillin. The tubes were then incubated in an incubator-shaker overnight at 37$^{o}$C. After the necessary growth was observed with turbidity, 1.5 ml of culture was taken into micro-centrifuge tubes and then was centrifuged for 5 min at 6500 rpm. The supernatant was removed and the remaining pellets were re-suspended in 200 µl of Solution I (50mM Glucose, 25mM Tris, 10mM EDTA). After re-suspension the tubes were incubated for 15 min at room temperature. 200 µl of Solution II

(0.2N NaOH, 1% SDS) was added following this incubation and the contents of the tube was mixed gently by inversions. The tube then was kept on ice for 5 min and it was followed with the addition of Solution III (3M Na-Acetate, pH: 4.8) which was kept cold. Again the tube was mixed gently with inversions for 10 sec and then it is kept on ice for 15 min. The tube was centrifuged at 13000 rpm and the supernatant was transferred into a new eppendorf tube. 2 volume of cold ethanol was added to the tubes in order to precipitate the plasmid and these were kept at -20$^{o}$C for 10 min. After the final centrifugation of 13000 rpm for 10 min, the liquid part was removed and the pellet was dissolved in distilled water after the drying process.

### 2.2.4 Primer Design

Primer design was performed taking the validated primers, previous studies that are conducted in the laboratory and the sequences from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) into consideration. New primers were designed, especially for gene and construct specific analysis. The program of Primer3 (http://frodo.wi.mit.edu/) was used for the primer design. The oligonucleotides used in this study, given in the table 2.1, were all synthesized by Iontek (Istanbul, Turkey).

For the species-specific control system, *patatin* gene was used for tomato. A new primer set was designed yielding a 237bp band. In the screening studies the control elements (P-35S, T-nos, *nptII*) were studied with the previously designed and/or validated primer pairs. Additionally, in the identification of the insect resistant tomato, an additional marker gene (*aad*) was taken into consideration in the screening studies. A primer pair yielding 373bp was used for this marker gene. For the gene-specific system, new primers were designed both for the *cry* and *sam* genes. For construct-specific studies, model transgenic constructs were used in order to design the primers. The primers used in this study can be seen in Table 2.1, which outlines the detection type, target gene, primer sequences (5'-3'), length of the expected fragment (bp), reference for the primers and accession number of the gene or the plasmid that was used for the design of the primers. In table 2.1, IR stands for insect resistant and DR stands for delayed ripening.

## Table 2.1 Primers Used in this Study

| Detection Type | Target | Prim. | Sequence (5'-3') | bp | Ref. | Access. |
|---|---|---|---|---|---|---|
| **Species** | *Pat.* | fp | TCCGGTGAAGGAGCTAGTGT | 237 | this study | X03932 |
| | | rp | GCTGCTGCTGTGGAATAACA | | | |
| | | | | | | |
| **Screen** | P-35S | fp | GCTCCTACAAATGCCATCA | 195 | JRC | AR656168 |
| | | rp | GATAGTGGGATTGTGCGTCA | | | |
| | | | | | | |
| | T-Nos | fp | GAATCCTGTTGCCGGTCTTG | 180 | JRC | U12540 |
| | | rp | TTATCCTAGTTTGCGCGCTA | | | |
| | | | | | | |
| | | fp | TCGTTCAAACATTTGGCAAT | 207 | Uckun(2007) | U12540 |
| | | rp | TTGCGCGCTATATTTTGTTTT | | | |
| | | | | | | |
| | *nptII* | fp | TTGCTCCTGCCGAGAAAG | 459 | Sonmezalp(2004) | AF274974 |
| | | rp | GAAGGCGATAGAAGGCGA | | | |
| | | | | | | |
| | | fp | GCCCTGAATGAACTGCAGGACGAGGC | 411 | CORESTA | U32991.1 |
| | | rp | GCAGGCATCGCCATGGGTCACGACGA | | | |
| | | | | | | |
| | | fp | GGATCTCCTGTCATCT | 175 | BgVV | U32991.1 |
| | | rp | GATCATCCTGATCGAC | | | |
| | | | | | | |
| | *aad* | fp | GAACATAGCGTTGCCTTGGT | 373 | Uckun(2007) | X02340 |
| | | rp | ATTTGCCGACTACCTTGGTG | | | |
| | | | | | | |
| **Gene** | *cry1ac* | fp | CGGGATTAGAACGTGTATGG | 329 | Uckun(2007) | AY225453 |
| | | rp | CATTATTTGATGCCCTGACC | | | |
| | | | | | | |
| | | fp | GCTTCTGTAACCCCGATTCA | 500 | this study | AY225453 |
| | | rp | CCACGTTCTGGTTGCCTATT | | | |
| | | | | | | |
| | | fp | ATTCGCTGGATGAAATACCG | 789 | this study | AY525369 |
| | | rp | TCTCGGACAATTCTCGCTTT | | | |
| | | | | | | |
| | *sam-k* | fp | GGTCTTTATGGCTCCGTTGA | 196 | this study | X04791 |
| | | rp | CAGCCGTGTGAGTCTGTGAT | | | |

**Table 2.1 continued.**

| Detect. Type | Target | Prim. | Sequence (5'-3') | bp | Ref. | Access. |
|---|---|---|---|---|---|---|
| **Constr.** | IR | fp | CCTACAAATGCCATCATT | | this study | PV-LEBK04 |
| | | rp | ATTTGCCGACTACCTTGGTG | | | |
| | | | | | | |
| | | fp | GAACATAGCGTTGCCTTGGT | | this study | PV-LEBK04 |
| | | rp | CTCCAAATGAAATGAAC | | | |
| | | | | | | |
| | DR | fp | GCCTGATGCGGTATTTTCTC | 914 | this study | pAG-5420 |
| | | rp | GGTCTTTATGGCTCCGTTGA | | | |
| | | | | | | |
| | | fp | GGAAAGCGGAAACCAGTACA | 573 | this study | pAG-5420 |
| | | rp | TACAACCTCCATGCCACTTG | | | |
| | | | | | | |
| **Inverse** | 1 | fp | TCAACGGAGCCATAAAGACC | | this study | X04791 |
| | | rp | TGCACGCCTAGCTTGTAATG | | | |
| | | | | | | |
| | 2 | fp | GGAAAGCGGAAACCAGTACA | | this study | X04791 |
| | | rp | GGTCTTTATGGCTCCGTTGA | | | |

### 2.2.5 Primer Preparation/Dilution

The primer oligonucleotides were obtained in the lyophilized form from Iontek (Istanbul, Turkey). Firstly 100 µM primer stocks were prepared with the addition of sterilized water according to the pmol values of the primers. Then the stock was incubated at 65$^{o}$C for 5 min and this incubation was repeated again for 3 min after the tubes were mixed. A dilution of 1:5 was obtained and finally 20µM aliquots were prepared (Querci et al, JRC, Session 9, http://ec.europa.eu/dgs/jrc/).

### 2.2.6 Amplification of Significant Fragments using Polymerase Chain Reaction (PCR)

The amplification reactions were performed in Biorad Mini-thermocycler, Techne TC-512 thermocycler and Applied Biosystems GeneAmp PCR System 9700. The total reaction volume was 30µl for each of the PCR.

Both the parameters like $MgCl_2$ and primer concentrations and the PCR cycling conditions were optimized for each of the primer pair. Each of the PCR system is given below with its PCR components and also cycling conditions (Table 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 2.10, 2.11, 2.12, 2.13, 2.14, 2.15).

#### 2.2.6.1 Optimization of PCR

It is important to optimize the components that are used in the amplification reactions in order to obtain accurate results. Taguichi optimization was used for the optimization of the primer pairs as explained in the study by Cobb and Clarkson, 1994. The factors affecting the PCR were used in different concentrations in order to check the effect of them in the resulting amplification. In this method, factors are arranged into an orthogonal array. In the PCR, each column represents individual reaction components like the concentrations of dNTP, primers and so on; and each row represents individual reaction vessels (Cobb and Clarkson, 1994). Each component of the PCR being tested occurs in the levels like A, B, C; so that their effects on the reaction can be determined. The concentrations were determined using the probable ranges that could affect the reaction. In table 2.2, it is possible to observe how each experiment tests the combination of the effects in different levels. Table 2.3 shows how each level is presented in terms of the factors of PCR.

**Table 2.2 Orthogonal Array for 4 variables of the PCR at 3 levels**

| Factors | Primer | DNA | MgCl2 | dNTP |
|---|---|---|---|---|
| **Experiment** | | | | |
| **1** | A | A | A | A |
| **2** | A | B | B | B |
| **3** | A | C | C | C |
| **4** | B | A | B | C |
| **5** | B | B | C | A |
| **6** | B | C | A | B |
| **7** | C | B | A | C |
| **8** | C | B | A | C |
| **9** | C | C | B | A |

**Table 2.3 Concentrations of the Factors that are tested for the Optimization**

| Level → | A | B | C |
|---|---|---|---|
| **Factors ↓** | | | |
| **Primer (µM)** | 0.5 | 1.5 | 3 |
| **Template DNA (ng)** | 50 | 100 | 150 |
| **MgCl2 (mM)** | 0.5 | 2 | 5 |
| **dNTP (mM)** | 0.1 | 0.2 | 0.4 |

Using different combinations of the factors effecting the amplification, it is possible to optimize the PCR conditions. Additional reactions might also be required in order to improve the gel image of the desired band.

**2.2.6.2 Control PCR (Species-Specific)**

For the tomato-specific reference system, *patatin* gene was used. For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR

Buffer (Fermentas), 3mM $MgCl_2$ (Fermentas), 0.2mM dNTP (Fermentas), 1µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$.

**Table 2.4 PCR Conditions for *Patatin* gene**

| | Temperature($^oC$) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 300 |
| | | |
| **Denaturation** | 95 | 30 |
| **Annealing** | 54 | 40 |
| **Extension** | 72 | 40 |
| **Number of Cycles: 40** | | |
| | | |
| **Final Extension** | 72 | 300 |

**2.2.6.3 Screening PCR**

For the amplification of the promoter 35S, the validated primers of JRC that yield a 195bp band were used. For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR Buffer (Fermentas), 3mM $MgCl_2$ (Fermentas), 0.2mM dNTP (Fermentas), 1µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$.

For the terminator nos, the JRC validated primer pair yielding 180bp was used in the amplification. For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR Buffer (Fermentas), 3.2mM $MgCl_2$ (Fermentas), 0.2mM dNTP (Fermentas), 1µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$.

**Table 2.5 PCR Conditions for P-35S**

| | Temperature($^{o}$C) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 300 |
| | | |
| **Denaturation** | 95 | 30 |
| **Annealing** | 54 | 40 |
| **Extension** | 72 | 45 |
| **Number of Cycles: 40** | | |
| | | |
| **Final Extension** | 72 | 300 |

**Table 2.6 PCR Conditions for T-Nos**

| | Temperature($^{o}$C) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 300 |
| | | |
| **Denaturation** | 95 | 30 |
| **Annealing** | 58 | 40 |
| **Extension** | 72 | 55 |
| **Number of Cycles: 40** | | |
| | | |
| **Final Extension** | 72 | 300 |

For the screening of the marker gene, kanamycin resistance gene, the primer pair that was previously designed in our laboratory was used and it yields a 459bp band. For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR Buffer (Fermentas), 3mM $MgCl_2$ (Fermentas), 0.2mM dNTP (Fermentas), 1µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$.

**Table 2.7 PCR Conditions for *nptII***

| | Temperature($^{o}$C) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 180 |
| | | |
| **Denaturation** | 95 | 30 |
| **Annealing** | 54 | 40 |
| **Extension** | 72 | 50 |
| **Number of Cycles: 40** | | |
| | | |
| **Final Extension** | 72 | 420 |

For the additional analyses of the *nptII* gene, the primer pairs yielding 411bp and 175bp fragments were also used in PCR analysis. These primer pairs were used in literature and in the previous studies conducted in our laboratory. The PCR conditions for these primer pairs were taken the same as the 459bp yielding primer pair (Table 2.7). For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR Buffer (Fermentas), 3mM $MgCl_2$ (Fermentas), 0.2mM dNTP (Fermentas), 1µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$.

For the screening of the other marker gene for the insect resistant tomato, *aad*, (3''(9)-O-aminoglycoside adenylyltransferase), the primer pair that was designed in our laboratory was used and it yields a 373bp band. For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR Buffer (Fermentas), 5mM $MgCl_2$ (Fermentas), 0.2mM dNTP (Fermentas), 3 µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$.

**Table 2.8 PCR Conditions for *aad***

| | Temperature($^{o}$C) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 420 |
| | | |
| **Denaturation** | 95 | 30 |
| **Annealing** | 55 | 40 |
| **Extension** | 72 | 60 |
| **Number of Cycles: 40** | | |
| | | |
| **Final Extension** | 72 | 300 |

#### 2.2.6.4 Gene-Specific PCR

In order to perform identification studies for the insect resistant and delayed ripening tomato events, amplifications of *cry1ac* and *sam-k* genes were performed.

For the insect resistant tomato event, which harbors *cry1ac* gene, the primer pairs yielding 329bp, 500bp and 789bp fragments were used. For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR Buffer (Fermentas), 3mM $MgCl_2$ (Fermentas), 0.2mM dNTP (Fermentas), 2 µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$.

**Table 2.9 PCR Conditions for *cry1ac***

| | Temperature($^{o}$C) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 420 |
| | | |
| **Denaturation** | 95 | 25 |
| **Annealing** | 58 | 40 |
| **Extension** | 72 | 45 |
| **Number of Cycles: 40** | | |
| | | |
| **Final Extension** | 72 | 420 |

One of the delayed ripening events of the transgenic tomatoes contains *sam-k* gene and this event was considered in this study. The primer pair yielding 196bp band was designed using the nucleotide sequence of the *sam-k* gene (Appendix E, Figure E.2). For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR Buffer (Fermentas), 3mM $MgCl_2$ (Fermentas), 0.2mM dNTP (Fermentas), 1 µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$

**Table 2.10 PCR Conditions for *sam-k***

| | Temperature($^oC$) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 300 |
| | | |
| **Denaturation** | 95 | 30 |
| **Annealing** | 60 | 40 |
| **Extension** | 72 | 45 |
| **Number of Cycles: 40** | | |
| | | |
| **Final Extension** | 72 | 300 |

### 2.2.6.5 Construct-Specific PCR

In order to perform construct studies for the delayed ripening tomato, the junction regions of the gene *sam-k* and terminator T-nos was aimed as well as the junction between the promoter P-E8 and the gene *sam-k*.

For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR Buffer (Fermentas), 5mM $MgCl_2$ (Fermentas), 0.2mM dNTP (Fermentas), 3 µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$.

**Table 2.11 PCR Conditions for the Construct Harboring *sam-k***

| | Temperature($^{o}$C) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 420 |
| | | |
| **Denaturation** | 95 | 30 |
| **Annealing** | 55 | 45 |
| **Extension** | 72 | 60 |
| **Number of Cycles: 40** | | |
| | | |
| **Final Extension** | 72 | 300 |

In order to amplify the junction regions of the gene cassette that was introduced to the insect resistant tomato, insect resistant cotton line was taken as the example because the same gene cassette was used in both of the events. The amplification was aimed at the junction region of the marker gene *aad* and the promoter region P-35S. Since the sequence of the gene cassette was not available, (only parts of the cassette was accessible) the band size was not determined prior to the amplification. The results were sent to sequencing for further analysis.

For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR Buffer (Fermentas), 3mM $MgCl_2$ (Fermentas), 0.4mM dNTP (Fermentas), 1.7 µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$.

**Table 2.12 PCR Conditions for the Construct Harboring *cry1ac***

| | Temperature(°C) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 420 |
| | | |
| **Denaturation** | 95 | 30 |
| **Annealing** | 54 | 40 |
| **Extension** | 72 | 60 |
| **Number of Cycles: 40** | | |
| | | |
| **Final Extension** | 72 | 300 |

In addition to these construct analysis, PREMasters that are prepared by Eurofins GeneScan GmbH (Germany) were used. The PREMasters include the primers, dNTPs, buffer and $MgCl_2$, which are required for the PCR amplification. In addition to these reagents, Taq DNA polymerase (Fermentas) is added to the final master mix that is prepared for the PCR. The PREMasters included the necessary primer sets in order to amplify the following junctions of the gene constructs (also applicable for delayed ripening and insect resistant tomato):

- 35-1-N(DR): PREMaster nos/nptII (Figure 2.1)
- 5345 (IR): PREMaster 35S/nptII (Figure 2.2)

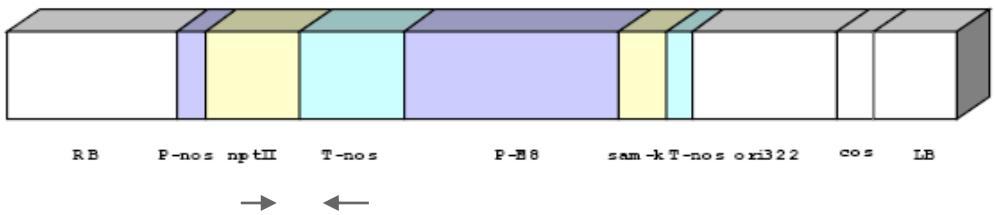



**Figure 2.1 Linear Map of DNA Construct used in the Transformation of 35 1N Tomato (Bruderer and Leitner 2003) and the Amplification Region**

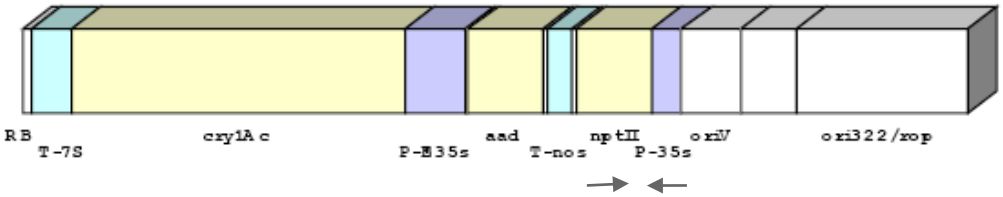



**Figure 2.2 Linear Map of DNA Construct used in the Transformation of 5345 Tomato (Bruderer and Leitner 2003) and the Amplification Region**

The PCR conditions of the construct analysis using the PREMasters can be observed in the table 2.13.

**Table 2.13 PCR Conditions for the Construct Analysis using PREMasters**

| | Temperature($^{o}$C) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 94 | 600 |
| | | |
| **Denaturation** | 95 | 25 |
| **Annealing** | 55 | 30 |
| **Extension** | 72 | 45 |
| **Number of Cycles: 50** | | |
| | | |
| **Final Extension** | 72 | 180 |

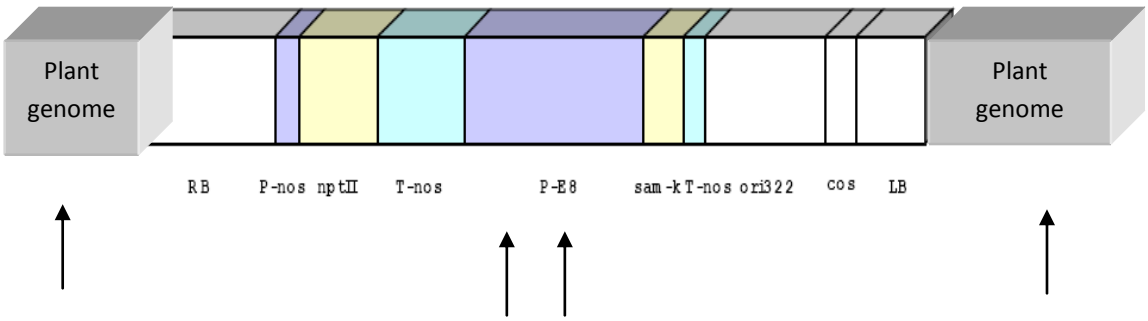
### 2.2.7 Inverse PCR

The inverse PCR analysis was initiated with the digestion of the tomato genomic DNA with restriction enzymes. The enzymes EcoRI, BamHI, HindIII and XbaI (Fermentas) were used in the restriction analysis. In the analysis, 1-2µg DNA, 1X restriction buffer, 10-20U of the enzymes were used and the samples were incubated at 37$^{o}$C overnight.

Additionally double digests were performed along with single restriction enzyme digestion. The restriction enzymes were inactivated with heat. Following digestion of the genomic DNA, self-ligation of the ends of the digested fragment (circularization) was performed using T4 DNA ligase (Fermentas). Ligation was performed with different trials in order to ensure circularization of the desired fragment rather than concatenation. Different concentrations of digested DNA were used for ligation trials (0.01µg, 0.05 µg, 0.1 µg, 0.2 µg) with 10U of T4 DNA ligase. The ligation was achieved with overnight incubation at 15 $^{o}$C. After the intended fragments were ligated, PCR amplification was done for the target regions using two pairs of primers that are directed to a region outside the known region. For the PCR, the following components with specified concentrations were prepared in 30µl: 1X PCR Buffer (Fermentas), 3mM $MgCl_2$ (Fermentas), 0.2mM dNTP (Fermentas), 3 µM forward and reverse primer, 1 unit Taq DNA Polymerase enzyme (Fermentas) and $ddH_2O$. The PCR conditions were given in Table 2.14. The steps of the inverse PCR could be observed in the following figure (Figure 2.3), which takes the *sam-k* gene cassette as the model of analysis. Although, *sam-k* gene cassette is taken as the model of the study, the crucial point is not to digest the *sam-k* gene fragment in order to amplify the flanking regions surrounding the gene.

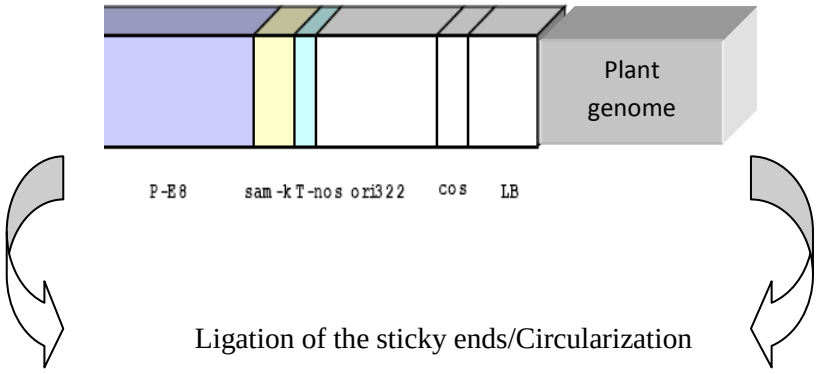
**Table 2.14 PCR Conditions for the Inverse PCR Analysis**

| | Temperature($^{o}$C) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 300 |
| | | |
| **Denaturation** | 95 | 30 |
| **Annealing** | 54 | 40 |
| **Extension** | 72 | 70 |
| **Number of Cycles: 40** | | |
| | | |
| **Final Extension** | 72 | 600 |




XbaI, HindIII EcoRI, BamHI HindII XbaI XbaI, HindIII, EcoRI, BamHI

(Random cuts in genome) (Random cuts in genome)




Ligation of the sticky ends/Circularization

(T4 DNA Ligase)

⇩

PCR Amplification (with Inverse Primers)

**Figure 2.3 Principles and Steps that were followed in Inverse PCR**

Inverse PCR was repeated for the optimization of the process. Chloroform extraction was applied following the restriction digestion, in order to optimize the circularization step. Additionally, long PCR enzyme mix (Fermentas) was used in the amplification studies, which is used for the long expected fragments and also for the problematic DNA sequences. The enzyme mix contains *taq* DNA polymerase and also another thermostable

polymerase which has proofreading activity (that is crucial for obtaining longer fragments). The cycling conditions of the PCR can be found in Table 2.15.

**Table 2.15 PCR Conditions for the Long PCR Analysis**

| | Temperature($^{o}$C) | Time (sec) |
|---|---|---|
| **Initial Denaturation** | 95 | 180 |
| | | |
| **Denaturation** | 95 | 20 |
| **Annealing** | 54 | 30 |
| **Extension** | 68 | 60 |
| **Number of Cycles: 10** | | |
| | | |
| **Denaturation** | 95 | 20 |
| **Annealing** | 54 | 30 |
| **Extension** | 68 | 80 |
| **Number of Cycles: 25** | | |
| | | |
| **Final Extension** | 72 | 600 |

### 2.2.8 Agarose Gel Electrophoresis

The results of the PCR were visualized under UV light using agarose gel electrophoresis. The concentration of agarose in the gel was adjusted according to size of the expected amplified fragment and it was taken as 1-1.25%.

The agarose gels were run at 100V (Biorad) and then stained with ethidium bromide in order to visualize the gels under UV light (Biorad UV Transilluminator).

### 2.2.9 Sequence Analysis of Significant Fragments

In order to confirm the fragments that were amplified using the specific primer pairs, DNA sequencing step was utilized. The fragments were sent to sequencing and the results were

checked using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) programs.

### 2.2.10 Screening for Genetic Modifications using Real-time PCR

For the screening of the P-35S and T-nos regions, LightCycler® GMO Screening Kit was used and real-time PCR reactions were performed with Roche LightCycler 1.5. The kit was mainly used for the qualitative detection of these common genetic modifications and at the same time it has the amplification mix for a plant specific gene, which enables to control the amplificability of the sample DNA. The PCR reagents that are used for the screening involve P35S/TNOS Detection Mix, Plant Gene Detection Mix, GMO Screening Enzyme Solution, GMO Screening Reaction Mix, and GMO Screening Control Template. The screening was performed with the primers and the hybridization probes that are specific for the 35 promoter and the nos terminator region that are commonly found in genetically modified organisms. It is asserted that the hybridization probe system "minimizes contamination risk and contains all reagents (except for template DNA) needed for screening genetically modified plants." The conditions that are followed in the screening analysis and the components that are used can be observed in Table 2.16 and Table 2.17 respectively.

**Table 2.16 Screening of P-35S and T-nos Elements using Real-time PCR**

| | Initial Denaturation | Denaturation | Annealing | Extension | Cooling |
|---|---|---|---|---|---|
| **Temp($^{o}$C)** | 95 | 95 | 60 | 72 | 40 |
| **Time(min.sec)** | 15.00 | 00.00 | 00.25 | 00.15 | 00.30 |
| **Acquisition Mode** | None | None | Single | None | None |
| **Cycle:45** | | | | | |

**Table 2.17 Real-time PCR Components for the Screening Analysis**

| **Component** | Volume | Final Concentration |
|---|---|---|
| **$H_2O$ PCR grade** | 11 | |
| **P35S/TNOS Detection Mix Or Plant Gene Detection Mix** | 2 | 1X |
| **GMO Screening Enzyme Master Mix** | 2 | 1X |
| **Total Volume** | 15 | |
| ***mixed carefully pipetting up and down.** | | |

### 2.2.11 Amplification of *nptII* gene using Real-time PCR

Different regions of the *nptII* gene were aimed in the qualitative detection with real-time PCR. Three different pairs of primers were used in the amplification reactions and the expected fragments have the following lengths: 459bp, 411bp, 175bp (Table 2.1). Real-time PCR reactions were performed with Roche LightCycler 1.5. SYBR Green I mix (LightCycler® FastStart DNA Master SYBR Green I) was used in the reactions. The components that were used in the reaction is given in Table 2.18. The cycling parameters for the *nptII* detection system can be observed in Table 2.19, which outlines the length of the fragment that is analyzed, denaturation, amplification, melting curve and cooling conditions.

**Table 2.18 Real-time PCR Components for the Detection of *nptII***

| **Component** | Volume | Final Concentration |
|---|---|---|
| **$H_2O$ PCR grade** | 11.6 | - |
| **$MgCl_2$ stock solution** | 2.4 | 4mM |
| **PCR Primer *2** | 1 | 0.5µM |
| **DNA Master SYBR Green I** | 2 | 1X |
| **Total Volume** | 18 | |
| ***mixed carefully by pipetting up and down.** | | |

**Table 2.19 LC-qPCR Cycling Parameters for the Detection of *nptII***

| Fragment | Denat. | Amplification (45cycles) | Melting Curve | Cooling |
|---|---|---|---|---|
| **459bp** | 95°C 10min, none | Denaturation: 95°C, 0s, none<br>Annealing: 54°C, 10s, none<br>Extension: 72 °C, 18s, single | Denaturation: 95 °C 0s, none<br>Annealing : 65°C, 15s, none<br>Melting:95 °C (slope: 0.1°C/sec), 0 s, continuous | 40 °C,30s, none |
| **411bp** | 95 °C 10min, none | Denaturation: 95°C, 10s, none<br>Annealing: 54°C, 5s, none<br>Extension: 72°C, 10s, single | Denaturation: 95°C, 0s, none<br>Annealing : 65°C, 15s, none<br>Melting: 95°C (slope: 0.1°C/sec), 0 s, continuous | 40 °C,30s, none |
| **175bp** | 95 °C 10min, none | Denaturation: 95°C, 10s, none<br>Annealing: 54°C, 5s, none<br>Extension: 72°C, 10s, single | Denaturation: 95°C, 0s, none<br>Annealing : 65°C, 15s, none<br>Melting: 95°C (slope: 0.1°C/sec), 0 s, continuous | 40 °C,30s, none |

### 2.2.12 Optimization of Real-time PCR Conditions for the Detection and Quantification of Samples Harboring *cry1ac*

In order to form a standard curve for the transgenic events harboring *cry1ac* gene, the plasmid harboring *cry1ac* gene was used as reference material (ECE53: *cry1Ac* cloned in pKK223-3; in *E. coli* JM103; ampicillin resistant). Real-time PCR reactions were performed with Roche LightCycler 1.5 and Applied Biosystems 7500 Real-Time PCR System. SYBR Green I mix (LightCycler® FastStart DNA Master SYBR Green I) was used in the reactions and each primer set was tested for its specificity.

The annealing temperature chosen for the amplification of quantification was determined with trials considering the melting temperature of the primers for the *cry1ac* gene (Table 2.1). The primers that yield 329bp, 500bp and 789bp bands were used in real-time PCR. The parameters and the conditions for this system can be observed in the Table 2.21. Furthermore, the components of the real-time PCR that are used in the reactions can be seen in Table 2.20.

The standard curves that are generated using different PCR systems were compared (Roche LightCycler 1.5 and Applied Biosystems 7500 Real-Time PCR System). The PCR cycling conditions and the components were tried to be kept the same in each unit, however according to the device that was used; the conditions were adjusted with respect to the system.

**Table 2.20 Real-time PCR Components for the Detection and Quantification of *cry1ac***

| **Component** | Volume | Final Concentration |
|---|---|---|
| **$H_2O$ PCR grade** | 11.6 | - |
| **$MgCl_2$ stock solution** | 2.4 | 4mM |
| **PCR Primer *2** | 1 | 0.5µM |
| **DNA Master SYBR Green I** | 2 | 1X |
| **Total Volume** | 18 | |
| ***mixed carefully by pipetting up and down.** | | |

**Table 2.21 LC- qPCR Cycling Parameters for the Detection and Quantification of *cry1ac***

| Fragment | Denat. | Amplification (45cycles) | Melting Curve | Cooling |
|---|---|---|---|---|
| **329bp** | 95°C 10min, none | Denaturation: 95°C, 0s, none<br>Annealing: 56°C, 10s, none<br>Extension: 72 °C, 10s, single | Denaturation: 95 °C 0s, none<br>Annealing : 65°C, 15s, none<br>Melting:95 °C (slope: 0.1°C/sec), 0 s, continuous | 40 °C,30s, none |
| **789bp** | 95 °C 10min, none | Denaturation: 95°C, 0s, none<br>Annealing: 58°C, 10s, none<br>Extension: 72°C, 20s, single | Denaturation: 95°C, 0s, none<br>Annealing : 65°C, 15s, none<br>Melting: 95°C (slope: 0.1°C/sec), 0 s, continuous | 40 °C,30s, none |
| **500bp** | 95 °C 10min, none | Denaturation: 95°C, 0s, none<br>Annealing: 56°C, 5s, none<br>Extension: 72°C, 10s, single | Denaturation: 95°C, 0s, none<br>Annealing : 65°C, 15s, none<br>Melting: 95°C (slope: 0.1°C/sec), 0 s, continuous | 40 °C,30s, none |

### 2.2.12.1 Standard Curve Formation

The plasmid was isolated and its concentration was determined for the initiation of the standard curve formation. The obtained DNA concentrations (ng/µL) were converted to copy numbers using the size of the transformed plasmid (8.4kbp). Serial dilutions were made to achieve genome copy numbers ranging from $10^4$-$10^{10}$ copies. Dilutions of the standards were then used to generate the standard curve. Firstly the mass of the plasmid was calculated according to the formula given below, (Eq.1), (Applied Biosystems,

http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_042486.pdf).

$$m = (n)(1.096e^{-21}\frac{g}{bp}) \text{ (Eq.1)}$$

Where n = plasmid size (bp), m = mass, $e^{-21} = 10^{-21}$

After calculating the mass of the single plasmid, mass of plasmid that is needed for a certain copy number was calculated (Eq.2).

$$Copy\ \#\ of\ interest \times mass\ of\ single\ plasmid = mass\ of\ plasmid\ DNA\ needed$$
(Eq.2)

From this value, it is possible to reach to the final concentration of the plasmid DNA using the volume that is going to be used in PCR. Finally serial dilutions were made using the plasmid stock. Using these dilutions in the real-time PCR, it was possible to generate a standard curve considering the copy number of the gene that was analyzed. The example of this calculation can be observed in the following table (Table 2.21).

**Table 2.22 Dilution Series of the Plasmid (ECE53)**

| **Source** | Concentration (g/µl) | Volume of plasmid (µl) | Volume of $dH_2O$ (µl) | Total Volume (µl) | Resulting Copy # of *cry1ac* |
|---|---|---|---|---|---|
| **Stock** | $2.6 \times 10^{-7}$ | - | - | - | - |
| **Dilution 1** | $4.6 \times 10^{-8}$ | 17.7 | 82.3 | 100 | $10^{10}$ |
| **Dilution 2** | $4.6 \times 10^{-9}$ | 10 | 90 | 100 | $10^{9}$ |
| **Dilution 3** | $4.6 \times 10^{-10}$ | 10 | 90 | 100 | $10^{8}$ |
| **Dilution 4** | $4.6 \times 10^{-11}$ | 10 | 90 | 100 | $10^{7}$ |
| **Dilution 5** | $4.6 \times 10^{-12}$ | 10 | 90 | 100 | $10^{6}$ |
| **Dilution 6** | $4.6 \times 10^{-13}$ | 10 | 90 | 100 | $10^{5}$ |
| **Dilution 7** | $4.6 \times 10^{-14}$ | 10 | 90 | 100 | $10^{4}$ |

### 2.2.12.2 Absolute Quantification Analysis and Melting Curve Analysis

Amplification curves and melting curves of the reactions were drawn and analyzed for each primer set. The fluorescence values versus cycle number were adjusted and specificity of the amplified PCR product was assessed by performing a melting curve analysis.

# CHAPTER 3

## RESULTS AND DISCUSSION

### 3.1 Experimental Strategies

In this study, the analyses of the genetic modifications were performed and developed based on two of the model transgenic tomatoes. The main steps that were followed included: DNA isolation, PCR amplification of significant fragments and verification of PCR products (Figure 3.1). In addition to conventional PCR analysis, real-time PCR analysis was also performed in the screening and the detection of genetic modifications and also in the formation of standard curves for the quantification of samples harboring *cry1ac*. The samples that were analyzed were tomato seeds that were collected in previous studies (Table 3.1) (Uckun, 2007), (Turkoglu, 2007).

**Table 3.1 The Seed Samples that are used in this Study**

| Seed Sample | Purchase Date | Information |
|---|---|---|
| **S1** | 2003 | Negative Control |
| **S6** | 2007 | Sample |
| **S9** | 2007 | Sample |
| **S13** | 2007 | Sample |
| **S17** | 2007 | Sample |
| **S19** | 2006 | Sample |
| **S27** | 2007 | Sample |
| **PC** | 2003 | Positive Control |

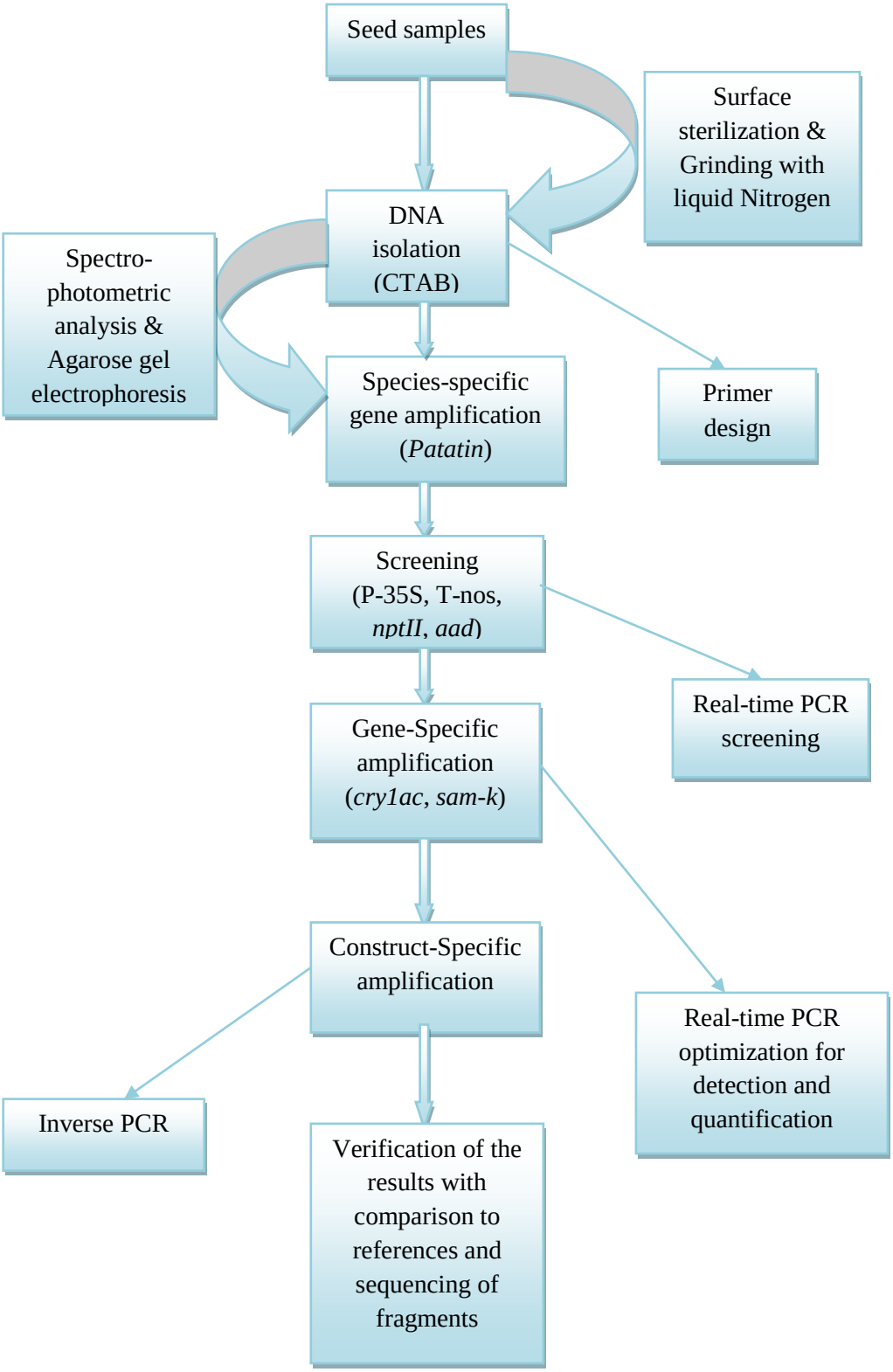



**Figure 3.1 Flow Chart of the Experimental Strategies**

### 3.2 DNA Isolation for GMO Analysis

The quality of the DNA, isolated from the samples, is essential for a reliable amplification analysis. The main aim of the isolation procedure is to obtain purified DNA from the samples that are to be analyzed using PCR studies.

DNA isolation was applied to the seed samples that are to be analyzed for genetic modifications. CTAB protocol was used since it is suitable for plant sources for the elimination of polysaccharides and polyphenolic compounds and also it has been used in the validation studies of GMO analysis. This protocol involves incubation in the presence of CTAB, extraction with chloroform and precipitation with isopropanol and ethanol (Somma, JRC, Session 4, http://ec.europa.eu/dgs/jrc/).

Firstly the seed samples were surface sterilized to remove the residues of soil, chemicals and to reduce the potential of bacterial contamination (Section 2.2.1.1). Then, the seed samples were grounded with liquid nitrogen using mortar and pestle in order to have powdered form of the seeds for the isolation. One of the most important points at this step is not to leave the powder to thaw in the mortar at room temperature. Otherwise, condensation of the atmospheric moisture would hydrate the sample and allow chemical and enzymatic reactions, such as the formation of polyphenolic compounds (Scarafoni and Duranti, 2001).

The factors related to handling in the isolation procedure, can also affect the quality of the isolated DNA, such as the unavoidable shearing of the DNA strands (Scarafoni and Duranti, 2001). The unwanted effects in the isolated DNA emerge from the grinding, handling and pipetting of the samples during the isolation procedure. Also the chemicals (chloroform, ethanol, isopropanol) that are used in the CTAB isolation procedure are the possible contaminants affecting the performance of the PCR (Somma, JRC, Session 4, http://ec.europa.eu/dgs/jrc/).

After the DNA isolation step, the samples were visualized in agarose gel electrophoresis (Figure 3.2). In addition to this, the concentration of the samples and the absorbance values at 260 and 280nm were measured (Table 3.2).

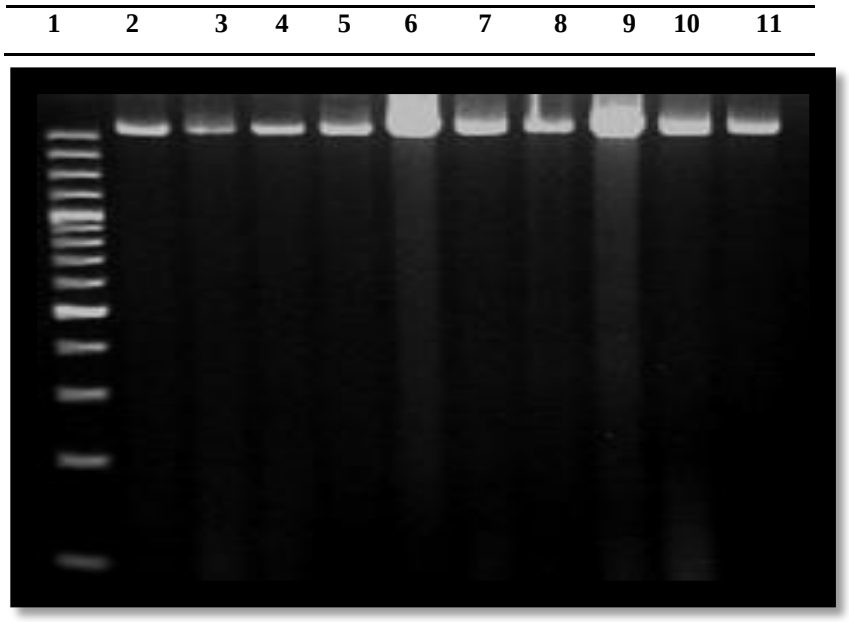



**Figure 3.2 DNA Isolation Results** Lane 1: 100bp DNA ladder, Lane 2: S1, Lane 3: S3, Lane 4: S6, Lane 5: S9, Lane 6: S13, Lane 7: S19, Lane 8: S27, Lane11: PC

The concentration and the quality of the DNA isolated from the samples were measured using AlphaSpec™ Spectrophotometer (Alpha Innotech, USA). The examples of the measurements can be observed in Table 3.2. The measurement of the absorbances at the wavelengths 260nm and 280nm are essential in order to use the absorbance ratio for commenting on the purity of the isolated DNA (Section 1.3.2.1). In addition, the readings taken at 260 nm allows calculation of the concentration of DNA (Concentration (ng/ml) = (A260 reading) x (50ng/µl) x dilution factor). Note that 1 O.D. at 260 nm for double-stranded DNA is 50 ng/ul of dsDNA (Mclaughlin, 2002).

The importance of the measurements at the specified wavelengths can be given as; A260nm: DNA absorbs light most strongly at 260nm; therefore this value is used to estimate the DNA concentration, A280nm: tyrosine and tryptophan residues absorb light strongly at this wavelength; therefore this value is used to estimate the protein contamination in the sample (Mclaughlin, 2002).

**Table 3.2 The Concentration of the Samples and the Absorbance Ratio at 260 and 280nm**

| Sample | A260/A280 | Concentration(ng/µl) |
|---|---|---|
| **S1** | 2.20 | 83.30 |
| **S6** | 1.97 | 123.68 |
| **S9** | 1.91 | 66.00 |
| **S13** | 2.00 | 143.00 |
| **S17** | 1.82 | 641.45 |
| **S19** | 2.10 | 129.10 |
| **S27** | 1.70 | 512.00 |
| **PC** | 2.10 | 95.73 |
| **Plasmid(ECE53)** | 1.75 | 691.09 |

A260/A280 ratio of 1.8 is required for a pure DNA sample and a decrease indicates contamination by proteins, while an increase in the ratio indicates RNA contamination (above 2.0) (Varma et al, 2007). Although some of the samples yielded a high A260/A280 ratio, other analyses (agarose-gel electrophoresis, Figure 3.2, species-specific amplification, Figure 3.3) showed that the DNA could be used for further analysis.

### 3.3 PCR Amplifications

In order to develop the detection and identification methods for the model transgenic tomato events, 18 different primer sets (Table 2.1) were used along with other primer pairs for the optimization studies.

Primer pairs were designed using the gene sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/) according to the transgenic event under study. In addition, the validated primer pair yielding 195bp fragment (JRC) was used for the screening of the promoter 35S (P-35S) and the validated primer pair yielding 180bp fragment (JRC) was used in the screening of terminator nos (T-nos). Note that JRC refers to Joint Research Centre, which is a body of European Commission and it is a research-based organization (http://ec.europa.eu/dgs/jrc/index.cfm).

The primer design was performed using the Primer3 program (http://frodo.wi.mit.edu/primer3/). The parameters that are considered for designing the primers are as follows: The percent of G/C bases in the primer, self-complementarity of the primer (tendency to anneal to itself or form secondary structure), 3' self-complementarity of the primer (tendency to form a primer-dimer with itself), melting temperature of the primer and also the length of the primer.

The concentrations of the sample DNAs were adjusted between 50ng and 200ng for each of the PCR amplifications throughout the study and the necessary fragments were obtained using the control and the sample DNAs.

Conventional PCR analyses were performed using Biorad Mini Gradient Thermal Cycler, Techne TC-512 and ABI GeneAmp PCR System 9700. Real-time PCR analyses were performed using Roche LightCycler 1.5 and Applied Biosystems 7500 Real-time PCR system.

### 3.3.1 *Patatin* Specific Control PCR for Tomatoes

In order to avoid the possibility of a false-negative result, species-specific amplification should be performed (Somma, JRC, Session 4, http://ec.europa.eu/dgs/jrc/). It is necessary to amplify an endogenous gene of the species under study for commenting on the amplificability of the DNA that has been isolated from the samples and to ensure no inhibitory reagent of PCR is present.

As it is shown in the study of Jaccaud et al, *patatin* gene can be used as a reference gene for the *Solanacea* family to which tomato belongs. *Patatin* gene codes for one of the major storage protein and it has been mapped genetically and physically in both the potato and tomato genomes (Ganal et al, 1991).

Using the gene sequence of *patatin*, X03932, in the Genbank of NCBI, (http://www.ncbi.nlm.nih.gov/) a primer pair was designed in this study, which yields a 237bp fragment.

All tomato seed samples that were analyzed showed the 237bp amplicon indicating the amplification capacity of the DNA that has been isolated (Figure 3.3). For the negative control, maize DNA was used and no amplification was seen in this case.

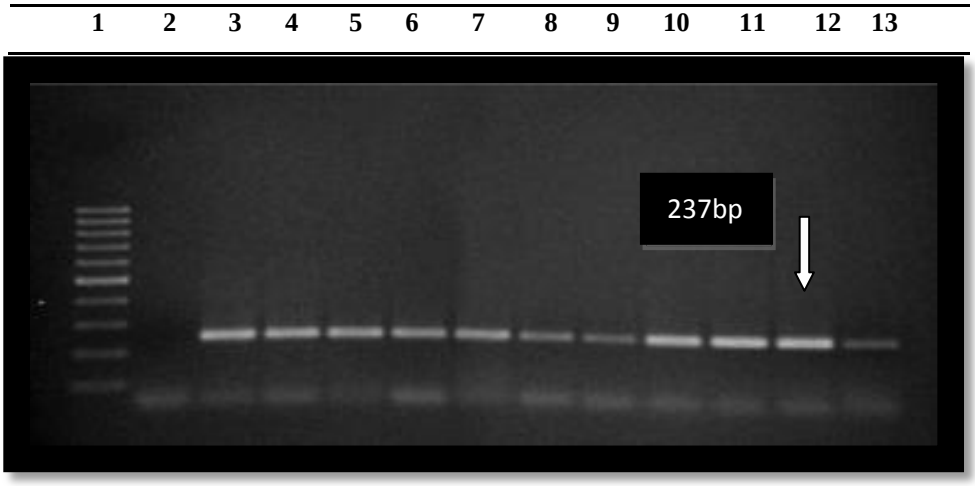



**Figure 3.3 *Patatin* specific PCR Amplification of Tomato samples (237bp)** Lane 1: 100bp DNA ladder, Lane 2: NTC, Lane 3: S1, Lane 4: S3, Lane 5: S6, Lane 6: S9, Lane 7: S13, Lane 8: S17, Lane9: S19, Lane10: S27, Lane13: PC

### 3.3.2 Screening PCR for the Detection of GM Tomatoes

The common elements used in the screening of GM tomatoes as well as other transgenic organisms include the promoter, terminator and marker gene regions.

In order to obtain the desired level of expression from the inserted gene constructs, the choice of the promoter region is essential. Different promoters induce different expression patterns for the same gene. In most of the transgenic events 35S promoter was used which is obtained from the Cauliflower mosaic virus (CaMV). According to the BATS report 2003, P-35S is the most frequently used promoter in the approved GM crops. Other promoters that are used in the transgenic events include P-E8 (*Solanum lycopersicum*), P-nos (*Agrobacterium tumefaciens*), P-Ssu (*Arabidopsis thaliana*), P-TA29 (*Nicotiana tabacum*), P-PEPC (*Zea mays*).

Although there is the common usage of P-35S promoter, the varieties of transgenic organisms also occur which use the promoter of the same species. For example, in Golden Rice, the transgenes are expressed under the control of the rice endosperm-specific glutelin promoter.

Similarly, in the High-Lysine Corn, which is in the pre-launch phase, the cordapA coding sequence is under the control of the maize Globulin 1 promoter (Corrado and Karali, 2009). Also in the delayed ripening tomato (35 1 N) that is taken as a model in this study, the promoter of the tomato is used (P-E8) in the gene construct (Figure 1.12).

Additionally in the gene construct, there is the terminator region that controls the ending of the expression of the gene that is inserted. The most frequently used terminator in approved GM crops is T-nos, that is from the nopaline synthase gene of *Agrobacterium tumefaciens* (Bruderer and Leitner, 2003). Other terminators that are used in the transgenic events include T-35S (CaMV), T-E9 (Pea), T-tml (*Agrobacterium tumefaciens*), T-ocs (*Agrobacterium tumefaciens*).

The other feature of the gene construct is the marker gene, which is also important in screening step. Bacteria have genes that make them resistant to antibiotics naturally produced by other microorganism. These genes are used in the gene constructs in the transformation process to mark the transformation (GMO-Compass, www.gmo-compass.org). The *nptII* gene confers resistance to the antibiotic kanamycin and neomycin, is the most often used marker gene of GMOs and it is widely used in the screening step. Other than *nptII*, *aad* marker gene, which confers streptomycin/spectinomycin resistance, is also used in the transgenic events (*Solanum lycopersicum* (5345), *Gossypium hirsutum* L. (1445, 1698), *Gossypium hirsutum* L. (15985), *Gossypium hirsutum* L. (Bollgard Cotton: 531, 757, 1076)) (Bruderer and Leitner 2003). The marker genes *nptII* and *aad* are both taken from *E.coli*.

In the screening studies for the detection of GMOs; P-35S, T-nos, *nptII* and *aad* genes are used in order to comment on the presence of a genetic modification (Barbau-Piednoir et al, 2010), (Hamels et al, 2009), (Reiting et al, 2007). Also in this study, these elements and marker genes are taken into account in the screening step of the detection system for the two transgenic tomato event, delayed ripening and insect resistant.

#### 3.3.2.1 Promoter 35S Amplification

For the amplification of the P-35 region, the primer pair that is validated by JRC was used and the expected amplicon was 195bp.

First of all, the optimization was tried to be accomplished using the positive control (PC) of tomato seed sample. Annealing temperature and the $MgCl_2$ concentration were the two variables that were changed in order to obtain the necessary conditions for having an intact amplicon. In the gradient PCR, the following annealing temperatures were used: 50, 54, 58, 60$^oC$. For each annealing temperature, both 2 and 4mM of $MgCl_2$ were used. As it can be seen from the Figure 3.4, for every annealing temperature and $MgCl_2$ concentration, the positive control yielded the necessary amplicon (195bp).

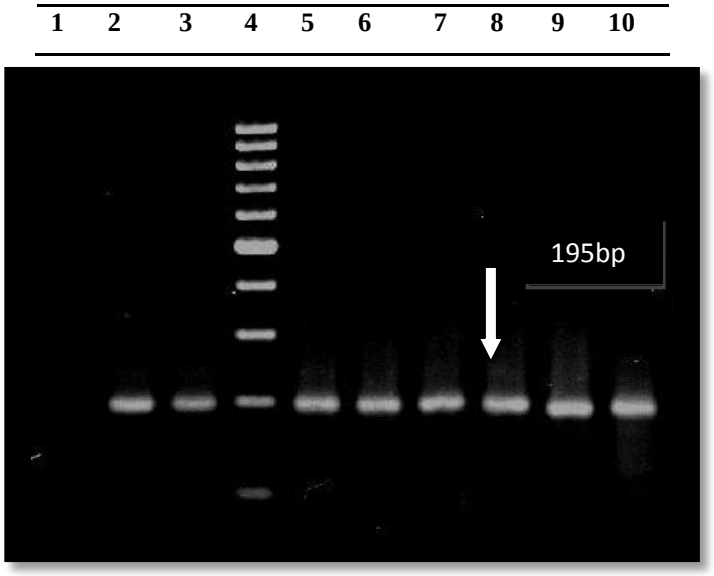



**Figure 3.4 P-35S Gradient PCR (195bp)** Lane 1: NTC, Lane 2: PC (50$^oC$, 2mM $MgCl_2$), Lane 3: PC (50$^oC$, 4mM $MgCl_2$), Lane 4: 100bp DNA ladder, Lane 5: PC (54$^oC$, 2mM $MgCl_2$), Lane 6: PC (54$^oC$, 4mM $MgCl_2$), Lane 7: PC (58$^oC$, 2mM $MgCl_2$), Lane 8: PC (58$^oC$, 4mM $MgCl_2$), Lane 9: PC (60$^oC$, 2mM $MgCl_2$), Lane 10: PC (60$^oC$, 4mM $MgCl_2$)

Following the gradient study for the amplification of the 35S promoter, different DNA concentrations from the positive control were also used in the analysis in order to check the DNA concentration that could be amplified regarding the primer pair for the 35S promoter (Figure 3.5).

Dilutions were made to obtain the DNA concentrations ranging from 100 ng to 1 ng in the final PCR reaction. The stock concentration of 260ng was used in the dilution series.

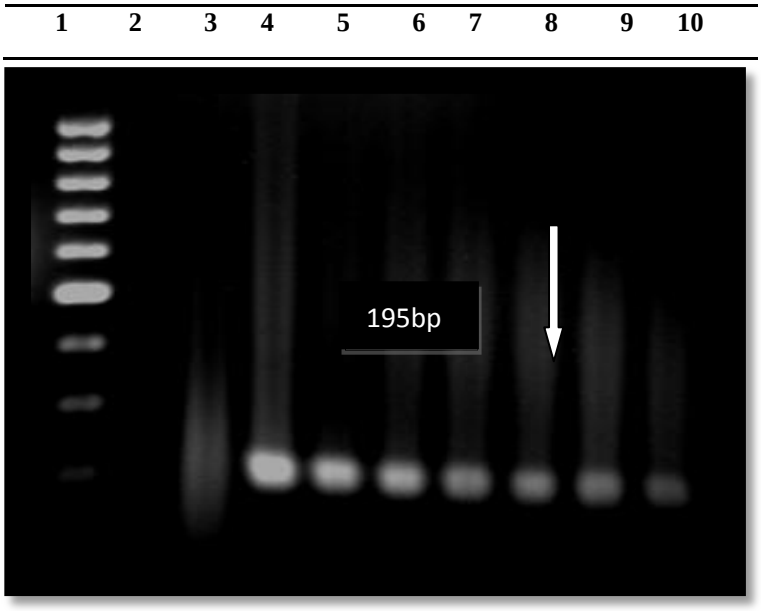



**Figure 3.5 P-35S specific PCR Amplification using Different DNA Concentrations of Positive Control (195bp)** Lane 1: 100bp DNA ladder, Lane 2: NTC, Lane 3: S1, Lane 4: PC (100ng), Lane 5: PC (65ng), Lane 6: PC (20ng), Lane 7: PC (10ng), Lane 8: PC (5ng), Lane9: PC (2ng), Lane 10: PC (1ng)

After the gradient PCR was performed, the samples were also used in the amplification reaction using 3mM $MgCl_2$ and the annealing temperature was taken as 54$^{o}$C considering both the gradient analysis and the previous studies into consideration (Figure 3.6).

Similarly, the P-35S amplification was performed using both the positive control tomato seed and a P-35S positive maize sample (Figure 3.7).

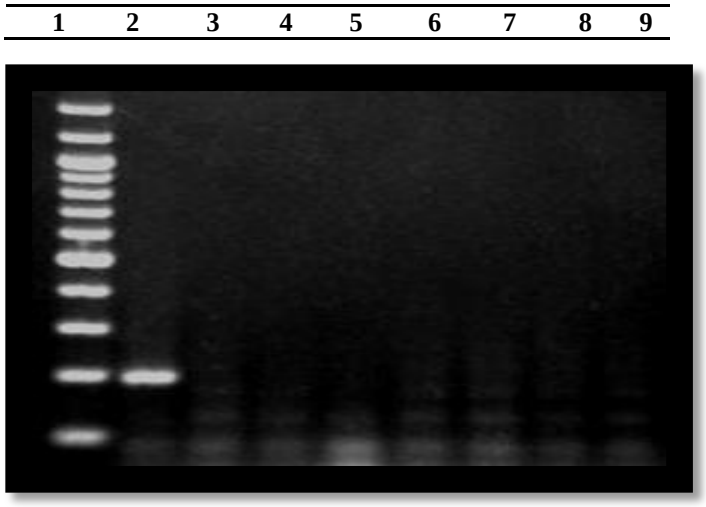



**Figure 3.6 P-35S specific PCR Amplification of the Samples (195bp)** Lane 1: 100bp DNA ladder, Lane 2: PC, Lane 3: S1, Lane 4: S3, Lane 5: S6, Lane 6: S9, Lane 7: S13, Lane 8: S19, Lane9: S27

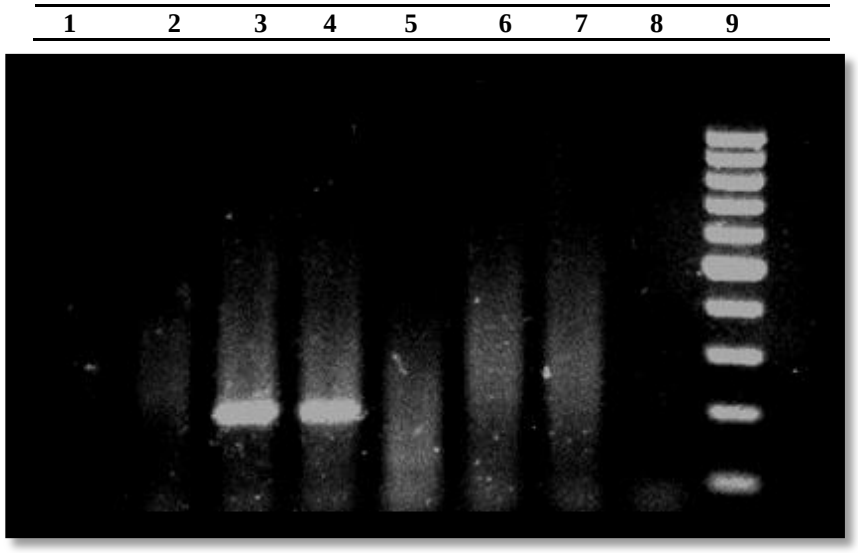



**Figure 3.7 P-35S specific PCR Amplification of Positive Controls and the Samples (195bp)** Lane 1: NTC, Lane 2: S1, Lane 3: PC, Lane 4: PC (Maize), Lane 5: S13, Lane 6: S13, Lane 7: S19, Lane 8: S19, Lane9: 100bp DNA ladder

No amplification results were obtained from the tomato seeds, samples under study, only the positive control tomato seed and maize control gave positive results. In order to comment on the presence of the P-35S region, additional optimization was performed. Taguchi optimization was tried for the P-35S amplification and the factors affecting the PCR were used in different concentrations in order to check the effect of them in the resulting amplification. The concentrations of primers, DNA, $MgCl_2$ and dNTP were changed. The seed sample 13 was used in Taguchi optimization of P-35S.

The seed sample 13 showed the weak amplification in the following concentrations of the PCR reagents:

| | | | |
|---|---|---|---|
| PCR buffer: | 1X | 1X | 1X |
| $MgCl_2$: | 2mM | 5mM | 5mM |
| dNTP: | 0.4mM | 0.1mM | 0.2mM |
| Forward primer: | 1.5µM | 1.5µM | 3µM |
| Reverse primer: | 1.5µM | 1.5µM | 3µM |
| Taq polymerase: | 1U | 1U | 1U |
| Template DNA: | 50ng | 100ng | 50ng |

However, the resulting band showed weak amplification and it was not reproducible, the result is shown in Figure 3.8. In order to confirm and repeat the results, real-time PCR screening was also performed and the results of this part can be observed in Section 3.4.1.

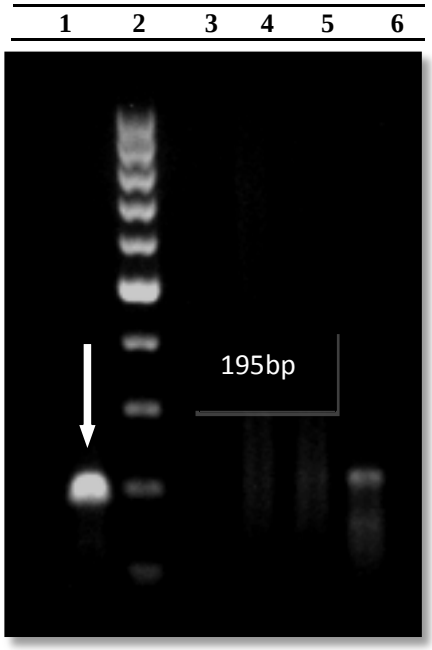



**Figure 3.8 P-35S Taguchi Optimization (195bp)** Lane 1: PC seed, Lane 2: 100bp DNA ladder, Lane 3: NTC, Lane 4-6: S13 Taquchi optimization

### 3.3.2.2 Terminator nos Amplification

For the amplification of the T-nos region of the gene cassette, the primer pair that yield a 180bp fragment was used in the optimization studies for the detection of the T-nos region. This primer pair is validated by JRC (http://ec.europa.eu/dgs/jrc/index.cfm) and the amplification studies using this primer pair was performed using DNA concentrations ranging from 130ng to 1 ng (Figure 3.9).

In the T-nos amplification, no amplification (180bp) was observed from sample DNAs (Figure 3.10), the only necessary amplification was observed in the positive control. This suggests the absence of the desired DNA fragment of nos terminator (180bp) in the samples.

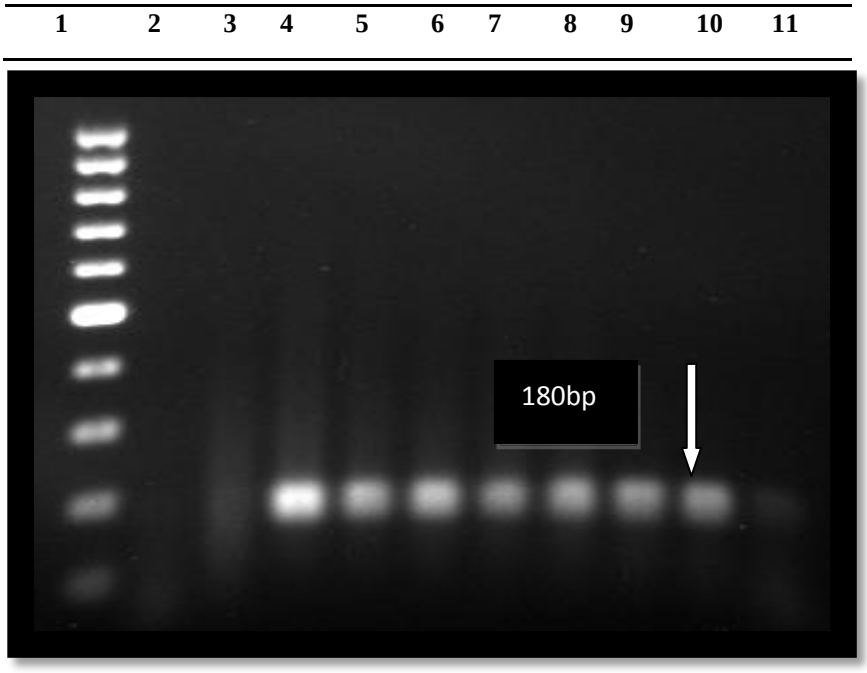



**Figure 3.9 T-nos specific PCR Amplification using Different Concentrations of Positive Control (180bp)** Lane 1: 100bp DNA ladder, Lane 2: NTC, Lane 3: S1, Lane 4: PC (130ng), Lane 5: PC (100ng), Lane 6: PC (65ng), Lane 7: PC (32ng), Lane 8: PC (16ng), Lane9: PC (5ng), Lane 10: PC (2ng), Lane 11(1ng)

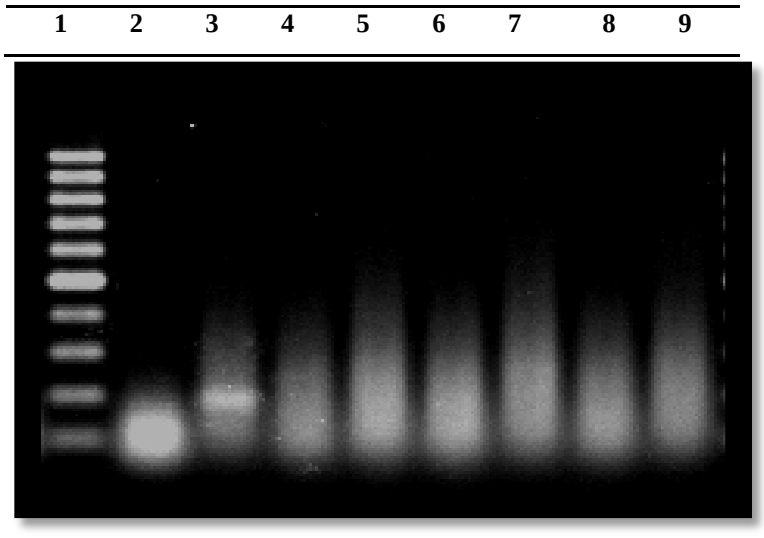



**Figure 3.10 T-nos specific PCR Amplification of the Samples (180bp)** Lane 1: 100bp DNA ladder, Lane 2: NTC, Lane 3: PC, Lane 4: S1, Lane 5: S6, Lane 6: S9, Lane 7: S13, Lane 8: S17, Lane9: S19

Since the result of the terminator nos amplification did not yield fragments in the samples analyzed, in order to further study the terminator nos region of the targeted construct, real-time PCR analyses were performed, in order to check for the presense of the T-nos in the samples, and the results of this part can be observed in Section 3.4.1.

#### 3.3.2.3 Marker gene (*nptII* and *aad*) Amplification

The antibiotic resistance genes that are present in GM plants and their derived products are mostly used for the detection of transformed plant cells.

The antibiotic resistance genes *nptII* and *aad* are present in the gene cassette of the delayed ripening and insect resistant tomato events that are considered in this study (Figure 1.12), (Figure 1.13). These resistance genes also occur at different frequencies in different bacterial species and strains, and also environments (EFSA, 2009, http://www.efsa.europa.eu/en/scdocs/doc/1108.pdf, EFSA-Q-2009-00589 and EFSA-Q-2009-00593). Therefore their presence in the sample does not always imply a genetic modification. However, screening of the samples for the marker gene gives an idea of the genetic modification for further analyses of the samples and it is also used in the screening studies (Guo et al, 2009), (Smith et al, 2008), (Jaccaud et al, 2003).

The tomato samples were tested for the presence of the *nptII* gene using the primer pair that was designed in a previous study (Sonmezalp, 2004) that was conducted in our laboratory and this primer pair yields a 459bp fragment (Figure 3.11). In addition to this analysis, the primer pairs yielding 411bp and 175bp were used for further study (Figure 3.12) (Figure 3.13). These primers can be observed in Table 2.1.

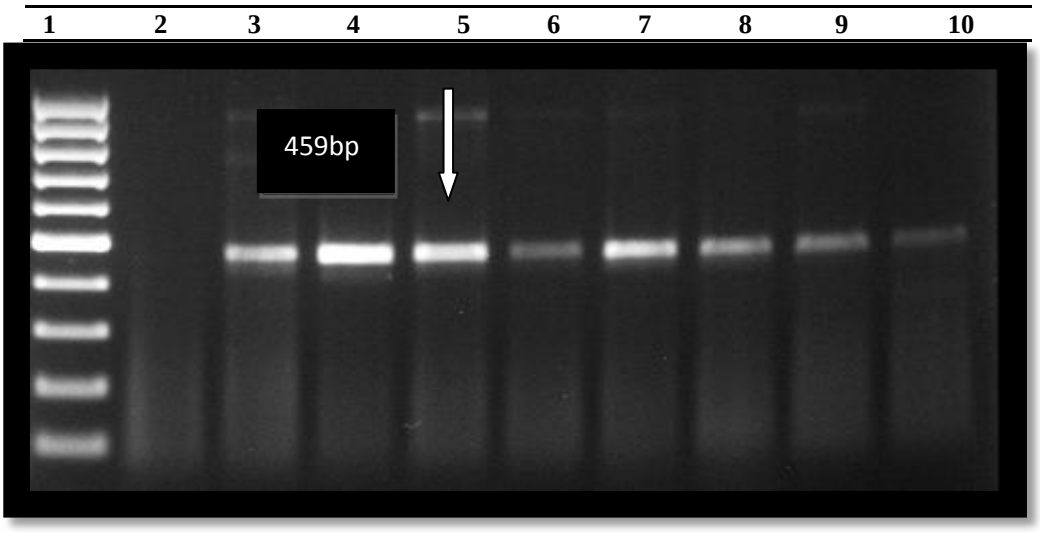



**Figure 3.11 *nptII* specific PCR Amplification (459bp)**. Lane 1: 100bp DNA ladder, Lane 2: NTC, Lane 3: S1, Lane 4: PC, Lane 5: S6, Lane 6: S9, Lane: 7: S13, Lane 8: S17, Lane 9: S19, Lane: 10: S27

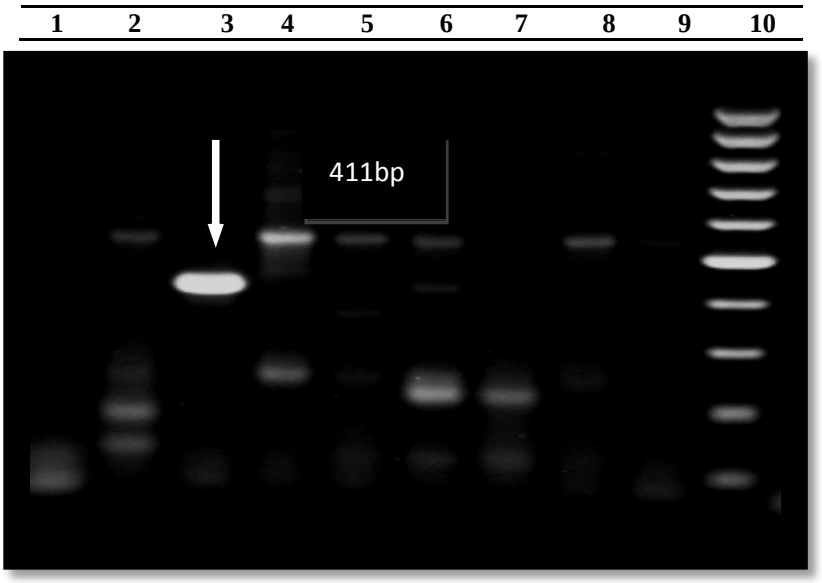



**Figure 3.12 *nptII* specific PCR Amplification (411bp).** Lane 1: NTC, Lane 2: S1, Lane 3: PC, Lane 4: S6, Lane 5: S9, Lane: 6: S13, Lane 7: S17, Lane 8: S19, Lane: 9: S27, Lane 10: 100bp DNA ladder

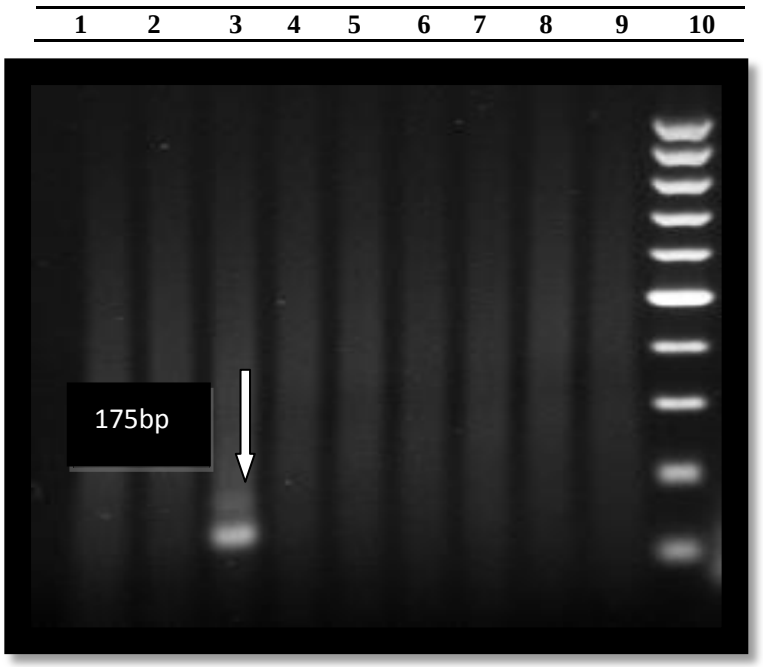



**Figure 3.13 *nptII* specific PCR Amplification (175bp).** Lane 1: NTC, Lane 2: S1, Lane 3: PC, Lane 4: S6, Lane 5: S9, Lane: 6: S13, Lane 7: S17, Lane 8: Lane 19, Lane: 9 Lane 27, Lane 10: 100bp DNA ladder

Three primer pairs were studied while analyzing the *nptII* gene of the samples. Positive control tomato seed sample showed the necessary fragments for each of the primer sets, suggesting the applicability of the primer sets to the screening study of the *nptII* gene. However the result of the 459bp primer set was misleading. Although the necessary fragments were produced in both the positive control and the tomato samples, significant amplification was also observed in the negative control seed sample. Therefore, the 459bp fragment of the positive control and the seed sample 13 was sent to sequencing for further comparison.

The sequence identity was compared using the BLAST alignment tool for the nucleotide sequence comparison (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Figure 3.14).

```
U32991.1     CTGCATACG-CTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGA  1281
             |||| |||| |  || ||||||||||||||||||||||||||||||||||||||||||
PC           CTGCCTACGACGAGAACCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGA  71

U32991.1     GCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCA  1341
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PC           GCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCA  131

U32991.1     TCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGA  1401
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PC           TCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGA  191

U32991.1     GGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCG  1461
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PC           TGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCG  251

U32991.1     CTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGC  1521
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PC           CTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGC  311

U32991.1     GTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGT  1581
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PC           GTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGT  371

U32991.1     GCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTAT-C-GCCTT  1633
             |||||||||||||||||||||||||||||||||||||||||||| || | |||||
PC           GCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTC-ATTCCGCCTT  424
```

**Figure 3.14 Nucleotide BLAST Alignment Result for the *nptII* Amplification using the *nptII* primer set (459bp) and the target sequence U32991.1**

For the positive control the sequencing result showed 97% sequence identity, when compared with the expected DNA sequence with the accession number: U32991.1. The sequence result of the *nptII* amplification was checked in the NCBI Database with the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 3.3). The result also showed the similarity between the amplified region with the vectors that harboring the *nptII* gene. Maximum identity of 98% was achieved with the cloning vector, pTDuExAn6, which also harbors *nptII* gene.

Note that the tables related to the BLAST results consist of the following elements: "Accession: The code of the gene in the NCBI database; Max Score/Total Score: bit score, the bit score gives an indication of how good the alignment is, higher scores suggest better alignments; Query Coverage: % length coverage for the query; E-value: expect value, lower E-values suggest the search is significant (An alignment with an E-value of 0.05 says that this search has a 5 in 100 chance of occurring by chance); Max identity: highest %identity of the high scoring pairs from database sequence."

**Table 3.3 Sequences Producing Significant Alignments with the *nptII* primer pair (459bp) for positive control**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| **GU370780.1** | Cloning vector pTDuExAn6, complete sequence | 717 | 717 | 91% | 0.0 | 98% |
| **GU327535.1** | Donor vector pTKIP-neo, complete sequence | 717 | 717 | 91% | 0.0 | 98% |
| **AB543179.1** | Gateway binary vector R4pGWB459 complete | 717 | 717 | 91% | 0.0 | 98% |
| **AB543175.1** | Gateway binary vector pGWB461 DNA, complete s | 717 | 717 | 91% | 0.0 | 98% |

For the seed sample 13, the comparison of the sequence result of the *nptII* amplification (459bp) and the expected sequence did not yield a significant similarity. The sequence identity was again compared using the BLAST alignment tool for the nucleotide sequence comparison (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the reference DNA sequence U32991.1. The alignment could not be shown by the program because only 2% of the reference sequence was found similar to the sample sequence. The DNA sequence of the sequenced fragment could be found in Figure 3.15.

GAAGAGTTTAACAGACAACAGTTAATTTGAGAATTTTCGACTCGA
CAACTCAAACGACATGCTATACTTTTATAGGTATCTCTTAAGAAA
TTGTACATAGATTCGTTACATACTCTATAACTTGTTTGATAAAAA
AAAGTAACTTCAGCCTGTATTGTTTGCACAAAAGATTGACGTATG
CTCTGTTTTTATGGCAATAGAGATCGAGGAAGAAGATAACGATTT
GCTGATTTTGAAGTTGCAGAATTAGTTTAGCGAGACGGCTGAGTG
AGTATAGTCAATAATTATATAAGTACCTTTTAGCTGTTATTACTTT
GGATTCGCACGCCTTTATTAGAAGAGCACATCGAAGGTCACACTT
GTTCTGGGATCTGGCCTATCGTCTTCGTCGTCATCAGCCTTCAGCT
TTGCTCCTGCCG

**Figure 3.15 The DNA Sequence of the Amplified Fragment from Sample 13 using *nptII* 459bp primer pair**

**Table 3.4 Sequences Producing Significant Alignments with the *nptII* primer pair (459bp) for the seed sample 13**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| **DQ002407.1** | *Zea mays* copia retrotransposon opie1, gypsy retrotransposon grande1, xilon1 retrotransposon, helitron B73_14578, gypsy retrotransposon huck1 and ruda retrotransposon, complete sequence | 46.4 | 46.4 | 4% | 0.27 | 87% |
| **AC006920.11** | *Arabidopsis thaliana* chromosome 2 clone F26H6 map mi398, complete sequence | 46.4 | 46.4 | 2% | 0.27 | 100% |
| **EU557673.1** | *Solanum lycopersicum* FAS protein (fasciated) gene, complete cds | 42.8 | 42.8 | 5% | 3.8 | 75% |

When the sequence from the seed sample was checked using the NCBI Database (http://www.ncbi.nlm.nih.gov/), the result only showed little similarity to the plant genome (Table 3.4). This low similarity could be observed with 87% identity only to the 4% of the given sequence.

In addition to the 459bp primer set, primer pairs yielding 411bp and 175bp fragments gave the necessary amplification for the positive control, however in 411bp primer set, other fragments were produced with different sizes in the tomato samples under study. The reason for this might be because of the primer characteristics and the ability of the undesired primer binding. The primers for the 411bp amplicon are 26 bases in length and the melting temperatures of them are 75 and 81$^{o}$C. Also high 3' stability and high end self complementarity is present for this primer pair; therefore undesired binding of the primers to the genome could be observed (Figure 3.12). In order to comment on the presence of the expected 411bp fragment, real-time data was compared with the conventional PCR results (Section 3.4.2).

In the PCR reaction, using the 175bp yielding primer pair, the only amplification was observed in the positive control sample (Figure 3.13). Positive control yielded the expected amplicon, however the samples did not show any amplification with this primer pair, suggesting that the targeted sequence (175bp) was not present in the sample.

In addition to the *nptII* marker gene detection, the tomato samples that were tested for the insect resistant tomato event (Figure 1.13) were analyzed for the presence of the *aad* gene, conferring streptomycin/spectinomycin resistance, using the primer pair that yields a 373bp amplicon (Figure 3.16). This primer pair was designed in a previous study conducted at our laboratory (Uckun, 2007). *aad* marker gene is present in 10 transgenic events according to AGBIOS database (http://www.agbios.com/) and it is used as a selectable marker gene.

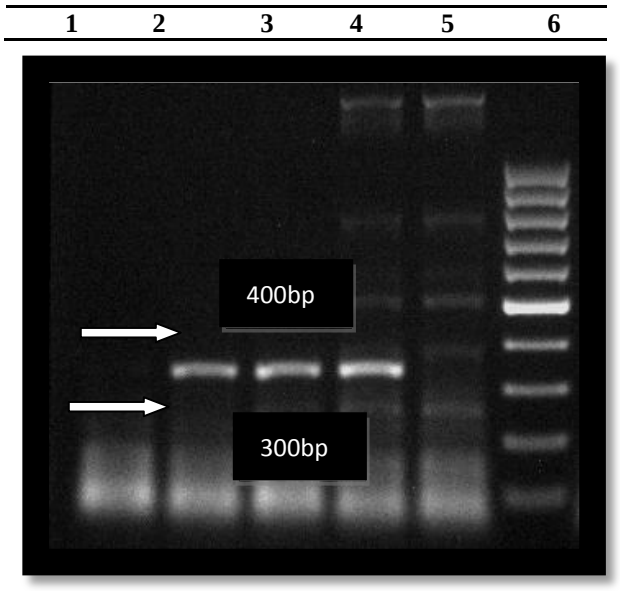



**Figure 3.16 *aad* specific PCR Amplification (373bp)** Lane 1: NTC, Lane 2:S13, Lane 3: S13, Lane 4:S13, Lane 5: S19, Lane 6: 100bp DNA ladder

*aad* gene amplification was sent to sequencing, the amplified fragment showed the expected size however the sequencing result showed only 41% similarity with the expected sequence (Figure 3.17). (The EMBOSS Pairwise Alignment Algorithm was used for the similarity calculation, http://www.ebi.ac.uk/Tools/emboss/align/). The similarity calculations can be given as follows: The Identity: 186/452 (41.2%), Similarity: 186/452 (41.2%), Gaps: 211/452 (46.7%)

In addition, the primer pair that was designed for the amplification of *aad* only gave amplification results for the sample 13. Sample 19 was also used in the *aad* amplification studies in order to check for the insect resistant gene construct. The similarity of the amplicon and the expected sequence is low and there are gaps between the compared sequenced (only 41% sequence similarity). The amplified sequence could be an altered version of the *aad* gene and this possibility was predicted before going into the BLAST analysis. The BLAST result for the sequence that was amplified with the *aad* primers can be observed in Table 3.5. According to this result, the highest value for the alignment was achieved from the *Gluconobacter oxydans* 621H plasmid pGOX4.

A possibility of the presence of a version of the *aad* gene in *Gluconobacter oxydans* lead to the comparison of the antibiotic resistance gene profiles of *Gluconobacter oxydans* and *E.coli*, to comment whether *aad* was present as the antibiotic resistance gene in both bacteria. According to ARDB-Antibiotic Resistance Genes Database (http://www.ardb.cbcb.umd.edu), no similarity exists between the antibiotic resistance gene profiles of *Gluconobacter oxydans* and *E.coli* (*aad* gene is taken from *E. coli*).

```
X02340             1              GA-AC-ATAGCGTTGCCTTGGTAGGTCCAGCGGCGGA     35
                                  || || |.|||..|||          .||| ||.||
sample 13          1 GCCCGGATTAGGGGATACTACAGCAATGC---------ACCA-CGCCG--     38

X02340            36 GGAACTCTTTGATCCGGTTCCTGAACAGGAT----CTATTTG---AGGCG     78
                             |||||           |.||    ||||..|   |||.|
sample 13         39 ------------TCCGG----------GTATACCGCTATACGCGCAGGAG     66

X02340            79 CTAAATGAAACCTTAACGCTATGGAAC----------TCGCC--------    110
                     |  |.|||..||.| ||.||.|.||||          |||||
sample 13         67 C--AGTGAGGCCCT-ACCCTTTTGAACATCGGCCTAGTCGCCATATAGAG    113

X02340           111 -GC-CCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCG    158
                      || ||.|.|.|.|| |||||.|.|   |||||  ||.| |.|.|..|||
sample 13        114 TGCTCCTATTTGTCT-GCGATTACC---ATGTA--GCAT-CCTGGATCCG    156

X02340           159 CATTTGGTACAGCGCAGTAACCG------GCAAAATCGCGCCGAA--GGA    200
                     ...|.|||.|.||..|..|||||      |.|.|||||..|.||  |||
sample 13        157 ACCTGGGTTCCGCCGACAAACCGGATTCCGAACAATCGCAACAAAGCGGA    206

X02340           201 T--------------GTCGCTGC--CGACTGGGCAATGGAGCGCCTGCCG    234
                     |               ||.|||.|  |||.| |.||   || || ||.|||
sample 13        207 TCAGCAAAACATCACGTTGCTCCCGCGAGT-GTCA---GA-CG-CTCCCG    250

X02340           235 GCCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTTATCTTGGACA    284
                     .|.|||   |.||||  ||||.|
sample 13        251 ACACAG---CCGCCC--CATATT--------------------------    268

X02340           285 AGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTTGTCC    334
                             ||||.|..|||  |||.||||                    |
sample 13        269 -------GATCTCCGGGC--CGCCCGCA--------------------C    288

X02340           335 ACTACGTGAAAGGCGAGA---TCACCAAGGTAGTCGGCAA----AT       373
                     |||             ||   |||||||       |||||    ||
sample 13        289 ACT-------------GACCCTCACCAA-------GGCAACGCTATGTTC    318

X02340           374       373

sample 13        319 AA    320
```

**Figure 3.17 EMBOSS Pairwise Alignment Result for the *aad* Amplification using the *aad* primer set (373bp) and the target sequence X02340**

**Table 3.5 Sequences Producing Significant Alignments with the *aad* primer pair (373bp)**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| **CP000007.1** | *Gluconobacter oxydans* 621H plasmid pGOX4, complete sequence | 53.6 | 53.6 | 37% | 6e-04 | 70% |
| **CU329670.1** | *Schizosaccharomyces pombe* chromosome I | 48.2 | 48.2 | 9% | 0.027 | 93% |
| **NM_001020107.1** | *Schizosaccharomyces pombe* 6-phosphofructo-2-kinase (SPAC144.17c) partial mRNA | 48.2 | 48.2 | 9% | 0.027 | 93% |
| **XM_001210618.1** | *Aspergillus terreus* NIH2624 hypothetical protein (ATEG_00532) partial mRNA | 44.6 | 44.6 | 16% | 0.32 | 77% |
| **CP000009.1** | *Gluconobacter oxydans* 621H, complete genome | 41.0 | 81.9 | 37% | 3.9 | 68% |
| **AE007870.2** | *Agrobacterium tumefaciens* str. C58 linear chromosome, complete sequence | 41 | 41 | 6% | 6.2 | 88% |

70% similarity of the amplified sequence with the *Gluconobacter oxydans* complete genome and its plasmid suggests the contamination of the sample seed. *G. oxydans* are found in flowers, fruits, garden soil (Gupta et al, 2001), suggesting the possible source of contamination. Although the seeds were surface-sterilized prior to DNA isolation, the sterilization might not have been enough to remove all the bacterial contamination from the sample 13.

### 3.3.3 Identification PCR

For the identification of the genetic modifications, additional analyses were performed. Gene-specific amplification is the starting point for the identification studies of the GMOs.

### 3.3.3.1 Gene-specific Amplification for Delayed Ripening Tomato 35 1 N line

In the gene cassette that is inserted in the 35 1 N event (Figure 1.12), there is the *sam-k* gene which codes for the S-adenosylmethionine hydrolase (SAMase), the enzyme capable of degrading S-adenosylmethionine. This gene is taken from *E. coli* bacteriophage T3 (GMDD, http://gmdd.shgmo.org/). The production of the SAMase alters the ethylene biosynthetic pathway and causes delayed ripening.

The DNA sequence of the *sam-k* gene has small differences from the native bacteriophage T3 *sam* gene. The gene is modified to have a consensus eukaryotic translation initiation site with the alteration of the nucleotide sequence surrounding ATG start codon of the *sam* gene (Kramer et al, 1995).

For the amplification of the *sam-k* gene in the sample 17, which is tested for the delayed ripening tomato event, a primer pair yielding 196bp was designed in this study using the DNA sequence of the S-adenosylmethionine hydrolase gene with the accession number X04791 (Appendix E, Figure E.2). This primer pair showed significant amplification in the sample 17 (Figure 3.18), however since no positive control was at hand for *sam-k* containing samples, the resulting band was sent to sequencing.

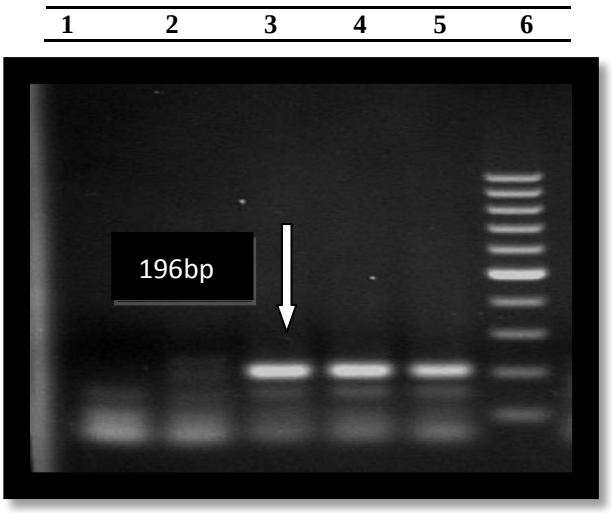



**Figure 3.18 *sam-k* specific PCR Amplification (196bp)** Lane 1: NTC, Lane 2: S1, Lane 3: S17, Lane 4: S17, Lane 5: S17, Lane 6: 100bp DNA ladder

The sequencing result showed 92% sequence identity when compared with the expected DNA sequence (Accession number: X04791, http://www.ncbi.nlm.nih.gov/). The sequence identity was compared using the BLAST alignment tool for the nucleotide sequence comparison (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Figure 3.19).

```
X04791       AGCGCACCAACTGAGGAAAAAACTGTTCGTGTACGCTGCAAGGACAAAGCGCAGGCACTC  344
             |||||  ||  |||||||  |||  |||||||||||||||||||||||||  ||||||||  |||
Sample 17    AGCGC-CCGACTGAGG-AAATACTGTTCGTGTACGCTGCAAGGACAAGGCGCAGGCTCTC  92

X04791       AATGTTGCACGCCTAGCTTGTAATGAGTGGGAGCAAGATTGCGTACTGGTATACAAATCA  404
              ||||  ||||||  |||||||  |||||||||||||||||||||||||||||||||||||
Sample 17    CATGTCGCACGCTTAGCTTGCAATGAGTGGGAGCAAGATTGCGTACTGGTATACAAATCA  152

X04791       CAGACTCACACGGCTG  420
             ||||||||||||||||
Sample 17    CAGACTCACACGGCTG  168
```

**Figure 3.19 Nucleotide BLAST Alignment Result for the *sam-k* Amplification using the *sam-k* primer set (196bp) and the target sequence X04791**

The sequence result of the *sam-k* amplification was also checked in the NCBI Database (http://www.ncbi.nlm.nih.gov/) (Table 3.6). The result also showed 93% similarity between the amplified region (196bp) and the T3 S-adenosyl-L-methionine hydrolase (also present in the genome of Bacteriophage phiYeO3-12, *Salmonella* phage phiSG-JL2).

**Table 3.6 Sequences Producing Significant Alignments with the *sam-k* primer pair (196bp)**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| **AJ251805.1** | Bacteriophage phiYeO3-12 complete genome | 211 | 211 | 50% | 1e-51 | 93% |
| **EU547803.1** | *Salmonella* phage phiSG-JL2, complete genome | 200 | 200 | 47% | 3e-48 | 93% |
| **AJ318471.1** | Bacteriophage T3 complete genome, strain Luria | 195 | 195 | 47% | 1e-46 | 92% |
| **X04791.1** | Coliphage T3 S-adenosyl-L-methionine hydrolase (AdoMetase, E.C. 3.3.1.2) | 195 | 195 | 47% | 1e-46 | 92% |

### 3.3.3.2 Gene-specific Amplification for Insect Resistant Tomato 5345 line

In the gene cassette that is inserted in the 5345 event (Figure 1.13), there is the *cry1ac* gene that codes for the insecticidal-active Cry1ac protein. The *cry1ac* gene that is introduced into the genome of tomato has been altered for the optimization of the expression; furthermore, the modified gene encodes an amino acid sequence that is 99.4% identical to the *Bacillus thruringiensis* subsp *kurstaki* gene (Gustafson et al, 1997).

The variants of crystal protein, δ endotoxin gene from *Bacillus thuringiensis* are the most frequently used genes in the transgenic crops after *nptII* marker gene. The *cry* genes are mostly modified and in some cases truncated forms of the native genes are utilized, so that the expression of the gene is improved in the host genome (Bruderer and Leitner, 2003)

For the analysis of the *cry1ac* in the samples, 3 different sets of primers were used. The primer pair yielding 329bp amplicon was studied in a previously in the laboratory. The primer pairs yielding 500bp and 789bp fragments were designed in this study using the *cry1ac* DNA sequence. The sequences that were considered during the design of the primers were: synthetic, *cry1Ac* gene from *Bacillus thuringiensis* (Accession number: AY525369, http://www.ncbi.nlm.nih.gov/) and the complete sequence of *Bacillus*

*thuringiensis* Cry1Ac (*cry1Ac*) gene (Accession number: AY225453, http://www.ncbi.nlm.nih.gov/). The primer pair yielding 329bp covered both the synthetic and the natural forms of the *cry1ac* gene. The other primer pairs yielding 500bp and 789bp covered the natural and the synthetic forms of the gene respectively. However, since there are only small nucleotide changes between the two forms of the gene, it was difficult to differentiate between the two forms (Appendix E, Figure E.1).

In the analysis of the *cry1ac* gene, the plasmid harboring the *cry1ac* gene (ECE53) was used as a positive control for the amplifications. The plasmid was kindly obtained from the *Bacillus* Genetic Stock Center (BGSC) (http://www.bgsc.org/).

The primer pairs were analyzed using the samples that were tested for insect resistant tomato event and also the plasmid, ECE53 as the positive control. The primer pairs yielding 329bp and 789bp fragments showed the necessary amplifications in the plasmid DNA, however they did not yield the necessary fragments in the samples that were analyzed (Figure 3.20), (Figure 3.21).

The primer pair yielding 500bp fragment, showed the necessary amplification for the sample 13 along with other bands. In order to analyze the fragment of 500bp in length, reamplification was performed using the section of the agarose gel that contains the necessary fragment. In the reamplification result, the fragment of 500bp was observed and it was sent to sequencing (Figure 3.22) and found to be 96% similar to the *cry1ac* gene (Figure 3.23). However, this result was not reproducible and the result could not be obtained again.

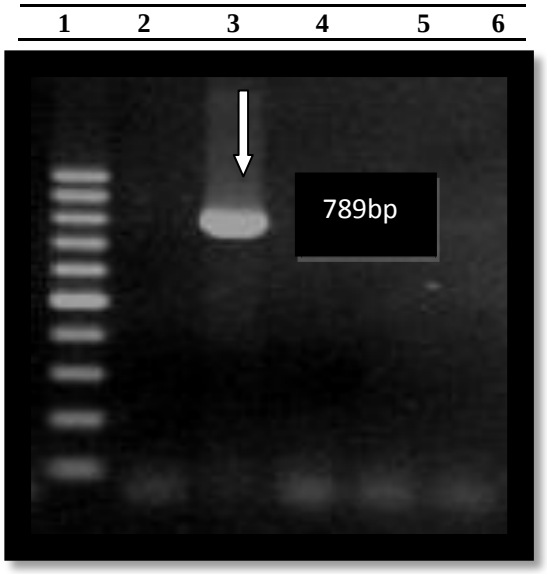



**Figure 3.20 *cry1ac* Specific PCR Amplification (789bp)** Lane 1: 100bp DNA ladder, Lane 2: NTC, Lane 3: ECE53, Lane 4: S13, Lane 5: S13, Lane 6: S13

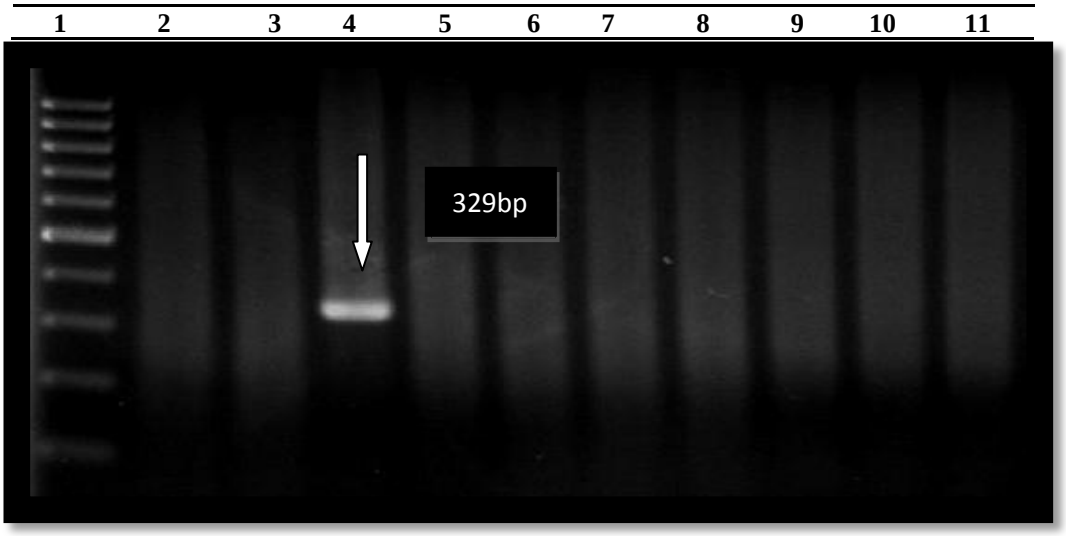



**Figure 3.21 *cry1ac* Specific PCR Amplification (329bp)** Lane 1: 100bp DNA ladder, Lane 2: NTC, Lane 3: S1 Lane 4: ECE53, Lane 5: S6, Lane 6: S9, Lane 7: S13, Lane 8: S13, Lane 9: S19, Lane 10: S19, Lane 11:S27

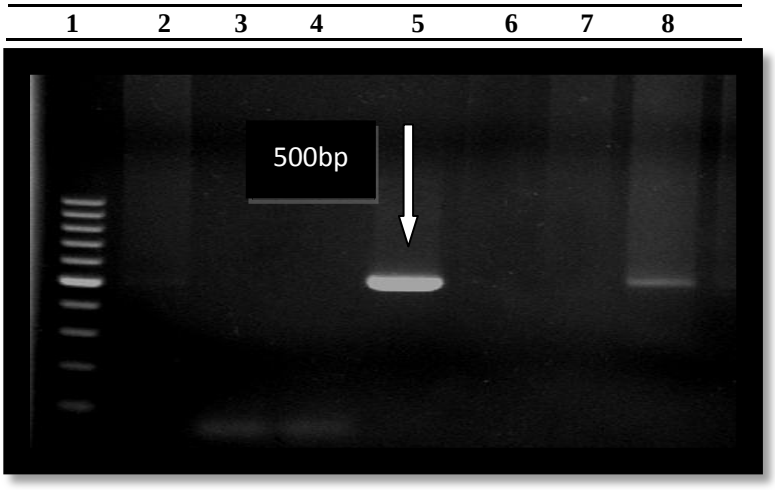



**Figure 3.22 *cry1ac* Specific PCR after Reamplification (500bp)** Lane 1: 100bp DNA ladder, Lane 2: S13(Reamp), Lane 3: NTC, Lane 4: S1, Lane 5: PC Plasmid, Lane 6: S13(Reamp), Lane 7: S13(Reamp), Lane 8: S13(Reamp)

```
AY225453    ATAC-AGTACC-AGC-TACAGCTACGTCA-TTAGATAATCTA-CAAT-CAA-GTGATTTT  111
            |||| |||||| ||| |||||  |||  |  |||||||||||| | || ||| ||||||||
Sample 13   ATACGAGTACCTAGCATACAGAGACG-AAGGTAGATAATCTATC-ATACAACGTGATTTT  81

AY225453    GGT-TATTTTGAAAGTGCCAA-TGCTTTTACATCTTCATTAGGTAATATAGTAGGTGTTA  169
            ||| |||||||||||||| ||| ||||||||||||||||||||||||||||||||||||
Sample 13   GGTATATTTTGAAAGTG-CAAGTGCTTTTACATCTTCATTAGGTAATATAGTAGGTGTTA  140

AY225453    GAAATTTTAGTGGGACTGCAGGAGTGATAATAGACAGATTTGAATTTATTCCAGTTACTG  229
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sample 13   GAAATTTTAGTGGGACTGCAGGAGTGATAATAGACAGATTTGAATTTATTCCAGTTACTG  200

AY225453    CAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGAAGGCGGTGAATGCGCTGTTTA  289
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sample 13   CAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGAAGGCGGTGAATGCGCTGTTTA  260

AY225453    CGTCTACAAACCAACTAGGGCTAAAAACAAATGTAACGGATTATCATATTGATCAAGTGT  349
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sample 13   CGTCTACGAACCAACTAGGGCTAAAAACAAATGTAACGGATTATCATATTGATCAAGTGT  320

AY225453    CCAATTTAGTTACGTATTTATCGGATGAATTTTGTCTGGATGAAAAGCGAGAATTGTCCG  409
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sample 13   CCAATTTAGTTACGTATTTATCGGATGAATTTTGTCTGGATGAAAAGCGAGAATTGTCCG  380

AY225453    AGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGCAATTTACTCCAAGATTCAAATT  469
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sample 13   AGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGCAATTTACTCCAAGATTCAAATT  440

AY225453    TCAAAGACATTAATAGGCAACCAGAACGTGG  500
            ||||||||||||||||||||||||||||||
Sample 13   TCAAAGACATTAATAGGCAACCAGAACGTGG  471
```

**Figure 3.23 Nucleotide BLAST Alignment Result for the *cry1ac* amplification using the *cry1ac* primer set (500bp) and the expected sequence from AY225453**

Although the result of the *cry1ac* gene amplification yielded the necessary fragment, the result was tried to be repeated with additional analyses in order to comment on the presence of the *cry1ac* gene, the result was not reproducible. Since reamplification was performed, the risk of contamination should be considered. When secondary amplification is required, the risk of carry-over contamination should be tried to be minimized. Therefore, in order to further analyze the presence of the *cry1ac* gene, while forming the standard curve for the samples harboring *cry1ac* gene, the sample 13 was also analyzed (Section 3.4.3).

In addition, the presence of the *cry1ac* gene indicates the presence of the cry toxin, which is either as a result of a genetic modification or (although the possibility is low) as a presence of the remains of the insecticidal spray that might have been used for the tomato crops. In order to have insecticidal effect liquid sprays containing cry toxins are applied on crop plants (Prieto-Samsonov et al, 1997). In order to obtain the crytal toxin product of the cry genes, transgenic bacteria are used for the expression. For example *cry1ac* gene is incorporated into the genome of *E.coli*, *Pseudomonas fluorescens*, *Clavibacter xyli* subsp *cynodontis*, *Bacillus subtilis* for the expression of cry toxin (Prieto-Samsonov et al, 1997). As the possibility of the contamination, this could also be taken into account.

#### 3.3.3.3 Construct Specific PCR System for Delayed Ripening Tomato 35 1 N line

In order to perform construct analysis to the sample harboring *sam-k* gene, the junction regions of the gene cassette was considered (Figure 3.24), (Figure 3.25) on the tomato sample 17. The junction region of the *sam-k* and T-nos was tried to be amplified using the primer pairs of the *sam-k* forward and T-nos reverse. For the other junction of P-E8 and *sam-k*, a primer set was designed using the T-DNA sequence of the plasmid pAG-5420 (Appendix C), which is introduced into the tomato genome (Kramer et al, 1995).

In the analysis of the samples, since no positive control was available, intact bands were tried to be obtained using the Taguchi optimization (Section 2.2.6.1) for the DNA sequencing analysis. For the amplification of the junction region of *sam-k* and T-nos, an amplicon of 559bp was expected, however in the amplifications a fragment between 400 and 500bp was obtained (Figure 3.26), which could be as a result of another terminator region in the construct. Although the 35 1N gene construct was taken as a model (Figure 1.12), the T-nos amplification could not be achieved in the screening (Section 3.3.2.2).

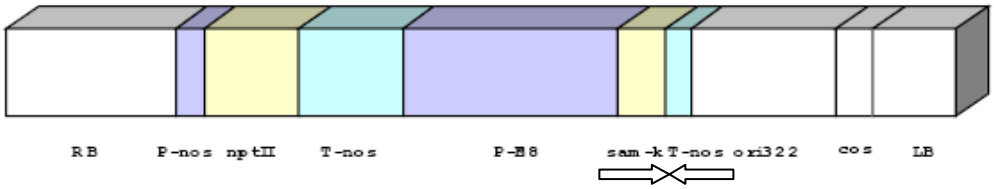



**Figure 3.24 Construct Analysis of the Delayed Ripening Tomato for the Amplification of the Junction between *sam-k* and T-nos**

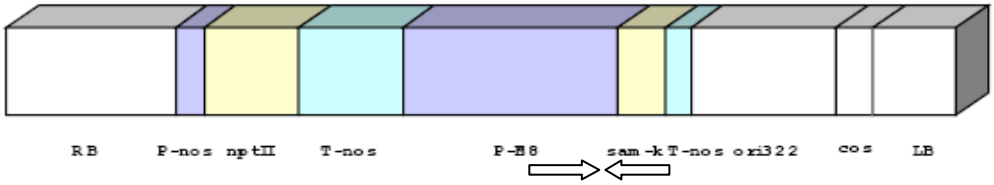



**Figure 3.25 Construct Analysis of the Delayed Ripening Tomato for the Amplification of the Junction between P-E8 and *sam-k***

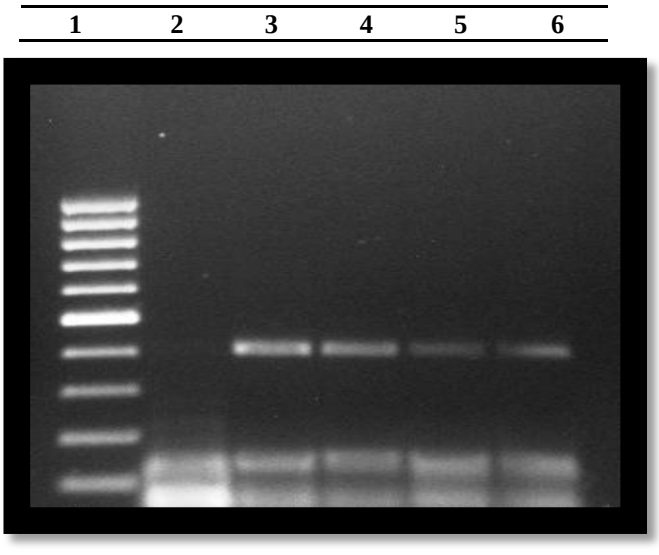



**Figure 3.26 Construct Specific PCR Amplification of the Junction between *sam-k* and t-nos** Lane 1: 100bp DNA ladder, Lane 2: NTC, Lane 3: S17, Lane 4: S17, Lane 5: S17

The sequencing result (Figure 3.27) of the construct analysis that was obtained from the tomato sample 17 showed similarity to the *Solanum lycopersicum* genome and the 25S, 26S ribosomal RNA gene from other species according to the BLAST result (Table 3.7), which shows the primer pair that was intended to amplify a region in the construct, amplified a region in the tomato genome.

The resulting sequence was also compared with the expected amplification region and only 38% similarity achieved with a high degree of gap regions (57.9%) (Figure 3.27). (The EMBOSS Pairwise Alignment Algorithm was used for the similarity calculation, http://www.ebi.ac.uk/Tools/emboss/align/). The similarity calculations can be given as follows: Identity: 242/810 (29.9%), Similarity: 242/810 (29.9%), Gaps: 469/810 (57.9%)

```
sample 17          1 ----------------------------------------CCTGC-----      5
                                                             .|.|.
Construct        151 ATGCATTACATGTTAATTATTACATGCTTAACGTAATTCAACAGAAATTA    200

sample 17          6 TGT-CTTA--A--GC--GACCAGCACCCTGTGTGGG--AT-CT---AGA-     41
                     |.| .|.|  |  ||  ||||.||| .|.|..|.    || .|   |.|
Construct        201 TATGATAATCATCGCAAGACCGGCA-ACAGGATT--CAATCTTAAGAAAC    247

sample 17         42 -TCAGCGCGCAAA---CT--AAGAT-----AAANTC----TGCGC-CGTC     75
                      |.|..|| ||||   .|  |.|||     ||| ||    |.|.. |  |
Construct        248 TTTATTGC-CAAATGTTTGAACGATCGGGGAAATTCGTTGTACTTGC--C    294

sample 17         76 CGCCGC-TCCGCCCTGGC-TC---------GCGCCCGAGGTTTTGCGGCG    114
                     .||.|| .|||..||  | ||         ||..||      ||||.|||
Construct        295 AGCGGCGACCGAACT--CATCaatagtgaagcagcc------ttgcaGCG    336

sample 17        115 ACCG-GCG-C-----G-----C-------C--CTC--CT-A----C-TCC    135
                        | ||| |     |     |       |  |.|  |. |    | .||
Construct        337 ---GTGCGCCTTTAGGAACCTCTTGGAAACTACCCGGCAGACGTTCAGCC    383

sample 17        136 TCG----CGGCC-TGGC---A-CTTGC-CCCG-CCGGCCGGGTGTGGGTC    174
                     |..    ||.|. |..|   | |.|.| ||.| ||.|||  |||||.|||
Construct        384 TTATACCCGTCGATACCTTTAGCGTACACCAGACCAGCC--GTGTGAGTC    431

sample 17        175 -GCGCGCT-T-T----G---GCGC-CTTCCGTTTTCGGGGCTCGTT--GA    211
                      |.|...| | |    |   ||.. |||  |.  ||.. .|||.||  .|
Construct        432 TGTGATTTGTATACCAGTACGCAATCTT--GC--TCCC-ACTCATTAGAA    476

sample 17        212 G-TCGGCAGGCG----T--GTGGTTGC-CC----ACTCCT----T-----    240
                     | |.|||..||.    |  ||||.||| |.    .||..|    |
Construct        477 GCTAGGCGTGCAACATTGAGTGGCTGCGCTTTGTCCTTGTAGCGTACACG    526

sample 17        241 AGC-G-----------GATT-T---C--------GACTTCC-ATGACCA-    264
                     |.| |           |.|. |   |        |.||..| || |.||
Construct        527 AACAGTTTTTTCCTCAGTTGGTGCGCTTGAGATTGCCTCACGAT-AGCAC    575
```

**Figure 3.27 EMBOSS Pairwise Alignment Result for the Construct Analysis of *sam-k* and t-nos region and pAG-5420 (Appendix C)**

```
sample 17       265 CC-GTC----CTGCTG--TCT-------C----A-A------TC-G-ACC    287
                    || |||    |.|.||  ||.       |    | |      .| | ||.
Construct       576 CCGGTCAAATCGGTTGACTCAACGGAGCCATAAAGACCCGGTGCGGCACG    625

sample 17       288 --AACA-C---CC---TT----TGTG-G-------------GA--GCT-A    307
                      ||.. |   ||   |.    |||. |             .|  ||. |
Construct       626 TAAAGTGCTTACCATGTGGCGGTGTCTGCTCATATTCACCTCATCGCAGA    675

sample 17       308 GGTTAGCGCGCAAACTAGGAAA----AATTTGGCCCTGTACACTTGTGGG    353
                    |||||    |  |||  |||||    ||.     ||.|||||       .
Construct       676 GGTTA----G--AAC--GGAAAGCGGAAA-----CCAGTACA-------T    705

sample 17       354 ACCAACTCGTATACGCCGAG---ATAGTGTCATTTATCCTGGACAGGCAT    400
                    |. ||..||  |.|||.|..   .|||||  |.. |.|.|||
Construct       706 AG-AAGACG--TTCGCAGGCTCTTTAGTG--AAA-ACCATGG--------    741

sample 17       401 ACTGCCGCGC    410

Construct       741 ----------    741
```

**Figure 3.27 continued.**

**Table 3.7 Sequences Producing Significant Alignments for the Construct Analysis of *sam-k* and t-nos region**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| **AC215459.2** | *Solanum lycopersicum* chromosome 2 clone C02SLe0089P21, complete | 352 | 881 | 58% | 9e-94 | 90% |
| **AF389260.1** | *Nepenthes* sp. *Nickrent* 3056 26S ribosomal RNA gene, partial sequence | 352 | 440 | 58% | 9e-94 | 90% |
| **AF479172.1** | *Nicotiana tabacum* 26S ribosomal RNA gene, complete sequence | 352 | 440 | 58% | 9e-94 | 90% |
| **EU161982.1** | *Solanum lycopersicum* 25S ribosomal RNA gene, complete sequence | 347 | 435 | 58% | 4e-92 | 90% |
| **AF479173.1** | *Schizanthus pinnatus* 26S ribosomal RNA gene, complete sequence | 347 | 435 | 58% | 4e-92 | 90% |

The other construct analysis that was performed for the delayed ripening tomato was aimed at the amplification of P-E8 and *sam-k* region. The designed primers for this region were expected to yield a 573bp fragment. In order to optimize the PCR conditions of the primer pair, Taguchi optimization was performed taking different concentrations of the PCR reagents (Figure 3.28).

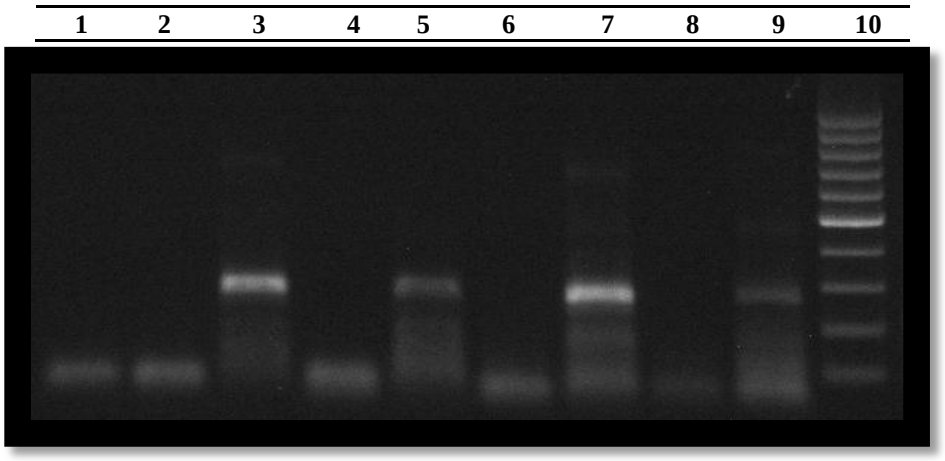



**Figure 3.28 Construct specific PCR Amplification of the Junction between P-E8 and *sam-k*** Lane1: NTC, Lane 2- 9: S13 Taquchi optimization, Lane 10: 100bp DNA ladder, the conditions of the lanes 3, 5, 7 and 9 produced the fragment to be analyzed

The fragment that was expected from the P-E8 and *sam-k* region was 573bp; however the resulting band was approximately 300bp. The sequencing analysis yield only 19% similarity with the expected DNA sequence, also with a high percentage of gaps are present in the comparison of the two sequences (Figure 3.29). (The EMBOSS Pairwise Alignment Algorithm was used for the similarity calculation, (http://www.ebi.ac.uk/Tools/emboss/align/) Identity: 230/1199 (19.2%), Similarity: 230/1199 (19.2%), Gaps: 871/1199 (72.6%).

Since the P-E8 promoter region was from the tomato genome, primer pair might anneal to another region in the genome. Consequently, the similarity results of the BLAST analysis only yield comparisons to some of the plant genomes (Table 3.8).

```
sample 17          1 --------------------------CAA----------AAA-TTAG---     10
                                               .||          ||. ||||
Construct        601 AGAAAGAGACAAAAGTAGTTTCAAACAAACTTCTCTTCTAAGTTTAGTCC    650

sample 17         11 CC---------ATG-------ATAC-TA-GATTGATTACA-AAAGCATTA     41
                     |.         |||       |||| |. ||||.|..|.| |||  |  |
Construct        651 CTTTTTAAAATATGAAACCCAATACGTCTGATTAAGAATAGAAA--A--A    696

sample 17         42 TCCATAATAATATT--ATATATCTCTTCCTCGTCACCTCATC--------     81
                     |  ||.| |||.||  ||||| .| ||.    | | ||.|||
Construct        697 T--ATCA-AATTTTCAATATA-AT-TTA----T-A-CTAATCGTTTTGAA    735

sample 17         82 TCCCTTCACGATCCAACT-AC-TACTAT--GTA-CATT-T--C--CCTC-    120
                     |   ||..| |   .||| |. ||.|.|  ||. |||. |  |  ||..
Construct        736 T---TTTTC-A---TACTGATATAGTGTACGTTTCATCATAACAACCAAA    778

sample 17        121 AC-TTGTCGCCGTG---------AAC-------CA--T--TCTAAGG-CT    148
                     || ||||.|  .|.         ||.       .|  |  |.||... .|
Construct        779 ACGTTGTTG--TTTCACAACAATAATATAGTAGTAGTTAATTTATTATTT    826

sample 17        149 A-TATT---T-GGCCC-------ACGCGCACGCATA--GCCAA-AT-ACG    182
                     | ||.|   | |.||.       | |. .|.  |||  .|.|. || |..
Construct        827 AGTAATAAGTGGTCCTAAAAATTA-GA-TAA--ATATTACTATGATAATA    872

sample 17        183 TAGAGATA----A--GAG---T----TGTTAGTTTACTGAACAGGCACGT    219
                     ||.|.|||    |  .||   |    ..||| ||||.|...||  .||.|
Construct        873 TAAAAATATTTGAGTCAGTCCTAAAAAATTA-TTTAGTATTCA--TACAT    919

sample 17        220 GAATTAGAACAACCTATACTCGCCCTCTTGTCTGACACAACTCGGTACGC    269
                     ||| |    |||.||| |.|.|     ||...||.  ||||..  |.
Construct        920 GAA-T----CAAACTA-ATTAG-----TTAAGTGT--CAACAA--TT---    951

sample 17        270 ACGACGAGCGGC----------TAGATGACTG---CTAGCGACCACTCCC    306
                      .|||.||.|||          ||.|.||.||   ..|||  |.||| .|
Construct        952 -GGACAAGTGGCATGGAGGTTGTAAAAGAATGACATAAGC--CAACT-GC    997

sample 17        307 ACATCTTGTATGCCCAACGCATATTAGATCTAGTCATTATA-TTCGCAAC    355
                      .| .||.|||  ||||   |.|..|||   ||  |. | | ||..||||
Construct        998 -TA-TTTTTAT--CCAA---AAAAAAGA---AG--AC-A-ACTTGACAAC   1033

sample 17        356 TATCGATCTTCCCCGCGAGCTCTCCCTTGTATGACCCACTTACTACTCTC    405
                       |  | |.|..|.      | |...||.||| |.  |.||||||.|.||
Construct       1034 --T--A-CATTTCT------T-TTATTTTTAT-AA--ATTTACTAATATC   1068

sample 17        406 TCGTA-------------------------------------------    410
                     |..||
Construct       1069 TTCTATGCAAAATTATTCGGTGCCTTTCTAAACTTTAAGGTTTTTATTT   1117
```

**Figure 3.29 EMBOSS Pairwise Alignment Result for the Construct Analysis of P-E8 and *sam-k* region and pAG-5420 (Appendix C)**

**Table 3.8 Sequences Producing Significant Alignments for the Construct Analysis of P-E8 and *sam-k* region**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| **AM487876.2** | *Vitis vinifera* contig VV78X155704.10, whole genome shotgun sequence | 42.8 | 42.8 | 5% | 2.2 | 86% |
| **AC183304.14** | *Medicago truncatula* chromosome 8 clone mth2-68f12, complete sequence | 41 | 41 | 3% | 7.6 | 100% |
| **AL049659.2** | *Arabidopsis thaliana* DNA chromosome 3, BAC clone | 41 | 41 | 5% | 7.6 | 90% |

In addition to the construct analysis around the *sam-k* gene, for further analysis of the gene cassette of the delayed ripening tomato event, PREMaster (prepared by Eurofins GeneScan GmbH (Germany)) was used. This analysis involves the junction region of *nptII*/t-nos (Figure 3.30). The expected fragment was 130-150bp, however no bands were observed in the analysis. Seed sample 17 was used in this analysis.

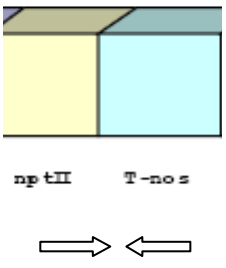



**Figure 3.30 Construct Analysis of the Delayed Ripening Tomato for the Amplification of the Junction between *nptII* and t-nos**

### 3.3.3.4 Construct Specific PCR System for Insect Resistant Tomato 5345 line

For the construct analysis of the insect resistant tomato, the amplification of the junction region of P-35S and *aad* gene is one of the suggested approaches (Figure 3.31). Since the DNA sequence of the gene construct is not available for this construct, the primer pairs that were used for the amplification of the individual elements were used for the construct study. (Only the DNA sequence of partial parts of the construct was available from the patent of the 5345 tomato line, which prevents the design of the primers for the short nucleotide sequences of the construct).

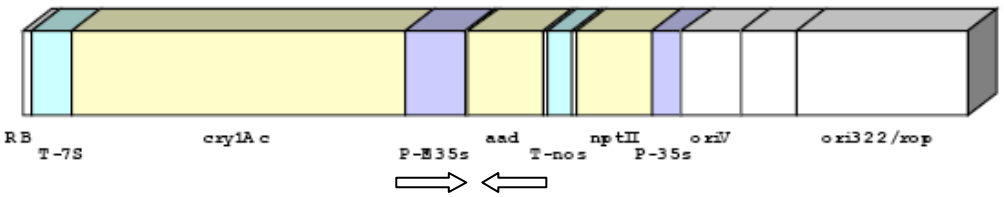



**Figure 3.31 Construct Analysis of the Insect Resistant Tomato for the Amplification of the Junction between P-E35S and *aad* (E35S: Enhanced 35S)**

However, the sample that was expected to harbor *cry1ac* gene and the genetic elements of the gene cassette of 5345 tomato line, did not show significant amplification in the studies. Nonetheless, in order to demonstrate the construct study of the 5345 gene construct, the primer pairs of the P-35S forward and *aad* reverse were used. The resulting banding pattern was from the DNA sequence of the *Solanum lycopersicum* genome (Figure 3.32), (Figure 3.33), (Figure 3.34), (Table 3.9), (Table 3.10).

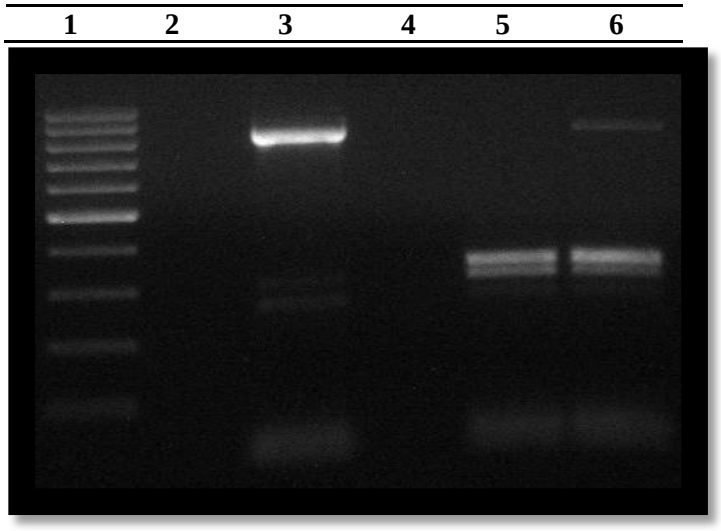



**Figure 3.32 Construct specific PCR Amplification of the Junction between P-35S and *aad*** Lane 1: 100bp DNA ladder, Lane 2: NTC, Lane 3:S13, Lane 4: NTC, Lane 5: S13, Lane 6: S13

ACACTTTTCTGATGAGAGACGACGATCTGGCATGAGTACCATGCCTTTGTATTATGTG
GTCGATCCTTTAAGAGAGAATCAAGTCAGCGAAAAGGAACATTCAACATATGGGAGA
AGTTATTTAGCAATGATACTCGTGATCACCGAAAGTTACAAAACGATGGAGCGAAAG
TCAACAAGATATAGACACAGGAGCATATAGGTAGGTGTATCGCCGACCAGGGTAACC
CTATGTTCATTAAGTTTAGGGGCACTAGTTCACCAACCAAGGCAACGCTATGTTCATTT
CTATCGGAGACGTAACAACCAACTTATTCCTTTAACTGATTGGTATGATCAGTTATCAC
ACAAAAGGTTTTCTTACTATGAACTTGTTCGAGGGACTAACAACTTTGACGAATCAAA
TTTGATTGGAAAGGGAAGCCTTGGTATGGTTTATAAGGGGACATTTACAAATGGGACT
ATAGCTGCTGTCAAGGTTTTCAATGCACAACTGCAAGATGCATTCAAGAGGTTTGATT
TGGAGTGTAAGGTTTTGCGTAACACTCGAAATAGGAATCTTGTTAAGGTGATAAGTAG
TTGTGCAAATCTTGATTTTTAAGGCATTGGTGTTTGAGTACATGCCTAATGGAGATGTT
GATTATTGGCTTTACTCACACAACAATTTCTTGGATTTACACAAGAGGCTGAAAATTA
TGTTTGATGTGGCTTGTGCCGTAGAGTATCTACACCAAGGCACGATTATGTTTCATTTA
TTTAGAAGAATATTGGGGGGGTAATTTTCAGGAGAGACCTCTTTTTTATTTTTTTTTTT
GGGGAGGGCGGAAACCANAGACAACCCAACAAAACCCACCACAACACAAACACACC
CACTCGACCCAAAAAAGAAAACACGACCCAAAAGGGCGGAGGAGGCGGCCAAACAA
CCAGAAAACGACGGAAAGCAT

**Figure 3.33 The DNA Sequence of the Amplified Fragment from Sample 13 for the Construct Analysis of P-35S and *aad* region (900bp band)**

GACAATATGAACATAGAGTTTTTCGTGGCTCATGCCCGACTCGAGGAAGATTCA
TCTTGTGTTAGATGTTCTTCATATAGTCGGCTTGATATCCCAGAGAAGCCTAAA
ATTATGAAGAGGAAGTCAACTCGAGTTCGTTCCGTGATTGCTAATTCGTAGTGA
TGATAGGGTGTCTAACGCTATGTCTAAAAAGATAAGGGGCACTAGTTCACCAA
CCAAGGCAACGCTATGTTCAGTAGCCCATCGCAACGCTATGTGCAGCAATTTGT
GGTATGCATCTTAGACATCCTTTTTGATCGTTTTGGAAGAGTGCATAGATATGA
ACCAACGAGACTCCACCGACAGGCCATTGAATCACCGTCGGCTCATCAATGTTT
ACTGTTAATTGAATACTTTTGATGTCCAATGTTGTGGTCTTCTTCATAGATCCTT
ATGGTGTTCCTTTGTGA

**Figure 3.34 The DNA Sequence of the Amplified Fragment from Sample 13 for the Construct Analysis of P-35S and *aad* region (350bp band)**

**Table 3.9 Sequences Producing Significant Alignments for the Construct Analysis of P-35S and *aad* region (900bp band)**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| **AP009297.1** | *Solanum lycopersicum* genomic DNA, chromosome 8, clone: C08SLe0082C24, complete sequence | 800 | 1685 | 47% | 0.0 | 99% |
| **AM467879.2** | *Vitis vinifera* contig VV78X216429.3, whole genome shotgun sequence | 174 | 174 | 36% | 8e-40 | 71% |
| **AM424198.2** | *Vitis vinifera* contig VV78X139377.9, whole genome shotgun sequence | 172 | 172 | 36% | 3e-39 | 71% |

**Table 3.10 Sequences Producing Significant Alignments for the Construct Analysis of P-35S and *aad* region (350bp band)**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| **CU457804.4** | *S.lycopersicum* DNA sequence from clone SL_MboI-121C14 on chromosome 4, complete sequence | 100 | 191 | 17% | 2e-17 | 73% |
| **AC232861.1** | *Solanum lycopersicum* chromosome 6 clone C06SLe0099J22, complete sequence | 98.7 | 202 | 17% | 5e-17 | 80% |
| **CU927996.3** | *S.lycopersicum* DNA sequence from clone LE_HBa-61A10, complete sequence | 93.3 | 148 | 15% | 2e-15 | 79% |

It is possible to observe that the primer pairs that are intended to be used for the amplification of the gene construct of the insect resistant tomato, amplified a region in the tomato genome, indicating that the expected gene construct was not present in the sample and the specificity of the primers was higher to a part of the genome.

In addition to the construct analysis using the primer pairs of the individual elements, PREMaster (prepared by Eurofins GeneScan GmbH (Germany)) was used, which was specific for the amplification of the 35S/*nptII* region of the gene cassette (Figure 3.35). The expected fragment was 200-300bp however no bands were observed in the analysis. Seed sample 13 was used in this study.

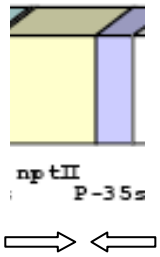



**Figure 3.35 Construct Analysis of the Insect Resistant Tomato for the Amplification of the Junction between *nptII* and P-35S**

After the real-time analysis (Section 3.4.1, Section 3.4.3) that were performed for the detection and quantification, contamination was taken as the possible source of the amplifications in Sample 13. Therefore the construct study of the model insect resistant tomato was not applicable to our sample.

### 3.3.4 Inverse PCR

In order to obtain the sequence of the flanking regions of a known DNA sequence, inverse PCR is one of the methods that is employed. In this study, in order to obtain the DNA sequence of the gene cassette, inverse PCR studies were initiated. Since the gene cassette from the approved tomatoes was not applicable to the samples under study, the analysis of the DNA sequence of the cassette is the next step for the identification system.

Especially in the sample that is tested for the delayed ripening, strong amplification of *sam-k* gene was observed. Therefore by taking this tomato sample as the model, inverse PCR analysis was tried to be performed.

The main steps of the inverse PCR involve: random restriction enzyme digestion, ligation/self-circularization of the produced fragments and PCR amplification using the primer pairs that are inversely oriented from the known DNA sequence. Using the *sam-k* gene sequence, two sets of primers were designed. The restriction enzymes EcoRI, BamHI, HindIII and XbaI were used according to the inverse PCR studies using tomato genome (Figure 3.36). These enzymes do not cut the DNA sequence of the *sam-k* gene.

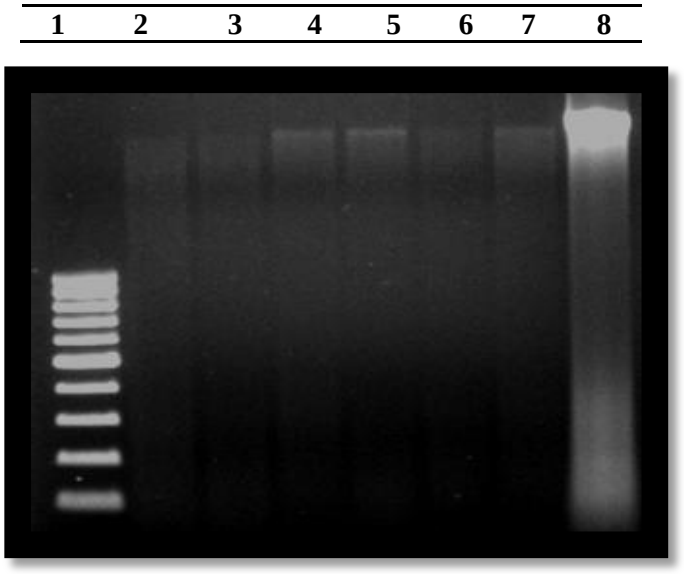



**Figure 3.36 Restriction Enzyme Digestion using Seed Sample 17.** Lane 1: 100bp DNA ladder, Lane 2: HindIII&XbaI Doubledigest, Lane 3: BamHI&XbaI Doubledigest, Lane 4: EcoRI, Lane 5: BamHI, Lane 6: HindIII , Lane 7: XbaI, Lane 8: Undigested genomic DNA

After restriction analysis, ligation step was performed. The concentration of the DNA that is used in this step is important in order to avoid linear concatamers to form. The concentrations of the digested DNA are summarized in the Table 3.11.

**Table 3.11 DNA Concentrations after Restriction Enzyme Digestions**

| Sample | A260/A280 | Concentration(ng/µl) |
|---|---|---|
| **1 HindIII&XbaI** | 2.25 | 19.63 |
| **2 BamHI&XbaI** | 2.60 | 11.42 |
| **3 EcoRI** | 1.92 | 8.90 |
| **4 BamHI** | 2.00 | 9.57 |
| **5 HindIII** | 1.78 | 12.62 |
| **6 XbaI** | 2.00 | 15.34 |

Various concentrations of the digested DNA were used in the ligation step (10ng, 50ng, 100ng and 200ng). As it is mentioned in the procedure outlined by Triglia 2000, DNA concentrations below 3μg/mL circles rather than the formation of the linear concatamers occurs. Therefore low DNA concentrations were tried to be achieved in the ligation step. After the ligation step, PCR amplifications were performed using the two sets of inverse primers that were designed (Figure 3.37).

In the PCR amplifications using the 2nd primer pair (Table 2.1), 500bp and other smaller-length amplicons fragments were observed. 500bp fragment was sent to sequencing (Figure 3.38), (Table 3.12). This fragment was achieved in the sample that was digested with HindIII.

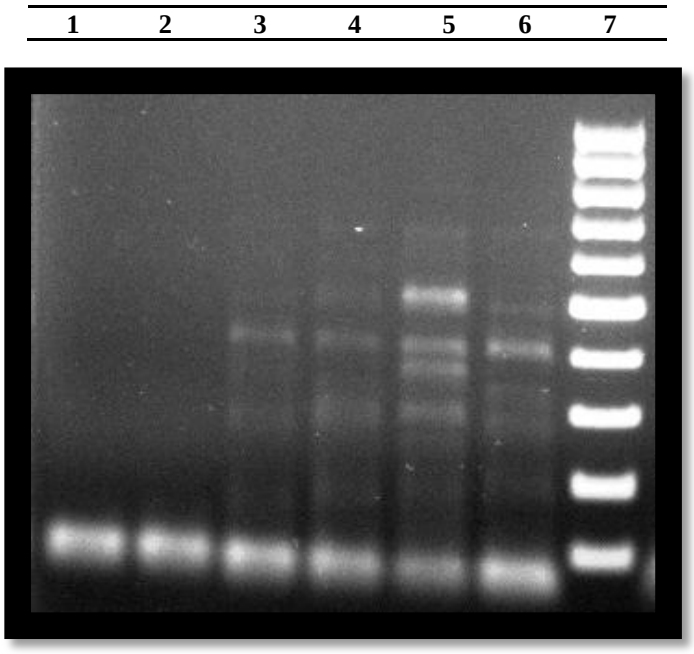



**Figure 3.37 Inverse PCR Amplification using 2nd primer pair.** Lane1: HindIII&XbaI, Lane 2: BamHI&XbaI, Lane 3: HindIII&XbaI, Lane 4: BamHI&XbaI, Lane 5: HindIII, Lane 6: XbaI, Lane 7: 100bp DNA ladder

AAGATCGGAGCGTTATCAAGGAGCCGCGGAGACGTTATTCGACGTTGCCTTCAT
GCTCTCCACATTGACGAGGAGTGGCGAGTTGTATGTGGCGTACTGTTTTCCGCTT
TCCACGGGTTCGGGATGCCACCTTATGCGGCCTTAGAAGGGGATTGCATATGCCT
CAAGAAAGTTGATAATCCACGAGAAGAATTAACCTACCCATGATCTTGAGTTAG
CTATTGTGGTATTTATGTTAAAACTTTGGAGACATTACTTATATGGAGTACATGTT
GATGTATTTCCTGACCATAAAAGTCTTCAGTATGTTTTTACAGAGAGAGAAATGA
ATCTTTGTTAGAGAAGATGGCTAAAGCTGATCAAGGATTATGACATGAGTGTTCA
CTATCACCCAGGTAAGGCCAATGTACTGGTTTCCGCTTTCCACGGACTGGTTTCC
GCTTTCCACAGTCCGAAGTTCGACCCTCCATCGAGCGGGGTCCACCCCTCCACCA
ATGCACTAAAGCGATGCCGAAGGTGAAAAAGGTAAGAGGGGACTTGCATAAGG
CCGCGGGGGCTCGTACCTTTGGACACGCGCCACAAGTTGCCAC

**Figure 3.38 The DNA Sequence of the Amplified Region using IPCR (500bp)**

**Table 3.12 Sequences Producing Significant Alignments for IPCR**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| **AC213007.2** | *Solanum lycopersicum* chromosome 12 clone LE_HBa-77H15, complete sequence | 348 | 348 | 55% | 3e-92 | 84% |
| **EU139075.1** | *Solanum lycopersicum* chromosome 9 clone C09SLm0094A22, complete sequence | 281 | 281 | 42% | 3e-72 | 85% |
| **CU457812.8** | *S.lycopersicum* DNA sequence from clone SL_MboI-58F7, complete sequence | 276 | 276 | 40% | 1e-70 | 85% |
| **CU927998.2** | *S.lycopersicum* DNA sequence from clone SL_MboI-78A8, complete sequence | 246 | 246 | 40% | 1e-61 | 84% |

No specific amplicon was expected from the IPCR results, since the gene cassette of the delayed ripening tomato might not be applicable to the sample seed 17 as predicted from previous analysis (T-nos amplification, construct-specific study). In this case, an unknown GMO event harboring *sam-k* gene might be considered. According to the circularization of the restricted fragments, higher band sizes were expected from the IPCR considering the genome size of the tomato (950mb). Additionally the resulting sequence of the amplified fragment should have covered a part of the *sam-k* sequence as the primers were designed accordingly (Appendix G, Figure G.1 and Figure G.2). The restriction digestion and the ligation steps were repeated. Additionally, an enzyme mix was used (Long PCR enzyme mix (Fermentas)), which is used for the synthesis of the longer PCR fragments. This ability was achieved by using an enzyme blend, consisting of DNA polymerases; *taq* DNA polymerase and an additional thermostable polymerase having proofreading activity. With this analysis, the previous result was repeated, 500bp fragment was again obtained.

### 3.4 Real-time PCR Systems

Real-time PCR studies involve the amplification reactions of conventional PCR and it enables the analysis of the reaction as it proceeds. It uses different chemistries for the analysis of the reaction and it measures the kinetics of the reaction starting from the early phases of PCR.

In this study, real-time PCR was applied for the screening part for the comparison of the results of the conventional PCR analyses for P-35S, T-nos and *nptII* marker gene. In addition to screening analysis, standard curves were tried to be established for the detection and the quantification of the samples harboring *cry1ac* gene. There are also other genetically modified organisms harboring *cry1ac* in their gene cassettes (Table 3.13).

In this study for the real-time PCR studies, Roche LightCycler 1.5 was used and Applied Biosystems 7500 Real-Time PCR System was used in the comparison of amplifications.

**Table 3.13 Examples of Transgenic Crops that Harbor *cry1ac* (AGBIOS, http://www.agbios.com/dbase.php)**

| Organism | Event | Company | Description |
|---|---|---|---|
| ***Zea mays* L.** | DBT418 | Dekalb Genetics Corporation | Insect-resistant and glufosinate ammonium herbicide tolerant maize achieved with the insertion of genes encoding Cry1AC protein from *Bacillus thuringiensis* subsp kurstaki and phosphinothricin acetyltransferase (PAT) from *Streptomyces hygroscopicus* |
| ***Solanum lycopersicum*** | 5345 | Monsanto Company | Resistance to lepidopteran pests with the *cry1Ac* gene from *B. thuringiensis* subsp. *Kurstaki*. |
| ***Gossypium hirsutum* L.** | 15985 | Monsanto Company | Insect resistant cotton derived by transformation of the DP50B parent variety, which contained event 531 (*cry1ac*), with plasmid DNA containing the *cry2Ab* gene from *B. thuringiensis* subsp. *kurstaki*. |
| ***Gossypium hirsutum* L.** | 31807/ 31808 | Calgene Inc. | Insect-resistant and bromoxynil herbicide tolerant cotton produced with the *cry1Ac* gene from *B. thuringiensis* and a nitrilase gene from *Klebsiella pneumoniae*. |

### 3.4.1 Screening of Promoter 35S and Terminator nos

For the screening of the samples for the common elements, LightCycler GMO Screening kit was used with Roche LightCycler 1.5. With this kit, it was possible to test the samples for the presence of the P-35S and T-nos, along with the plant gene specific detection, which is required as the control point of the amplification capacity of the sample DNAs as well as for the demonstration of lack of an inhibition. This system uses the primers and the hybridization probes specific to each detection system.

The plant gene specific detection demonstrated the necessary amplifications in all of the samples that were tested (Figure 3.39).

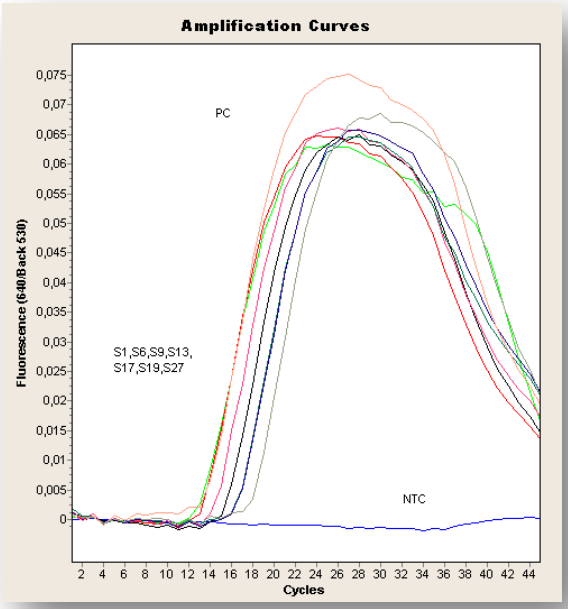



**Figure 3.39 Amplification Curves of the Samples for the Plant gene specific Detection**

In the screening of the samples for P-35S, strong amplification was present in the PC. However, only weak amplification curves were observed for the S6, S27 and S13 which were beyond the crossing point of 30 cycles (Figure 3.40).

Similarly, in the screening result for the T-nos (Figure 3.41), PC gave the strong amplification curve. The other samples gave weak amplifications (S6, S17) or no amplifications at all (S9, S13, S19, S27).

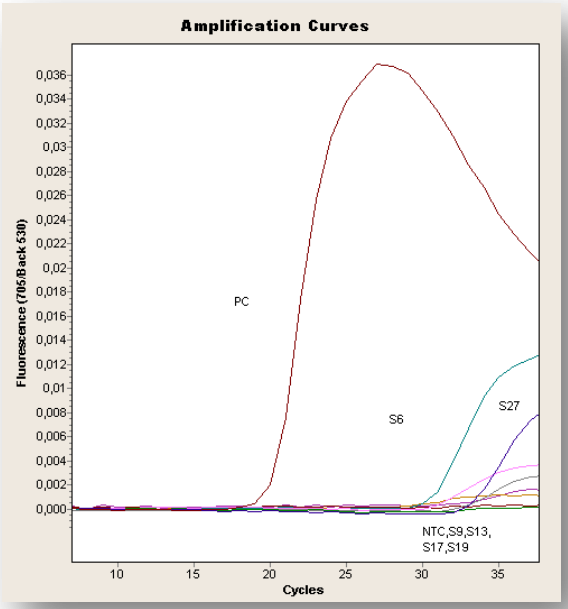



**Figure 3.40 Amplification Curves of the Samples for the P-35S Detection**

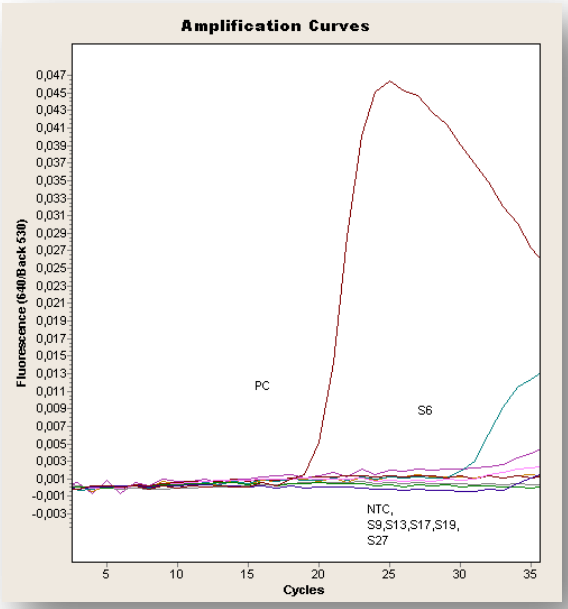



**Figure 3.41 Amplification Curves of the Samples for the T-nos Detection**

According to the gene constructs that were analyzed and the gene-specific analysis that was performed with conventional PCR, seed sample 13 was expected to harbor P-35S and T-nos and seed sample 17 was expected to harbor T-nos region. Consequently the amplification was expected with a strong positive signal; however it was not observed in the real-time analysis with the hybridization probes, therefore the result does not suggest the expected gene constructs from the samples under study. The possibility of an unknown GMO in the samples analyzed was considered in further steps.

### 3.4.2 Amplification of *nptII* gene

In the amplification of *nptII* gene with real-time PCR, the three primer sets used in conventional PCR studies were used (Table 2.1). Firstly the PCR conditions were optimized for each of the primer set using the positive control seed sample. Then the samples were analyzed using the conditions obtained. Roche LightCycler 1.5 was used as the instrument for Real-time PCR analysis.

Different concentrations of the positive control were used in order to observe the amplification curve pattern of each primer set. Dilutions were made for obtaining the following concentrations 100ng, 50ng, 20ng and 10ng. The stock concentration was 260ng, which was also used in the amplification reactions. For the primer pair yielding 459bp amplicon, the amplification curve (Figure 3.42) gave the expected result. The amplification was observed earliest (lowest crossing point) in the highest concentration used, which is the highest copy number containing sample, the stock solution.

In order to check the specificity of the amplification, melting curve analysis was performed. The primer pair yielding 459bp fragment in the *nptII* gene, produced the specific amplicon in the positive control sample and the melting temperature of this fragment is observed as 91$^{o}$C (Figure 3.43).

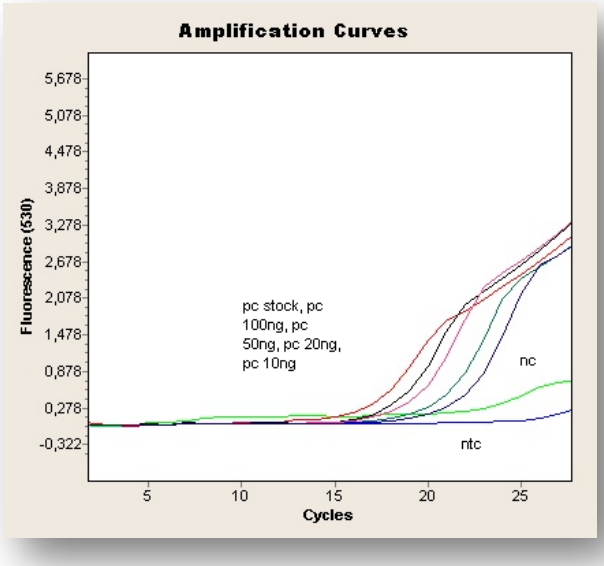



**Figure 3.42 Amplification Curves of the Positive Control for *nptII* (459bp)**

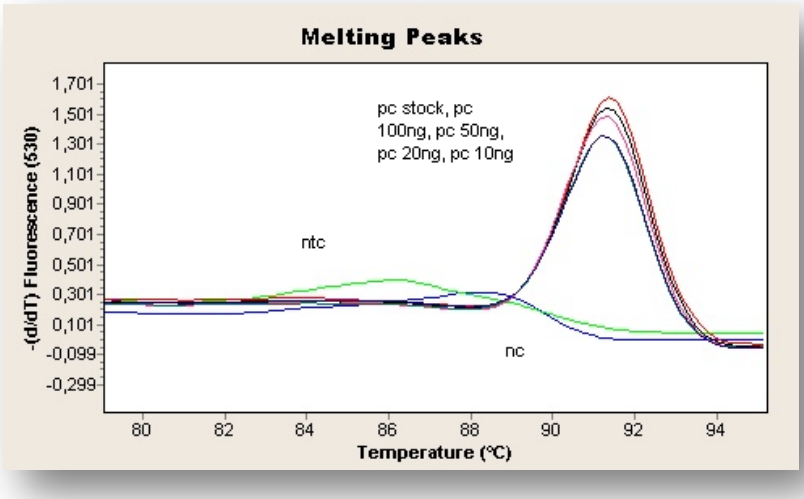



**Figure 3.43 Melting Curve Analysis of the Positive Control (459bp)**

The same procedure of the real-time amplification was performed for the primer pairs yielding 411bp and 175bp. The amplification curve of the 411bp can be observed in Figure 3.44.

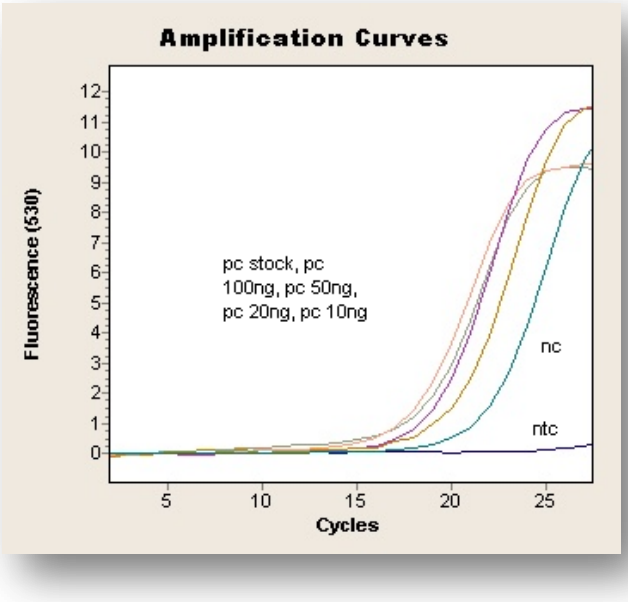



**Figure 3.44 Amplification Curves of the Positive Control for *nptII* (411bp)**

Although there is amplification in the negative control (S1), it is not the same amplicon as the one yielded by the positive control seed sample. This was checked by performing melting curve analysis, which enables the differentiation of the produced fragments. As the melting temperatures of the fragments were compared, it was observed that the fragments were indeed different from each other. While S1 yielded a banding pattern having 86$^{o}$C as the melting temperature, positive control yielded 92$^{o}$C (Figure 3.45).

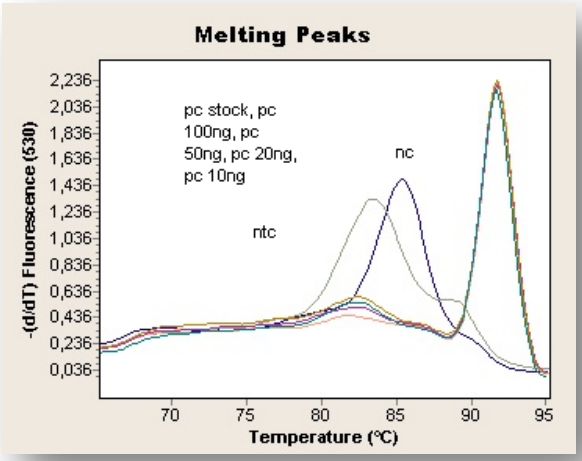



**Figure 3.45 Melting Curve Analysis of the Positive Control (411bp)**

The amplification curve of the 175bp can be observed in Figure 3.46. Similar to the primer yielding 411bp, the unspecific fragment formation was also observed in this primer set and the result was further analyzed with melting curve analysis (Figure 3.47).

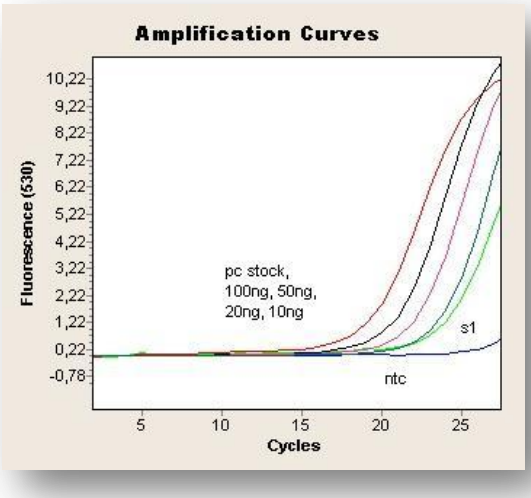



**Figure 3.46 Amplification Curves of the Positive Control for *nptII* (175bp)**

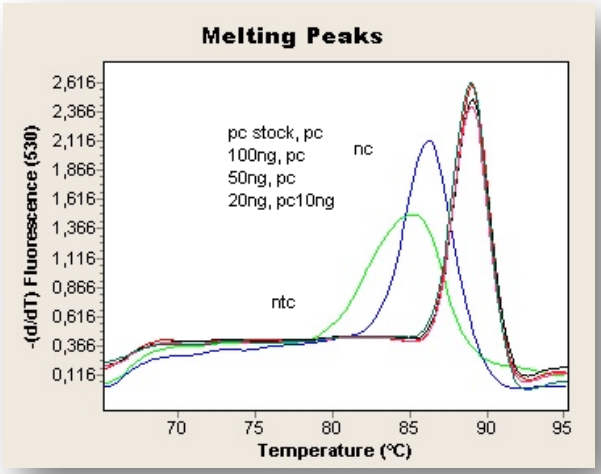



**Figure 3.47 Melting Curve Analysis of the Positive Control (175bp)**

After the conditions for each of the primer set were determined, the samples were analyzed for each of the primer set and the results were compared with the conventional PCR results.

In the detection of *nptII* gene, the primer pair yielding 459bp fragment gave positive results for all the seed samples that were analyzed together with positive and negative control. Therefore in addition to the sequencing analysis, real-time PCR amplification was also performed in order to comment on the presence of the *nptII* gene. The amplification curve of the analysis could be observed in Figure 3.48. Positive control was used in this analysis along with the samples. Since there is a need for differentiating the fragments that were formed with this primer pair, melting curve analysis was performed (Figure 3.49). It was observed that the positive control sample had a different amplification than the other samples. The products of this analysis were also ran on 1% agarose gel and showed the same banding pattern as the conventional PCR analysis.

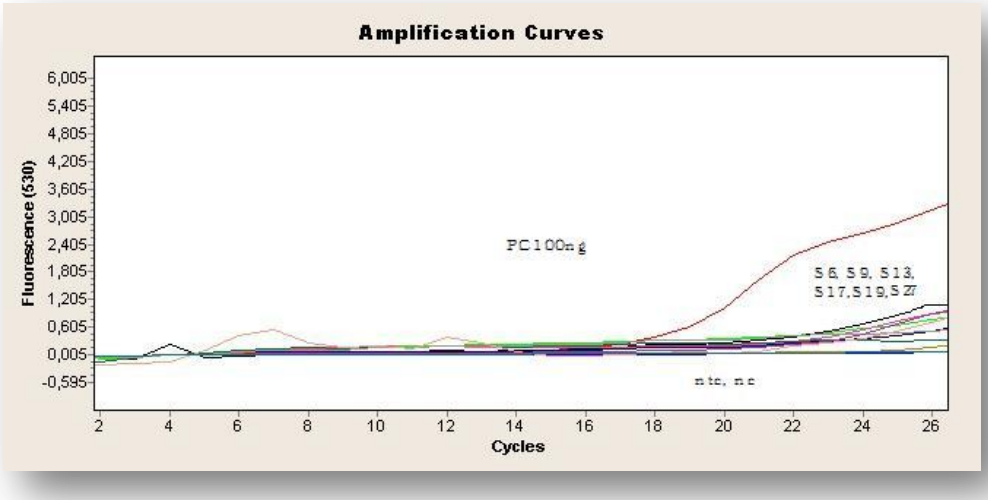



**Figure 3.48 Amplification Curves of the Samples for *nptII* (459bp)**

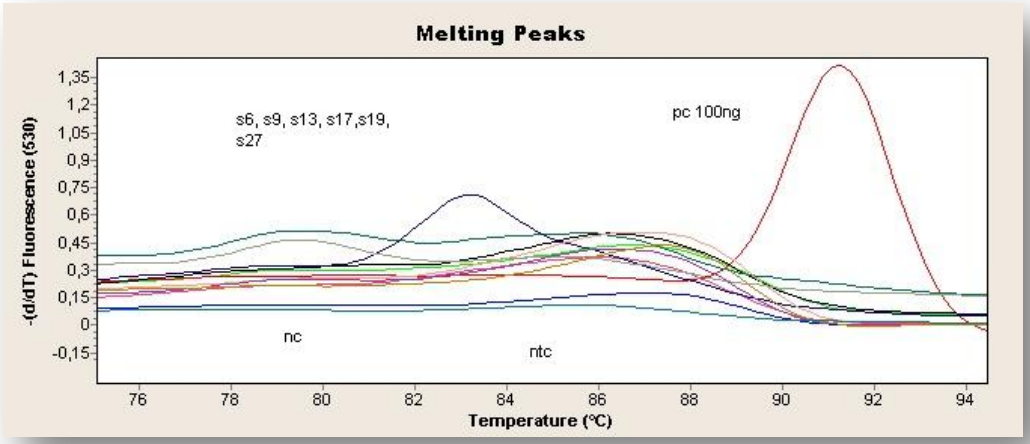



**Figure 3.49 Amplification curves of the Samples (459bp)**

The other primer sets (411bp, 175bp) also gave similar results in melting curve analysis. The samples had different melting curves than the positive control seed sample.

### 3.4.3 Development of Detection and Quantification Methods for the Samples harboring *cry1ac* gene

The detection system that was obtained with quantitative real-time PCR depends on the use of calibrator and reference materials. For GMO analysis, along with genomic DNA, plasmid DNA is also being used in the studies for obtaining a standard curve (Taverniers et al, 2004), (Mattarucchi et al, 2005) (Taverniers et al, 2005), (Zhang et al, 2008). Indeed, Taverniers et al showed that the plasmids having the target sequences form perfect alternative calibrators in GMO quantification. Therefore, the main aim of this part of the study was to develop the detection and quantification for the samples harboring *cry1ac* gene by obtaining a standard curve. The plasmid (pKK223-3, Appendix D) containing the target sequence was used as the calibrator. With the help of the standard curve, measurement of absolute amounts of target copies is possible.

Standards of the plasmid were made so that the intended amount of the copies of target sequence is present. In this study, $10^4$-$10^{10}$ copies of the *cry1ac* gene were tried to be established making serial dilutions of the plasmid stock solution (Section 2.2.12.1). There are also other important points to consider while preparing the standard curve. Plasmid DNA must be from a single and pure species. For example, the plasmid DNA that is prepared from *E. coli* can be contaminated with RNA, which increases the A260 and interferes with the copy number that is calculated for the plasmid (Applied Biosystems, http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf).

For the amplification of *cry1ac* gene, three primer sets were used that yield 329bp, 500bp and 789bp amplicons (Table 2.1), to analyze the tomato samples for the presence of *cry1ac*. The presence of *cry1ac* in transgenic events and its modified form that is employed was given in Section 3.3.3.2. Using each of the primer sets, optimization of the reaction conditions was performed.

The amplification curves were obtained using the LightCycler DNA master SYBR Green I mix and the fluorescence values versus cycle number are displayed for each primer set. Roche LightCycler 1.5 was used as the instrument for real-time PCR analysis.

### 3.4.3.1 Replicate Analysis

To obtain the standard curve for the *cry1ac* gene, replicate analysis was performed. Each standard solution was used three times to control the amplification and the amplifications of the replicates were compared for each of the primer set (Table 3.14), (Table 3.15), (Table 3.16). The tables consist of the following elements: Samples (ntc: No template control, std: Standard, Rstd: replicate of standard), Cp: Crossing point, Measured Conc.: Measured concentration of the standard, Expected Conc.: Calculated concentration of the standard. Expected Concentrations, copy numbers were determined before performing the dilution of the stock solution (Section 2.2.12.1). Copy numbers refers to the *cry1ac* sequence that is present in the plasmid (ECE53) that was used in the generation of the standard curve. The standard curve was tried to be formed in which the *cry1ac* sequence is present at $10^4$-$10^{10}$ copies.

**Table 3.14 Replicate Analysis of the Primer yielding 329bp**

| Sample | Cp | Measured Conc. | Expected Conc. |
|---|---|---|---|
| **ntc** | - | - | - |
| **std1** | 8 | $1.05*10^{10}$ | $10^{10}$ |
| **Rstd1** | 7.99 | $1.05*10^{10}$ | $10^{10}$ |
| **Rstd1** | 8.17 | $9.43*10^{9}$ | $10^{10}$ |
| **std2** | 11.81 | $1.00*10^{9}$ | $10^{9}$ |
| **Rstd2** | 11.98 | $9.01*10^{8}$ | $10^{9}$ |
| **Rstd2** | 12.19 | $7.89*10^{8}$ | $10^{9}$ |
| **std3** | 15.44 | $1.06*10^{8}$ | $10^{8}$ |
| **Rstd3** | 15.44 | $1.06*10^{8}$ | $10^{8}$ |
| **Rstd3** | 15.51 | $1.01*10^{8}$ | $10^{8}$ |
| **std4** | 18.18 | $9.45*10^{6}$ | $10^{7}$ |
| **Rstd4** | 18.21 | $9.18*10^{6}$ | $10^{7}$ |
| **Rstd4** | 17.97 | $1.19*10^{7}$ | $10^{7}$ |
| **std5** | 19.88 | $1.02*10^{6}$ | $10^{6}$ |

**Table 3.15 Replicate Analysis of the Primer yielding 500bp**

| Sample | Cp | Measured Conc. | Expected Conc. |
|---|---|---|---|
| **ntc** | - | - | - |
| **std1** | 9.66 | $1.01*10^{10}$ | $10^{10}$ |
| **Rstd1** | 9.67 | $1.00*10^{10}$ | $10^{10}$ |
| **Rstd1** | 9.78 | $9.47*10^{9}$ | $10^{10}$ |
| **std2** | 14.18 | $1.04*10^{9}$ | $10^{9}$ |
| **Rstd2** | 14.04 | $1.11*10^{9}$ | $10^{9}$ |
| **Rstd2** | 14.38 | $9.37*10^{8}$ | $10^{9}$ |
| **std3** | 19 | $9.17*10^{7}$ | $10^{8}$ |
| **Rstd3** | 19.03 | $9.01*10^{7}$ | $10^{8}$ |
| **Rstd3** | 18.91 | $9.57*10^{7}$ | $10^{8}$ |
| **std4** | 23.33 | $1.04*10^{7}$ | $10^{7}$ |
| **Rstd4** | 23.11 | $1.16*10^{7}$ | $10^{7}$ |
| **Rstd4** | 23.31 | $1.05*10^{7}$ | $10^{7}$ |
| **std5** | 27.18 | $2.21*10^{6}$ | $10^{6}$ |

**Table 3.16 Replicate Analysis of the Primer yielding 789bp**

| Sample | Cp | Measured Conc. | Expected Conc. |
|---|---|---|---|
| **ntc** | - | - | - |
| **std1** | 6.43 | $6.15*10^{9}$ | $10^{10}$ |
| **Rstd1** | 6.43 | $6.15*10^{9}$ | $10^{10}$ |
| **Rstd1** | 6.83 | $4.91*10^{9}$ | $10^{10}$ |
| **std2** | 9.70 | $9.93*10^{8}$ | $10^{9}$ |
| **Rstd2** | 9.78 | $9.51*10^{8}$ | $10^{9}$ |
| **Rstd2** | 9.67 | $1.01*10^{9}$ | $10^{9}$ |
| **std3** | 13.94 | $9.41*10^{7}$ | $10^{8}$ |
| **Rstd3** | 13.76 | $1.04*10^{8}$ | $10^{8}$ |
| **Rstd3** | 13.57 | $1.15*10^{8}$ | $10^{8}$ |
| **std4** | 17.94 | $1.01*10^{7}$ | $10^{7}$ |
| **Rstd4** | 18.21 | $8.76*10^{6}$ | $10^{7}$ |
| **Rstd4** | 17.72 | $1.19*10^{7}$ | $10^{7}$ |
| **std5** | 21.62 | $1.31*10^{6}$ | $10^{6}$ |

It is observed in the replicate analysis that the designed primers gave efficient results for the copy numbers of $10^6$-$10^{10}$. Indeed lower copy numbers of the target gene was aimed, however possibly due to integrity and the purity of the plasmid DNA; up to $10^6$ copy number could be achieved.

### 3.4.3.2 Absolute Quantification Analysis

As SYBR Green dye is an intercalating dye, it has an affinity to the double stranded DNA. With its ability to bind double stranded DNA, fluorescence signal is detected upon its DNA binding. Continuous monitoring of the fluorescence signal enables the amplification curves to be generated as the reaction proceeds (Figure 3.50), (Figure 3.51), (Figure 3.52). Amplification curve indicates the cycle in which the sample exerts a significant fluorescence signal, meaning that a significant amount of the product is started to be accumulated; it is given as the threshold cycle or the crossing point (Ct or Cp). No amplification curve is expected from the negative control in the ideal case; however, due to primer self-binding, there may be primer-dimers producing as the reaction carry on to its final cycles. The primer-binding can be detected with the melting curve analysis (Section 3.4.3.3).

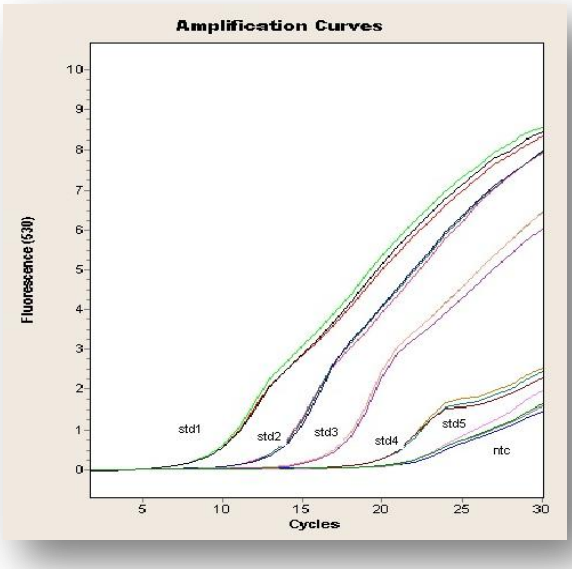



**Figure 3.50 Amplification Curves of the Standards (329bp)**

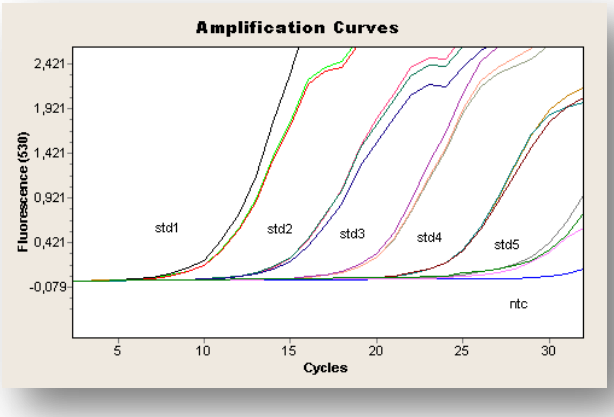



**Figure 3.51 Amplification Curves of the Standards (500bp)**

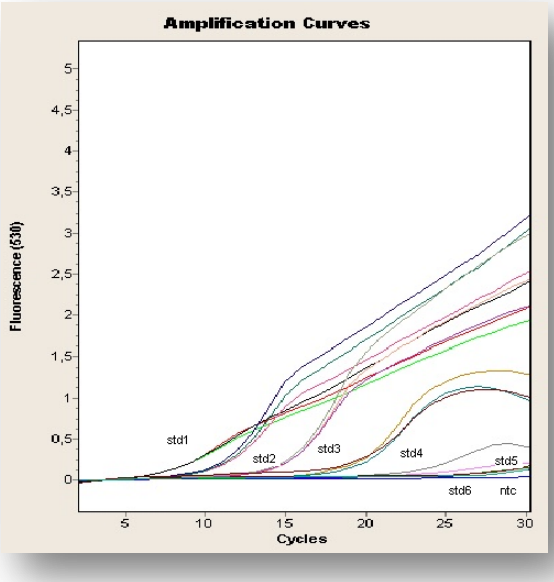



**Figure 3.52 Amplification Curves of the Standards (789bp)**

As it was expected from the standard solutions, the amplification started earliest in the standard having the highest copy number of the target gene, standard 1 (std1). Consequently, other standard solutions entered the amplification as it can be observed from the amplification curves. The replicate studies also gave consistent results when their crossing points are considered (Table 3.14), (Table 3.15), (Table 3.16). Among the primer pairs, the one yielding 789bp amplicon, generates an amplification curve of the standards to the $10^5$ copy number and that is the lowest copy number that was achieved among the primer pairs that were used

### 3.4.3.3 Melting Curve Analysis

Specificity of the amplified PCR product was assessed by performing a melting curve analysis (Figure 3.53), (Figure 3.54), (Figure 3.55). The resulting melting curves allow discrimination between the non-specific products (like primer-dimers) and the specific product. The specific *cry1ac* product melts at a different temperature than the primer-dimers which can be observed in the melting curves when compared with the no template control. The melting curves display the specific amplifications of the *cry1ac* gene for the $10^{10}$ to $10^6$ copies. Less than $10^6$ copies lead to unspecific binding of the primers together with the desired one. In order to be able to detect and quantify samples containing less than $10^6$ copies, the integrity and the quality of the plasmid DNA should be controlled.

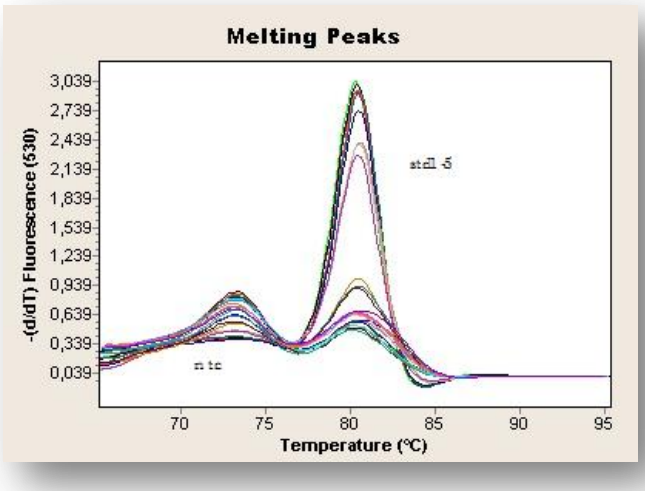



**Figure 3.53 Melting Curve Analysis of the Standards (329bp)**

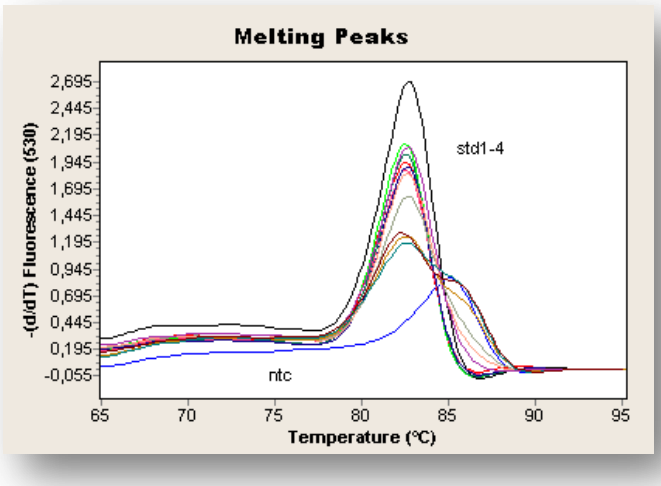



**Figure 3.54 Melting Curve Analysis of the Standards (500bp)**

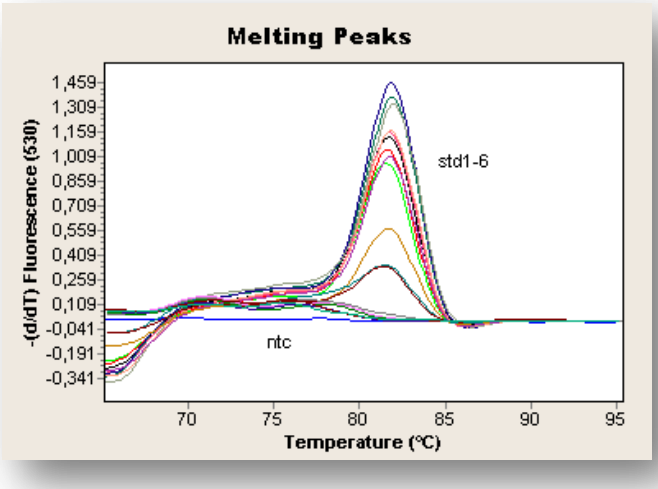



**Figure 3.55 Melting Curve Analysis of the Standards (789bp)**

Among the primer sets, the pair yielding a 789bp fragment was the most specific to the target sequence without the occurrence of significant primer-dimer formation or unspecific amplification. While in the 329bp amplification, the unspecific product was produced having a lower melting temperature than the expected amplicon (which can be detected from the extent of the melting curves), opposingly in the 500bp amplification, it is the unspecific product which has the higher melting point. With the comparison of the curves with the no template control, it is possible to comment on the produced fragments. In the 329bp amplification, there seems to be specific product amplification in the no template control, which was not expected. A possibility is the contamination of the no template control. However, the pattern was repeated with trials with the change of the reagents and the primer solution, therefore it can be commented that the unspecific product that was produced in the negative template control has a similar or close melting point to the expected *cry1ac* amplification.

### 3.4.3.4 Testing Samples

Standard curves were formed for each of the primer pair to test the samples harboring *cry1ac* gene (Figure 3.56), (Figure 3.57), (Figure 3.58). The curve that was obtained by linear regression relates the crossing point to the log concentration or copy number of the target. The equation of the line and the square of the correlation coefficient were calculated for each of the primer sets.

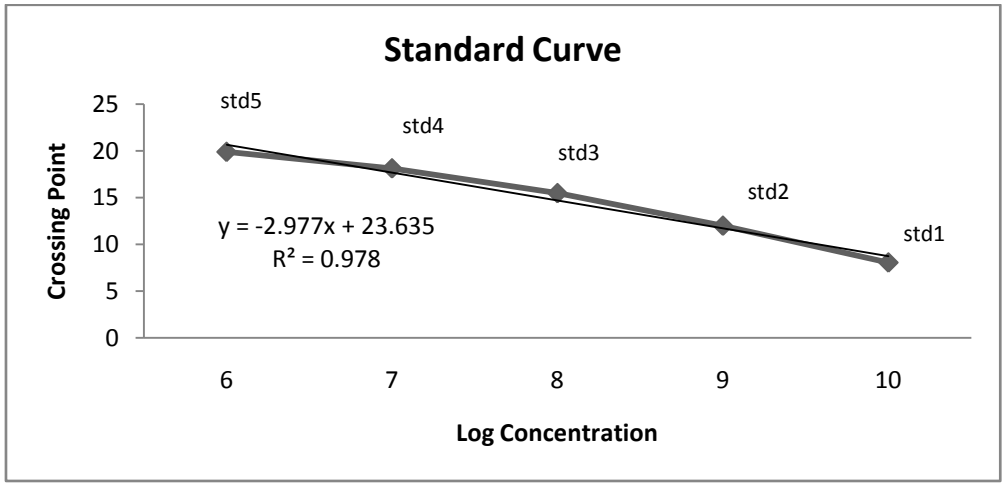



**Figure 3.56 Standard Curve of the Dilution Series of Standards (329bp)**

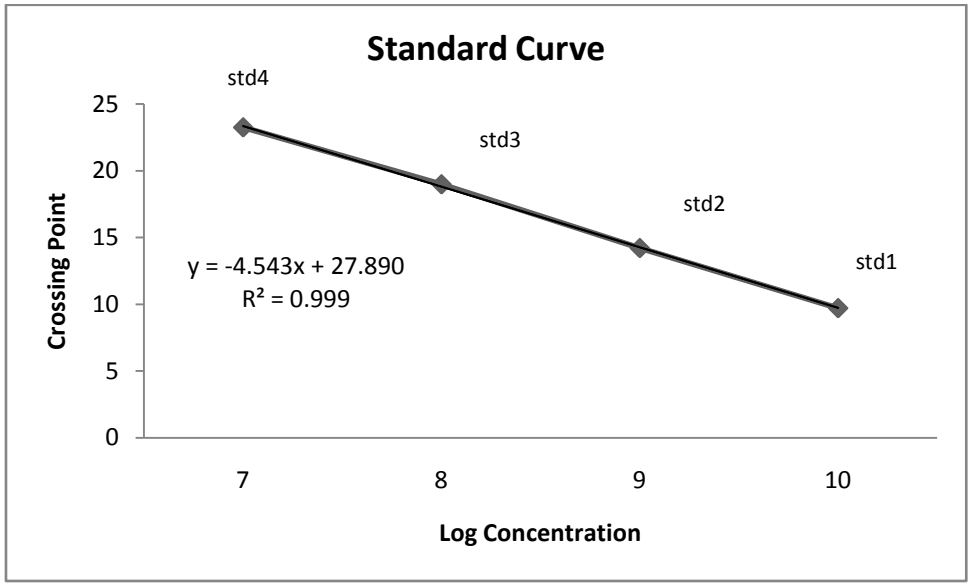



**Figure 3.57 Standard Curve of the Dilution Series of Standards (500bp)**

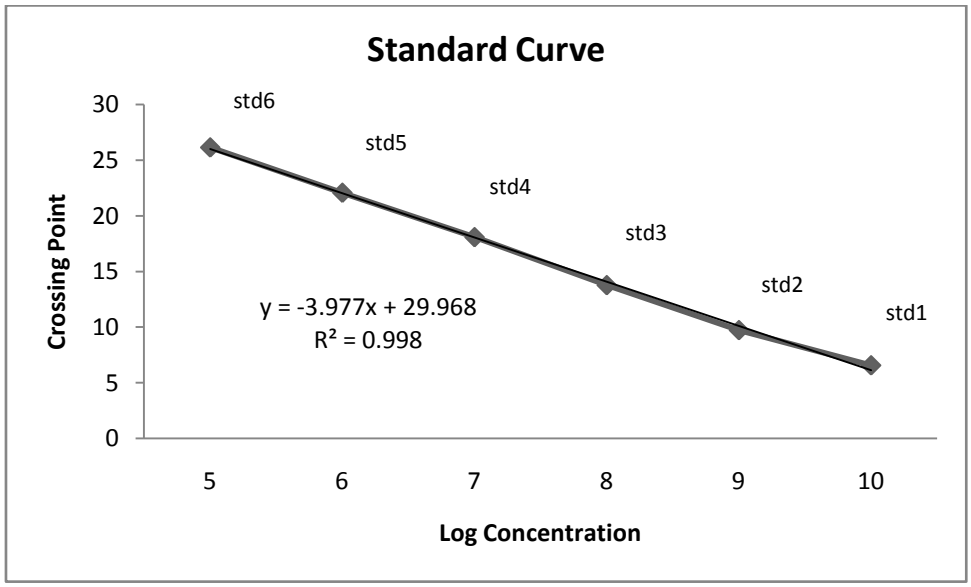



**Figure 3.58 Standard Curve of the Dilution Series of Standards (789bp)**

The applicability of the standard curves was checked by using them with known concentrations of standards. Only one of the standard solutions is enough to use the standard curve for the measurement of the other samples (Table 3.17), (Table 3.18), (Table 3.19). The tables consist of the following elements: std: standard, Cp: Crossing point, Measured Conc.: Measured concentration of the sample by the standard curve generated, Standard: Copy number of the standard that was used as a control for the standard curve, Expected Conc.: The actual calculated copy number of the sample. The tomato sample 13 was also checked for the amplification of 500bp; however no significant amplification was achieved. This result could be due to the copy number of the target that is analyzed in the seed sample 13 (could be lower than $10^5$). Also, it is possible that the expected fragment of *cry1ac* (500bp) does not exist in our sample. Therefore the standard curves should be improved for testing a wider range of target copy number.

**Table 3.17 External Measurement using the Standard Curve 329bp**

| Sample | Cp | Measured Conc. | Standard | Expected Conc. |
|---|---|---|---|---|
| **Ntc** | - | - | - | - |
| **std1** | 8.86 | $1.11*10^{10}$ | - | $10^{10}$ |
| **std2** | 12.76 | $9.96*10^{8}$ | - | $10^{9}$ |
| **std3** | 16.36 | $1.00*10^{8}$ | $10^{8}$ | $10^{8}$ |
| **std4** | 19.15 | $5.15*10^{6}$ | - | $10^{7}$ |
| **Plasmid** | 9.56 | $7.19*10^{9}$ | - | - |

**Table 3.18 External Measurement using the Standard Curve 500bp**

| Sample | Cp | Measured Conc. | Standard | Expected Conc. |
|---|---|---|---|---|
| **Ntc** | - | - | - | - |
| **std1** | 9.75 | $1.00*10^{10}$ | $10^{10}$ | $10^{10}$ |
| **std2** | 13.93 | $1.28*10^{9}$ | - | $10^{9}$ |
| **std3** | 18.42 | $1.39*10^{8}$ | - | $10^{8}$ |
| **Plasmid** | 6.51 | $4.95*10^{10}$ | - | - |

**Table 3.19 External Measurement using the Standard Curve 789bp**

| Sample | Cp | Measured Conc. | Standard | Expected Conc. |
|---|---|---|---|---|
| **Ntc** | - | - | - | - |
| **std1** | 9.80 | $3.99*10^{10}$ | - | $10^{10}$ |
| **std2** | 14.32 | $2.14*10^{9}$ | - | $10^{9}$ |
| **std3** | 19.07 | $1.00*10^{8}$ | $10^{8}$ | $10^{8}$ |
| **std4** | 21.18 | $3.12*10^{7}$ | - | $10^{7}$ |
| **std5** | 25.78 | $5.52*10^{6}$ | - | $10^{6}$ |

### 3.4.3.5 Comparison of Different Real-time Systems

The standard curves for the three primer sets were also checked using the ABI 7500 real-time PCR system. The following graphs were drawn for the quantification of the *cry1ac* gene (Figure 3.59), (Figure 3.60), (Figure 3.61).

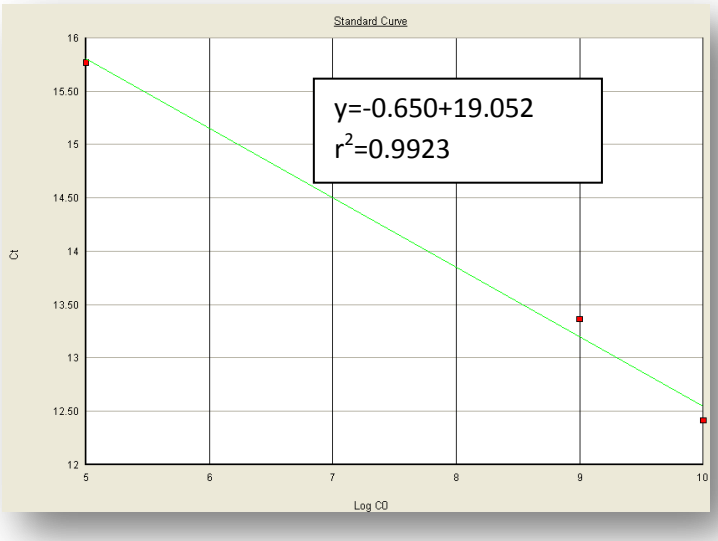



**Figure 3.59 Standard Curve of the Dilution Series of Standards (329bp) using ABI 7500**

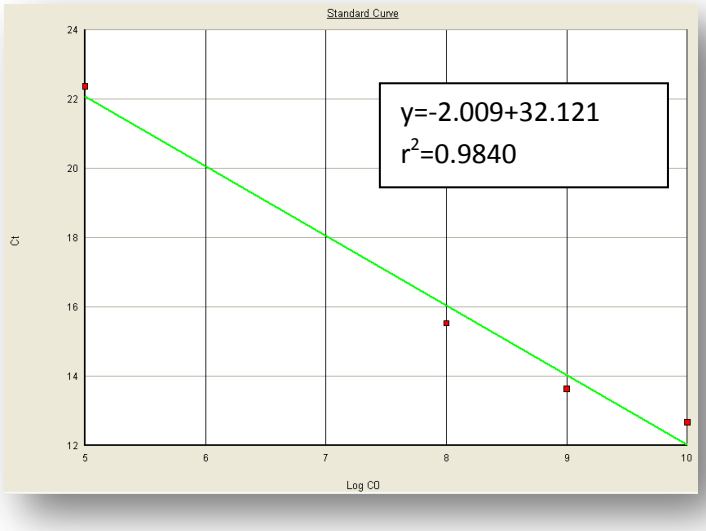



**Figure 3.60 Standard Curve of the Dilution Series of Standards (500bp) using ABI 7500**

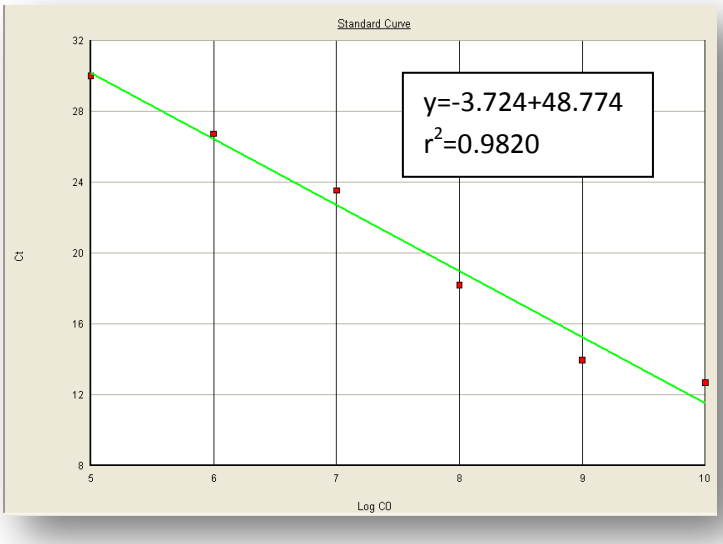



**Figure 3.61 Standard Curve of the Dilution Series of Standards (789bp) using ABI 7500**

The standard curves generated using LightCycler and ABI gave consistent results. The differences in the equations (Table 3.20) of the standard curves can be taken due to the change in the device. The primer pair yielding 789bp generated the best result for the quantification of a wider range of sample concentration. In addition to this, melting curve analysis of this primer pair also gave the optimum result, with the elimination of undesired products of NTC (Figure 3.62).

**Table 3.20 The Equations of the Standard Curves for the *cry1ac* gene that are Generated using Roche LightCycler 1.5 and ABI 7500**

| Primer Pair | Roche LightCycler 1.5 | ABI 7500 |
|---|---|---|
| **329bp** | y= -2.977x + 23.635<br>$r^2$= 0.978<br>5 stds | y= -0.650 + 19.052<br>$r^2$= 0.992<br>4 stds |
| **500bp** | y= -4.543x + 27.890<br>$r^2$= 0.999<br>4 stds | y= -2.009 + 32.121<br>$r^2$= 0.984<br>4 stds |
| **789bp** | y= -3.977x + 29.968<br>$r^2$= 0.998<br>6 stds | y= -3.724 + 48.774<br>$r^2$= 0.982<br>6 stds |

The external measurements were also applied using the standard curves generated using the ABI real-time PCR system. The result that was obtained using the primer set 789bp is given in the following table (Table 3.21). The table consists of the following elements: std: standard, Cp: Crossing point, Measured Conc.: Measured concentration of the sample by the standard curve generated, Expected Conc.: The actual calculated copy number of the sample.

Comparisons of different real-time systems were also performed in various studies. (Criado-Fornelio et al, 2007), (Deback et al, 2009), (Grohmann et al, 2009). These studies also showed the similarity in the real-time analysis of the two systems.

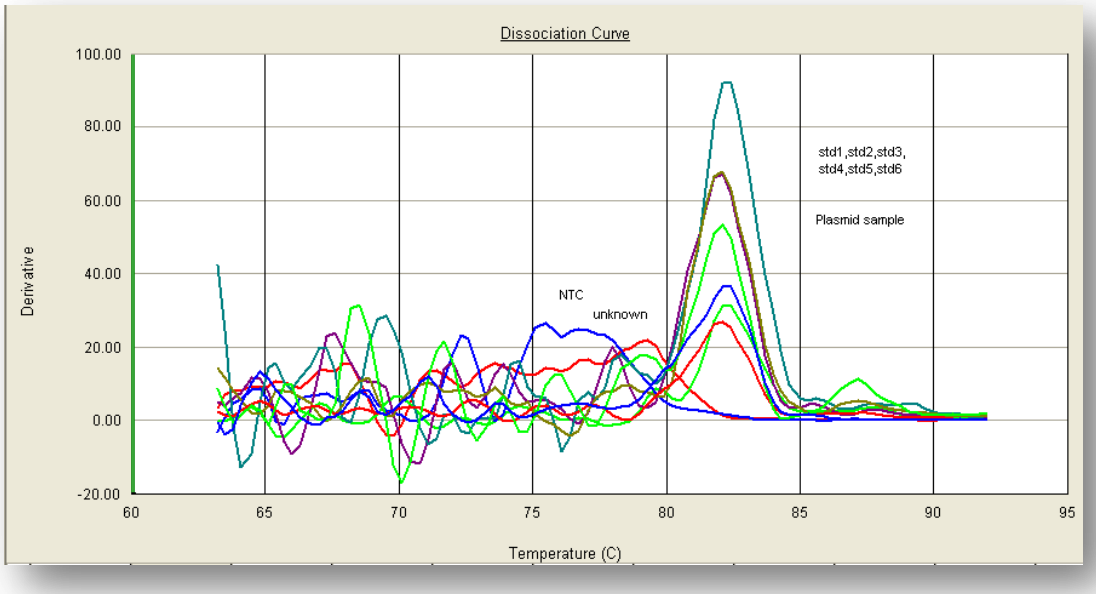



**Figure 3.62 Melting Curve Analysis of Standards (789bp) using ABI 7500**

**Table 3.21 External Measurement using the Standard Curve 789bp for ABI 7500**

| Sample | Cp | Measured Conc. | Expected Conc. |
|---|---|---|---|
| **ntc** | - | - | - |
| **std1** | 12.67 | - | $10^{10}$ |
| **std2** | 13.96 | - | $10^{9}$ |
| **std3** | 18.18 | - | $10^{8}$ |
| **std4** | 23.53 | - | $10^{7}$ |
| **std5** | 26.73 | - | $10^{6}$ |
| **std6** | 30.00 | - | $10^{5}$ |
| **Plasmid sample** | 12.74 | $6.49*10^{9}$ | $2*10^{9}$ |

# CHAPTER 4

## CONCLUSION

The main aim of this study was to demonstrate and apply the detection and identification methods for two of the transgenic events of the tomato: delayed ripening and insect resistant and additionally apply these methods to tomato samples. Previously collected tomato seed samples were used for the adoption of the GMO assessment method. In addition, methods of detection and identification were developed for the real-time PCR analysis.

As more transgenic events are commercialized and used worldwide, an established and validated detection and identification system is necessary for the analysis of genetic modifications. This is required for the evaluation of the food chain and transparency of the products that are consumed by the public.

For the development of the detection methods of the transgenic tomatoes, considered in this study, DNA-based detection was performed. PCR was used as the method of detection, identification and quantification. In the experimental strategy, the analysis was started with surface sterilization of the material analyzed (Koc et al, 2007), which was done for eliminating any source of contamination before performing DNA isolation. DNA isolation was performed and it is a critical step in GMO analysis, since the quality and purity of the DNA directly affect the further steps of the system, these parameters should be checked before PCR analysis. In this study, the concentration and the purity of the isolated DNA were controlled using spectrophotometric techniques (Table 3.2) and the integrity of the isolated DNA was checked using agarose gel electrophoresis (Figure 3.2).

After the DNA was isolated from the material to be analyzed, the workflow of GMO analysis was done with increase in the specificity of the detection. Screening was performed for the promoter, terminator and marker gene regions and gene-specific

detection was applied for the gene that is introduced in the construct. In addition to this, for the construct-specific detection, methods were suggested.

The main requirement for the adoption of a detection system is to have the reference materials. When the positive control is lacking, as in the case of *sam-k* gene amplification, the amplified fragment should be sequenced and the result should be compared to the known gene sequences. In this study, the positive control tomato seeds that harbor the common genetic elements were used in the screening analysis. In the model transgenic tomatoes that were analyzed the following studies were performed according to the gene constructs taken as models (Figure 1.12) (Figure 1.13):

35 1N Delayed Ripening Tomato: T-nos, *nptII*, *sam-k*, construct

5345 Insect Resistant Tomato: P-35S, T-nos, *nptII*, *cry1ac*, construct

Another requirement for the establishment of the detection system is to acquire the DNA sequence information of the transgene. The DNA sequence of the gene construct of the GMOs is usually kept by the developers and the regulatory agencies with some exceptions of the widely used and studied GMO crops (Bt10, Mon810). In this case, the sequence and the construct similarity of the events is the starting point of the analysis. For the events considered in this study, the construct sequence that was used in 35 1N Delayed Ripening tomato was given by Kramer et al, 1995, therefore it was possible to design the specific primers accordingly. However, the construct sequence of the 5345 Insect Resistant tomato was not available; therefore the similarity of the inserted genetic elements to a widely used transgenic event MON531 cotton was employed.

For establishing the screening analysis of the promoter and the terminator regions of the model transgenic tomatoes, validated primer pairs (P-35S:195bp, T-nos: 180bp) were used and the necessary amplifications were obtained for the positive control. In the screening of the samples, no positive signal was obtained. Only in sample 13, 195bp fragment was obtained for P-35S screening as a weak signal and it was not reproducible. The reason for this amplification could be due to possible contamination or low copy number of the promoter region resulting in inconsistent results.

For the marker gene amplification, three primer pairs were used for the *nptII* gene analysis, these were yielding 459bp (Sonmezalp, 2004), 411bp (CORESTA, Cooperative

Centre for the Scientific Research Relative to Tobacco) and 175bp (BgVV, German institute for consumer health protection and veterinary medicine). The primer pairs gave the necessary amplicons for the positive control. The samples did not yield the necessary fragments in the primer sets 411bp and 175bp. Contrary to those primer sets, the primer pair yielding 459bp produced amplifications in every sample as well as in the negative control. The possibility of the presence of the 459bp fragment of *nptII* gene was eliminated with the real-time PCR analysis of the samples and the sequencing results of the conventional PCR analysis. Additionally, in the marker gene detection, *aad* gene was also considered in the insect resistant tomato line. However, although the necessary fragment was obtained, the sequencing result only gave 41% sequence similarity with the expected DNA sequence.

For the gene-specific identification of the 35 1N Delayed Ripening tomato, a primer pair yielding 196bp was designed in this study for the amplification of the *sam-k* gene. No positive control was available for this detection and the sample that was collected in previous studies (Turkoglu, 2007) was used in the amplification of the *sam-k* gene. 93% sequence similarity with the T3 S-adenosyl-L-methionine hydrolase was achieved in the analysis and the results were reproducible.

For the gene-specific identification of the 5345 Insect Resistant tomato, *cry1ac* gene was analyzed in the samples using three primer pairs yielding 329bp (Uckun, 2007), 500bp and 789bp fragments. These primers were designed using the *Bacillus thuringiensis cry1Ac* gene, natural and synthetic, truncated gene versions. The primer pairs 329bp and 500bp amplified a region of the natural *cry1ac* gene, while 789bp was designed using the synthetic version of the gene. It was observed that the 789bp fragment did not differentiate the *cry1ac* gene, because the change in the DNA sequence was small (Appendix E). Although the differentiation was not performed, the detection of the transgenic events are possible with the given primer pairs since the insect resistant events harbor the *cry1ac* gene which is similar to the natural *Bacillus thuringiensis cry1Ac* gene, only harboring small nucleotide changes for codon optimization. In PCR studies, each primer set produced the necessary amplicons in the positive control, which is a plasmid (ECE53) harboring natural *cry1ac* gene.

In the samples the necessary fragments were not obtained, suggesting that they do not harbor *cry1ac* gene. However, in sample 13, 500bp fragment was obtained along with

other multiple bands. Performing reamplification, 500bp fragment was sent to sequencing and 96% similarity was found with the *cry1ac* sequence AY225453. However, this amplification could not be repeated, therefore it was also studied using real-time PCR and concluded that no strong amplification of the *cry1ac* gene was possible in the sample that was analyzed.

Following gene-specific studies, construct specific studies were suggested and demonstrated for the *sam-k* and *cry1ac* harboring constructs, 35 1N and 5345 (Note that the construct analysis was performed considering the known and authorized transgenic events). The junction regions that were suggested were as follows:

35 1N: *sam-k* /T-nos, P-E8/*sam-k*, *nptII*/T-nos

5345: P-35S /*aad*, P-35S/*nptII*

In the construct study, the amplifications did not yield the related sequences of the junctions and this result was expected as a result of the conventional PCR analysis of the samples (no fragments, weak signals). However, in order to check the possibility of the model transgenic events and to suggest the possible regions for construct study, this part was performed in the study. The importance of the construct-specific study was tried to be emphasized in the establishment of a GMO identification system. It is important to note that the model transgenic events that were considered in this study could not be traced in the samples analyzed. Especially in the sample harboring *sam-k* gene, an unknown transgenic event is considered. The summary of the results could be observed in the following table (Table 4.1).

Since the suggested construct studies could not be applied to the samples, the DNA sequence surrounding the gene of the construct was tried to be analyzed with the inverse PCR technique. It was commented that the *sam-k* gene was possibly introduced to the tomato with a different gene cassette.Unknown and unauthorized GMOs may enter the market intentionally or unintentionally. With inverse PCR, the amplification of the unknown flanking regions beside the known gene fragment is possible. However, there is the necessity of the optimization and additional genome walking techniques in order to comment on the construct that was introduced in the delayed ripening tomato. Although the procedure was repeated with different conditions, additional study is necessary to obtain the flanking regions of the *sam-k* gene.

**Table 4.1 Summary of the Conventional PCR Results**

| Sample/Event | P-35S | T-nos | *nptII* | Gene (*sam-k*, *cry1ac*) |
|---|---|---|---|---|
| **35 1N** | - | + | + | *sam-k* |
| **5345** | + | + | + | *cry1ac* |
| **S1 (NC)** | - | - | - | - |
| **PC** | + | + | + | - |
| **S6** | - | - | - | - |
| **S9** | - | - | - | - |
| **S13** | not reproducible | - | - | *cry1ac* (not reproducible) |
| **S17** | - | - | - | *sam-k* |
| **S19** | - | - | - | - |
| **S27** | - | - | - | - |

Apart from conventional amplification studies, real-time PCR was also performed for the screening and the gene-specific detection. Screening of the positive control and the samples for the presence of P-35S and T-nos was performed using the probes that were specifically employed for the transgenic regions. Also for the *nptII* marker gene detection, real-time PCR studies were performed with the three primer sets (459bp, 411bp, 175bp) and this part of the study was compared with the conventional PCR analysis.

Additionally, standard curves were generated using three sets of primers (329bp, 500bp, 789bp) for the quantification of the samples harboring *cry1ac* gene. This part of the study demonstrated the quantification of the samples for the presence of the *cry1ac* gene. Absolute quantification was demonstrated using the plasmid DNA. It was essential to use plasmid DNA because reference genomic DNA is not available for all transgenic events.

The major problems that were faced during this study were related to the amplification of the samples; the weak amplifications. These could be produced as false-positive results due to possible contamination or as a result of presence of a low copy number transgene.

This is especially the case of P-35 and *cry1ac* amplification of the sample 13. It is essential to adopt a detection system that is reproducible and repeatable for both reference and the sample material. Therefore, in the analyses, different approaches were performed; for example different sets of primers were used, real-time analyses and conventional analyses were evaluated and compared.

Another point was that in the real-time PCR analysis, unspecific amplifications were also present in amplifications (observed in negative control and no template control), but this did not cause difficulties for the recognition of the desired gene fragment. This recognition was achieved with the melting curve or dissociation curve analysis of the produced fragment. Additionally, in real-time PCR systems, three primer pairs were used both in the generation of the standard curve and in the detection of the *nptII* gene, in order to choose the optimum amplification conditions.

In this study it was observed that a combination of techniques should be used while detecting the genetic modifications. It was not sufficient to employ a single detection and identification method in order to comment on the sample as containing a transgenic construct or not. Especially if the sample at hand contains a low copy number of genetic modification, it is difficult to establish the detection system. At low target-DNA copy number levels, the presence of GMOs would not be detected, or weak signals could be obtained as indicated in the article of Morisset et al, 2009. Additionally, collaboration of the laboratories is also necessary in order to demonstrate the consistency of the method.

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# APPENDIX A

## CHEMICALS ENZYMES AND SUPPLIERS

**Table A.1 Chemicals, Enzymes, Reagents and Suppliers**

| Chemicals | Suppliers |
|---|---|
| Agarose | Prona |
| CTAB | Applichem |
| Sodium Acetate (NaAc) | Applichem |
| Chloroform | Applichem |
| Isopropanol | Applichem |
| TAE buffer | Applichem |
| TBE buffer | Applichem |
| Sodium Chloride (NaCl) | Merck |
| Sodium Hydroxide(NaOH) | Merck |
| SDS | Merck |
| Ethanol | Sigma |
| Ethidium Bromide(EtBr) | Sigma |
| Tris | Sigma |
| EDTA | Sigma |
| $ddH_2O$ | Dr.Zeydanli |
| DNA *taq* polymerase | Fermentas |

**Table A.1 continued.**

| Chemicals | Suppliers |
|---|---|
| Ribonuclease A | Fermentas |
| Proteinase K | Fermentas |
| 100bp DNA ladder | Fermentas |
| dNTP | Fermentas |
| PCR buffer | Fermentas |
| $MgCl_2$ | Fermentas |
| Restriction Enzymes (EcoRI, HindIII, XbaI, BamHI | Fermentas |
| T4 DNA ligase | Fermentas |
| Long PCR Enzyme Mix | Fermentas |
| Primers | Iontek |
| LightCycler FastStart DNA Master SYBR Green I | Roche |
| LightCycler® GMO Screening Kit | Roche |

# APPENDIX B

## BUFFERS AND SOLUTIONS

### 1. Solutions used in DNA Isolation

1.1 CTAB Buffer

| | |
|---|---|
| CTAB | 20g/l |
| NaCl | 1.4M |
| Tris HCl | 100mM |
| EDTA | 20mM |

The components are brought to a last volume of 1liter with $dH_2O$. The pH is then adjusted to 8. The buffer is autoclaved ($121^oC$, 15min).

1.2 CTAB Precipitation Buffer

| | |
|---|---|
| CTAB | 5g/l |
| NaCl | 0.04M |

The components are brought to a last volume of 1liter with $dH_2O$. The pH is then adjusted to 8. The buffer is autoclaved ($121^oC$, 15min).

1.3 NaCl Solution (1.2M)

| | |
|---|---|
| NaCl | 70g |
| $dH_2O$ | 1liter |

NaCl is dissolved in 1liter of $dH_2O$ and the solution is autoclaved ($121^oC$, 15min).

1.4 Washing Buffer

| | |
|---|---|
| $dH_2O$ | 300mL |
| EtOH | 700mL |

**2. Buffers used in Agarose Gel Electrophoresis**

2.1 Electrophoresis buffer

TAE Buffer and TBE buffer of Amplichem

2.2 Loading Buffer

Loading buffer of Fermantas 100bp DNA ladder

2.3 Ethidium Bromide Solution

10mg/ml EtBr was dissolved in $dH_2O$

**3. Solutions used in Plasmid Isolation**

3.1 Solution I

| | |
|---|---|
| Glucose | 50mM |
| Tris(pH:8) | 25mM |
| EDTA | 10mM |

The components are brought to a last volume of 1liter with $dH_2O$.

3.2 Solution II

| | |
|---|---|
| NaOH | 0.2N |
| SDS | 1% |

The components are brought to a last volume of 1liter with $dH_2O$.

3.3 Solution III

| | |
|---|---|
| Na-Ac | 3M (pH is adjusted to 4.8 by adding acetic acid) |

# APPENDIX C

## PLASMID MAPS OF INSERTED GENE CONSTRUCTS AND *CRY1AC* POSITIVE CONTROL

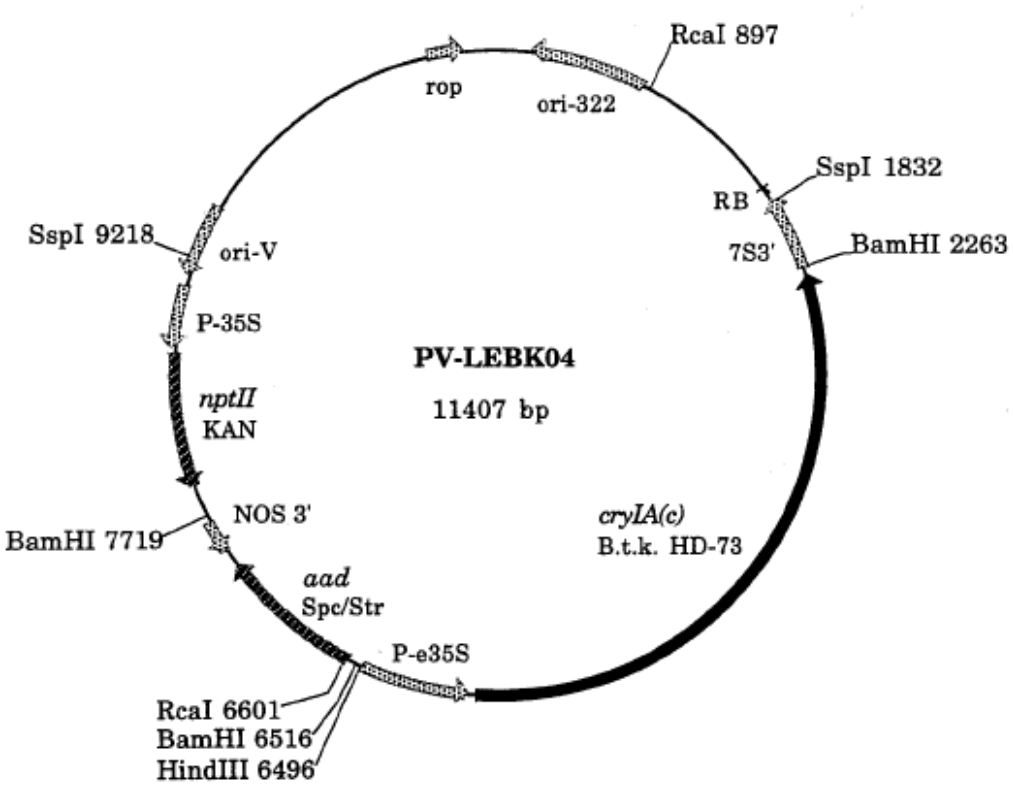


**Figure C.1 Plasmid map of PV-LEBK04 (harboring *cry1ac* gene), Vector for 5345 Tomato, (From Monsanto Petition for Determination of Non-Regulated Status for Insect Resistant Tomato Line 5345, (Retrieved from GMO Detection method Database (GMDD), http://gmdd.shgmo.org))**

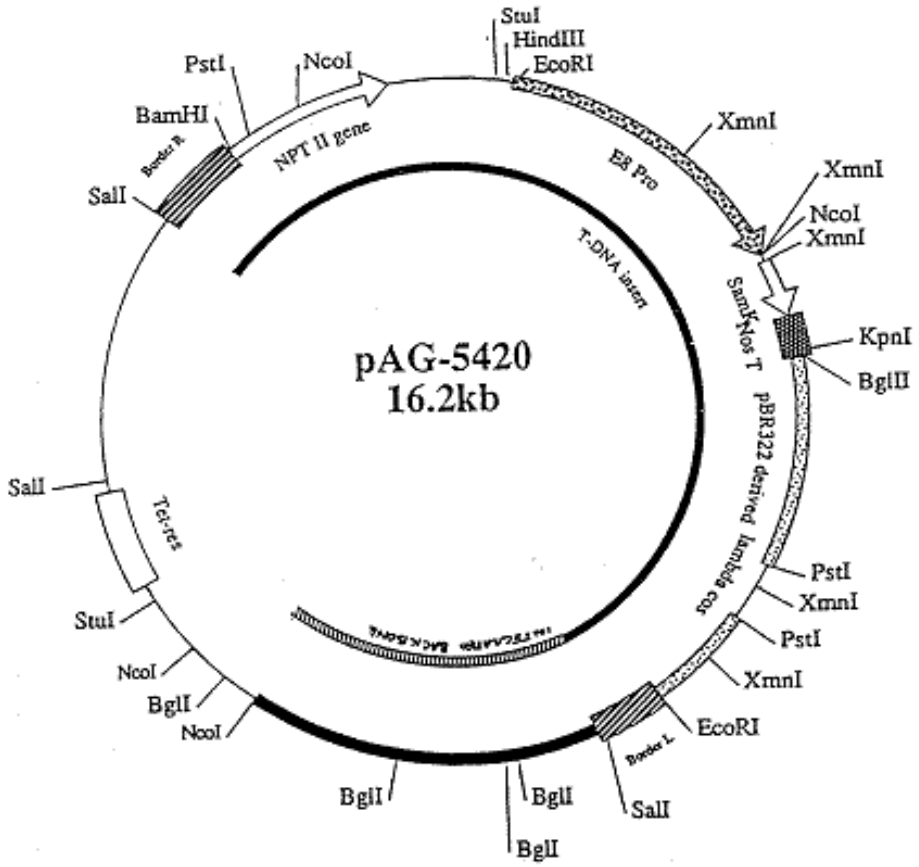


**Figure C.2 Plasmid map of pAG-5420 (harboring *sam-k* gene) Vector for 35 1 N tomato, (From Petition for Determination of Regulatory Status: Cherry Tomatoes with a S-adenosylmethlonine hydrolase gene (Retrieved from GMO Detection method Database (GMDD), http://gmdd.shgmo.org))**

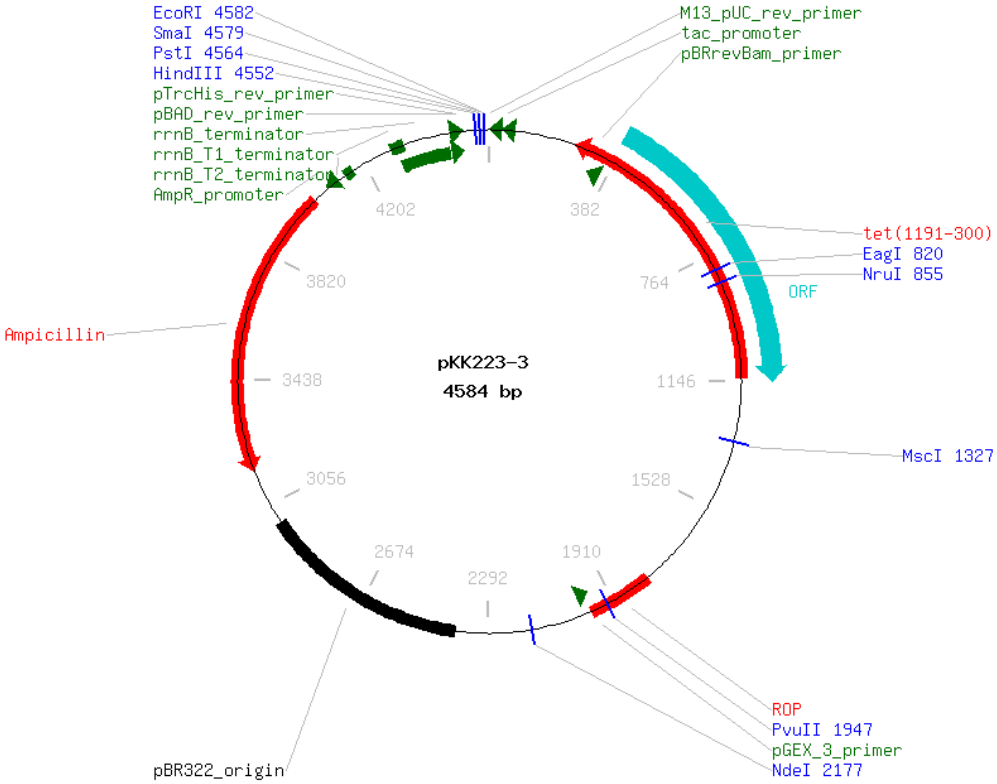


**Figure C.3 Plasmid Map of pKK223-3, (harboring *cry1ac* gene) (*cry1Ac* is cloned in pKK223-3; in E. coli JM103 with ampicillin resistance (Addgene, Inc.: http://www.addgene.org))**

# APPENDIX D

## SEQUENCES OF THE EXPECTED AMPLIFIED REGIONS

GCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTG
AAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCC
ACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTC
AAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATG
ACGCACAATCCCACTATC

**Figure D.1 195bp from P-35S, NCBI Accession No: AR656168**

GAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTG
AATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTA
TTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTT
AATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAA

**Figure D.2 180bp from T-nos, NCBI Accession No: U12540**

TCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGT
TGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTT
AAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGA
TGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGC
GATAGAAAACAAAATATAGCGCGCAA

**Figure D.3 207bp from T-nos, NCBI Accession No: U12540**

TTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGC
GGCTGCATACGCTTGATCCGGCTACCTGCCCATACGACCACCAAG
CGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGT
CTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGC
GCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACG
GCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATA
TCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCC
GGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACC
CGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTC
CTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGGATCGCC
TTCTATCGCCTTC

**Figure D.4 459bp from *nptII*, NCBI Accession No: AF274974**

GCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCT
GGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCAC
TGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGC
AGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCA
TCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTA
CCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCA
CGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGAC
GAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCT
CAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATG
GCGATGCCTGC

**Figure D.5 411bp from *nptII*, NCBI Accession No: U32991.1**

GGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCAT
CATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTAC
CTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCAC
GTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTG

**Figure D.6 175bp from *nptII*, NCBI Accession No: U32991.1**

GAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTT
GATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAACC
TTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCG
AAATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAAC
CGGCAAAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAATGG
AGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGAC
AGGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCTCGCGCGCA
GATCAGTTGGAAGAATTTGTCCACTACGTGAAAGGCGAGATCAC
CAAGGTAGTCGGCAAAT

**Figure D.7 373bp from *aad*, NCBI Accession No: X02340**

GGTCTTTATGGCTCCGTTGAGTCAACCGATTTGACCGGGTGCTATC
GTGAGGCAATCTCAAGCGCACCAACTGAGGAAAAAACTGTTCGT
GTACGCTGCAAGGACAAAGCGCAGGCACTCAATGTTGCACGCCTA
GCTTGTAATGAGTGGGAGCAAGATTGCGTACTGGTATACAAATCA
CAGACTCACACGGCTG

**Figure D.8 196bp from *sam-k*, NCBI Accession No: X04791**

CGGGATTAGAACGTGTATGGGGACCGGATTCTAGAGATTGGG
TAAGGTATAATCAATTTAGAAGAGAATTAACACTAACTGTAT
TAGATATCGTTGCTCTGTTCCCGAATTATGATAGTAGAAGATA
TCCAATTCGAACAGTTTCCCAATTAACAAGAGAAATTTATACA
AACCCAGTATTAGAAAATTTTGATGGTAGTTTTCGAGGCTCGG
CTCAGGGCATAGAAAGAAGTATTAGGAGTCCACATTTGATGG
ATATACTTAACAGTATAACCATCTATACGGATGCTCATAGGG
GTTATTATTATTGGTCAGGGCATCAAATAATG

**Figure D.9 329bp from *cry1ac*, NCBI Accession No: AY225453**

GCTTCTGTAACCCCGATTCACCTCAACGTTAATTGGGGTAATTCA
TCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGAT
AATCTACAATCAAGTGATTTTGGTTATTTTGAAAGTGCCAATGCT
TTTACATCTTCATTAGGTAATATAGTAGGTGTTAGAAATTTTAGT
GGGACTGCAGGAGTGATAATAGACAGATTTGAATTTATTCCAGTT
ACTGCAACACTCGAGGCTGAATATAATCTGGAAAGAGCGCAGAA
GGCGGTGAATGCGCTGTTTACGTCTACAAACCAACTAGGGCTAA
AAACAAATGTAACGGATTATCATATTGATCAAGTGTCCAATTTAG
TTACGTATTTATCGGATGAATTTTGTCTGGATGAAAAGCGAGAAT
TGTCCGAGAAAGTCAAACATGCGAAGCGACTCAGTGATGAACGC
AATTTACTCCAAGATTCAAATTTCAAAGACATTAATAGGCAACCA
GAACGTGG

**Figure D.10 500bp from *cry1ac,* NCBI Accession No: AY225453**

ATTCGCTGGATGAAATACCGCCACAGAATAACAACGTGCCACCT
AGGCAAGGATTTAGTCATCGATTAAGCCATGTTTCAATGTTTCGT
TCAGGCTTTAGTAATAGTAGTGTAAGTATAATAAGAGCTCCTATG
TTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCAT
CGGATAGTATTACTCAAATCCCTGCAGTGAAGGGAAACTTTCTTT
TTAATGGTTCAGTAATTTCAGGACCAGGATTTACTGGTGGGGACT
TAGTTAGATTAAATAGTAGTGGAAATAACATTCAGAATAGAGGG
TATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGATAT
CGAGTTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCTCAAC
GTTAATTGGGGTAATTCATCCATTTTTTCCAATACAGTACCAGCT
ACAGCTACGTCATTAGATAATCTACAATCAAGTGATTTTGGTTAT
TTTGAAAGTGCCAATGCTTTTACATCTTCATTAGGTAATATAGTA
GGTGTTAGAAATTTTAGTGGGACTGCAGGAGTGATAATAGACAG
ATTTGAATTTATTCCAGTTACTGCAACACTCGAGGCTGAATATAA
TCGTGAAAGAGCGCAGAAGGCGGTGAATGCGCTGTTTACGTCTA
CAAACCAACTAGGGCTAAAAACAAATGTAACGGATTATCATATT
GATCAAGTGTCCAATTTAGTTACGTATTTATCGGATGAATTT

**Figure D.11 789bp from *cry1ac,* NCBI Accession No: AY525369**

# APPENDIX E

## SEQUENCES OF *CRY1AC* GENES AND *SAM-K* GENE

ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) Alignment Results of:

1) *Bacillus thuringiensis* Cry1Ac (*cry1Ac*) gene, NCBI Accession No: AY225453

2) Synthetic construct *cry1Ac*AT modified toxin gene, NCBI Accession No: AY525369

3) Synthetic, truncated *cry1Ac* gene from *Bacillus thuringiensis*, NCBI Accession No: U63372

```
1               ----------ATGGATAACAATCCGAACATCAATGAATGCATTCCTTATAATTGTTTAAG 50
2               ----------ATGGATAACAATCCGAACATCAATGAATGCATTCCTTATAATTGTTTAAG 50
3               GGATCCAACAATGGACAACAATCCCAACATCAACGAGTGCATTCCTTACAACTGCCTGAG 60
                          ***** ******** ******** ** *********** ** **  * **

1               TAACCCTGAAGTAGAAGTATTAGGTGGAGAAAGAATAGAAACTGGTTACACCCCAATCGA 110
2               TAACCCTGAAGTAGAAGTATTAGGTGGAGAAAGAATAGAAACTGGTTACACCCCAATCGA 110
3               CAACCCTGAGGTTGAGGTGCTGGGTGGAGAACGGATTGAGACTGGTTACACACCTATCGA 120
                 ******** ** ** **  * ********* * ** ** *********** ** *****

1               TATTTCCTTGTCGCTAACGCAATTTCTTTTGAGTGAATTTGTTCCCGGTGCTGGATTTGT 170
2               TATTTCCTTGTCGCTAACGCAATTTCTTTTGAGTGAATTTGTTCCCGGTGCTGGATTTGT 170
3               CATCTCGTTGTCACTTACCCAATTCCTTTTGTCAGAGTTCGTGCCCGGTGCTGGATTCGT 180
                 ** ** ***** ** ** ***** ******   ** ** ** ************** **

1               GTTAGGACTAGTTGATATAATATGGGGAATTTTTGGTCCCTCTCAATGGGACGCATTTCT 230
2               GTTAGGACTAGTTGATATAATATGGGGAATTTTTGGTCCCTCTCAATGGGACGCATTTCT 230
3               GCTTGGACTTGTCGATATCATTTGGGGAATCTTTGGTCCCTCTCAATGGGACGCCTTTCT 240
                * * ***** ** ***** ** ******** ********************** *****

1               TGTACAAATTGAACAGTTAATTAACCAAAGAATAGAAGAATTCGCTAGGAACCAAGCCAT 290
2               TGTACAAATTGAACAGTTAATTAACCAAAGAATAGAAGAATTCGCTAGGAACCAAGCCAT 290
3               TGTACAGATAGAGCAACTTATCAACCAAAGGATTGAAGAGTTCGCTAGGAACCAAGCCAT 300
                ****** ** ** **  * ** ******** ** ***** ********************

1               TTCTAGATTAGAAGGACTAAGCAATCTTTATCAAATTTACGCAGAATCTTTTAGAGAGTG 350
2               TTCTAGATTAGAAGGACTAAGCAACTTGTACCAGATCTACGCAGAATCTTTTAGAGAGTG 350
3               CTCAAGGTTAGAAGGCCTCAGCAACCTTTACCAGATTTACGCAGAATCTTTTCGAGAGTG 360
                 ** ** ******** ** *****  * ** ** ** *************** *******

1               GGAAGCAGATCCTACTAATCCAGCATTAAGAGAAGAGATGCGTATTCAATTCAATGACAT 410
2               GGAAGCAGATCCTACTAATCCAGCATTAAGAGAAGAGATGCTGATTCAATTCAATGACAT 410
3               GGAAGCAGACCCGACCAATCCTGCCTTAAGAGAGGAGATGCGCATTCAATTCAATGACAT 420
                ********* ** ** ***** ** ******** *******  *****************

1               GAACAGTGCCCTTACAACCGCTATTCCTCTTTTTGCAGTTCAAAATTATCAAGTTCCTCT 470
2               GAACAGTGCCCTTACAACCGCTATTCCTCTTTTTGGTGTTCAAAATTATCAAGTTCCTCC 470
3               GAACAGCGCGCTGACGACCGCAATTCCGCTCTTCGCCGTTCAGAATTACCAAGTTCCTCT 480
                ****** ** ** ** ***** ***** ** ** *  ***** ***** **********
```

```
1               TTTATCAGTATATGTTCAAGCTGCAAATTTACATTTATCAGTTTTGAGAGATGTTTCAGT 530
2               TTTATCAGTATATGTTCAAGCTGCAAACTTACACTTATCAGTTCTGAGAGATGTTTCAGT 530
3               TTTATCCGTGTACGTGCAGGCTGCCAACCTGCACTTGTCGGTGCTCCGCGATGTCTCCGT 540
                ****** ** ** ** ** ***** **  * ** ** ** **  *  * ***** ** **

1               GTTTGGACAAAGGTGGGGATTTGATGCCGCGACTATCAATAGTCGTTATAATGATTTAAC 590
2               GTTTGGACAAAGGTGGGGATTTGATGCCGCGACTATCAATAGTCGTTACAACGACTTGAC 590
3               GTTCGGACAACGGTGGGGCTTTGATGCCGCAACTATCAATAGTCGTTATAATGATCTGAC 600
                *** ****** ******* *********** ***************** ** **  * **

1               TAGGCTTATTGGCAACTATACAGATTATGCTGTACGCTGGTACAATACGGGATTAGAACG 650
2               TAGGCTTATTGGCAACTATACAGATTATGCTGTACGCTGGTACAATACGGGATTAGAACG 650
3               TAGGCTTATTGGCAACTATACCGATTATGCTGTTCGCTGGTACAACACGGGTCTCGAACG 660
                ******************** *********** *********** *****  * *****

1               TGTATGGGGACCGGATTCTAGAGATTGGGTAAGGTATAATCAATTTAGAAGAGAATTAAC 710
2               TGTATGGGGACCGGATTCTAGAGATTGGGTAAGGTATAATCAATTTAGAAGAGAATTAAC 710
3               TGTCTGGGGACCGGATTCTAGAGATTGGGTCAGGTACAACCAGTTCAGGCGAGAGTTGAC 720
                *** ************************** ***** ** ** ** **  **** ** **

1               ACTAACTGTATTAGATATCGTTGCTCTGTTCCCGAATTATGATAGTAGAAGATATCCAAT 770
2               ACTAACTGTATTAGATATCGTTGCTCTGTTCCCGAATTATGATAGTAGAAGATATCCAAT 770
3               ACTAACTGTCCTAGACATTGTCGCTCTCTTTCCCAACTACGACTCTAGGCGCTACCCAAT 780
                *********  **** ** ** ***** ** ** ** ** **   ***  * ** *****

1               TCGAACAGTTTCCCAATTAACAAGAGAAATTTATACAAACCCAGTATTAGAAAATTTTGA 830
2               TCGAACAGTTTCCCAATTAACAAGAGAAATTTATACAAACCCAGTATTAGAAAATTTTGA 830
3               CCGTACTGTGTCACAATTGACCCGGGAAATCTACACAAACCCAGTCCTCGAGAACTTCGA 840
                 ** ** ** ** ***** **  * ***** ** ***********  * ** ** ** **

1               TGGTAGTTTTCGAGGCTCGGCTCAGGGCATAGAAAGAAGTATTAGGAGTCCACATTTGAT 890
2               TGGTAGTTTTCGAGGCTCGGCTCAGGGCATAGAAAGAAGTATTAGGAGTCCACATTTGAT 890
3               CGGTAGCTTTCGAGGCTCGGCTCAGGGCATAGAGAGAAGCATCAGGTCTCCACACCTGAT 900
                 ***** ************************** ***** ** ***  ******  ****

1               GGATATACTTAACAGTATAACCATCTATACGGATGCTCATAGGGGTTATTATTATTGGTC 950
2               GGATATACTTAACAGTATAACCATCTATACGGATGCTCATAGGGGTTATTATTATTGGTC 950
3               GGACATATTGAACAGTATCACGATCTACACCGATGCGCACCGCGGTTATTACTACTGGTC 960
                *** *** * ******** ** ***** ** ***** **  * ******** ** *****

1               AGGGCATCAAATAATGGCTTCTCCTGTCGGTTTTTCGGGGCCAGAATTCACGTTTCCGCT 1010
2               AGGGCATCAAATAATGGCTTCTCCTGTAGGGTTTTCGGGGCCAGAATTCACTTTCCGCT 1010
3               AGGGCATCAGATCATGGCATCACCCGTTGGGTTCTCTGGACCAGAATTCACTTTCCCACT 1020
                ********* ** ***** ** ** ** ** ** ** ** ** *********** ** ** **

1               ATATGGAACCATGGGAAATGCAGCTCCACAACAACGTATTGTTGCTCAACTAGGTCAGGG 1070
2               ATATGGAACTATGGGAAATGCAGCTCCACAACAACGTATTGTTGCTCAACTAGGTCAGGG 1070
3               TTACGGGACTATGGGCAATGCAGCTCCACAACAACGTATTGTTGCTCAACTCGGTCAGGG 1080
                 ** ** ** ***** ********************************** ********

1               CGTGTATAGAACATTATCGTCCACTTTATATAGAAGACCTTTTAATATAGGGATAAATAA 1130
2               CGTGTATAGAACATTATCGTCCACTTTATATAGAAGACCTTTTAATATAGGGATAAATAA 1130
3               CGTGTATAGAACCTTGTCCAGCACTCTATATAGGAGACCTTTCAACATCGGCATCAACAA 1140
                ************ ** **   **** ******* ******** ** ** ** ** ** **

1               TCAACAACTATCTGTTCTTGACGGGACAGAATTTGCTTATGGAACCTCCTCAAATTTGCC 1190
2               TCAACAACTATCTGTTCTTGACGGGACAGAATTTGCTTATGGAACCTCCTCAAATTTGCC 1190
3               TCAACAATTGTCTGTGCTTGACGGGACAGAATTTGCCTATGGAACCTCCTCAAATCTGCC 1200
                ******* * ***** ******************** ****************** ****

1               ATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCGCTGGATGAAATACCGCCACAGAA 1250
2               ATCCGCTGTATACAGAAAAAGCGAAACGGTAGATTCGCTGGATGAAATACCGCCACAGAA 1250
3               ATCCGCTGTCTACAGAAAGAGCGGAACAGTTGATAGCTTGGATGAGATCCCTCCACAGAA 1260
                ********* ******** **** *** ** ***    ******* ** ** ********
```

**Figure E.1 Alignment Results of *cry1ac* genes, comparison of *cry1ac* sequences (1, 2, 3 as outlined above)**

AGCTCTTGAGTGTACCATATAAGCGAATAACTCAAGGTCGCACTG
AAAGCGTGGCCTTTATGATATTCACTTAACGAACTAACATGAGGT
AACACCAAATGATTTTCACTAAAGAGCCTGCGCACGTCTTCTATG
TACTGGTTTCCGCTTTCCGTTCTAACCTCTGCGATGAGGTGAATAT
GAGCAGACACCGCCACATGGTAAGCACTTTACGTGCCGCACCG**G**
**GTCTTTATGGCTCCGTTGAGTCAACCGATTTGACCGGGTGCT**
**ATCGTGAGGCAATCTCAAGCGCACCAACTGAGGAAAAAACTG**
**TTCGTGTACGCTGCAAGGACAAAGCGCAGGCACTCAATGTTG**
**CACGCCTAGCTTGTAATGAGTGGGAGCAAGATTGCGTACTGG**
**TATACAAATCACAGACTCACACGGCTG**GTCTGGTGTACGCTAA
AGGTATCGACGGGTATAAGGCTGAACGTCTGCCGGGTAGTTTCCA
AGAGGTTCCTAAAGGCGCACCGCTGCAAGGCTGCTTCACTATTGA
TGAGTTCGGTCGCCGCTGGCAAGTACAATAAGTGTTAAACTCAAG
GTCATGCACGATGCGTGGCCTTTATGATTAACATTCTCTCTATAG
AGGTATTGAATG

**Figure E.2 DNA Sequence of *sam-k*, from Coliphage T3 S-adenosyl-L-methionine hydrolase (AdoMetase, E.C. 3.3.1.2) (The primer pair yielding 196bp were underlined)**

# APPENDIX F

## EXAMPLES OF SEQUENCING RESULTS

GAAGAGTTTAACAGACAACAGTTAATTTGAGAATTTTCGACTCGA
CAACTCAAACGACATGCTATACTTTTATAGGTATCTCTTAAGAAA
TTGTACATAGATTCGTTACATACTCTATAACTTGTTTGATAAAAA
AAAGTAACTTCAGCCTGTATTGTTTGCACAAAAGATTGACGTATG
CTCTGTTTTTATGGCAATAGAGATCGAGGAAGAAGATAACGATTT
GCTGATTTTGAAGTTGCAGAATTAGTTTAGCGAGACGGCTGAGTG
AGTATAGTCAATAATTATATAAGTACCTTTTAGCTGTTATTACTTT
GGATTCGCACGCCTTTATTAGAAGAGCACATCGAAGGTCACACTT
GTTCTGGGATCTGGCCTATCGTCTTCGTCGTCATCAGCCTTCAGCT
TTGCTCCTGCCG

**Figure F.1 459bp fragment from *nptII* amplified from Sample 13**

# APPENDIX G

## INVERSE PRIMER PAIRS

ATGGTTTTCACTAAAGAGCCTGCGAACGTCTTCTATGTACTGGTTTCCGCTTTC
CGTTCTAACCTCTGCGATGAGGTGAATATGACAGACACCGCCACATGGTAAGC
ACTTTACGTGCCGCACCG**GGTCTTTATGGCTCCGTTGA**GTCAACCGATTTGA
<<<<<<<<<<<<<<<<<<<<<<<<<<<<
CCGGGTGCTATCGTGAGGCAATCTCAAGCGCACCAACTGAGGAAAAAACTGTT
CGTGTACGCTACAAGGACAAAGCGCAGGCACTCAATGT**TGCACGCCTAGCTT**
>>>>>>>>>>>>>>>>
**GTAATG**AGTGGGAGCAAGATTGCGTACTGGTATACAAATCACAGACTCACAC
>>>>>>>
GGCTGGTCTGGTGTACGCTAAAGGTATCGACGGGTATAAGGCTGAACGTCTGC
CGGGTAGTTTCCAAGAGGTTCCTAAAGGCGCACCGCTGCAAGGCTGCTTCACT
ATTGATGAGTTCGGTCGCCGCTGGCAAGTACAATAA

**Figure G.1 Inverse PCR 1st Primer Pair**

ATGGTTTTCACTAAAGAGCCTGCGAACGTCTTCTA**TGTACTGGTTTCCGCTTT**
<<<<<<<<<<<<<<<<<<<<<<
**CC**GTTCTAACCTCTGCGATGAGGTGAATATGACAGACACCGCCACATGGTAAG
<<<
CACTTTACGTGCCGCACCG**GGTCTTTATGGCTCCGTTGA**GTCAACCGATTTG
>>>>>>>>>>>>>>>>>>>>>>>>
ACCGGGTGCTATCGTGAGGCAATCTCAAGCGCACCAACTGAGGAAAAAACTG
TTCGTGTACGCTACAAGGACAAAGCGCAGGCACTCAATGTTGCACGCCTAGCT
TGTAATGAGTGGGAGCAAGATTGCGTACTGGTATACAAATCACAGACTCACAC
GGCTGGTCTGGTGTACGCTAAAGGTATCGACGGGTATAAGGCTGAACGTCTGC
CGGGTAGTTTCCAAGAGGTTCCTAAAGGCGCACCGCTGCAAGGCTGCTTCACT
ATTGATGAGTTCGGTCGCCGCTGGCAAGTACAATAA

**Figure G.2 Inverse PCR 2nd Primer Pair**