# SCREENING OF TOMATO SEEDS FOR GENETIC MODIFICATION AND IDENTIFICATION OF GENETICALLY MODIFIED RIPENING DELAYED TOMATO SEEDS

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## ABSTRACT

### SCREENING OF TOMATO SEEDS FOR GENETIC MODIFICATION AND IDENTIFICATION OF GENETICALLY MODIFIED RIPENING DELAYED TOMATO SEEDS

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Tomato has been genetically modified for providing properties such as insect-resistance or delayed-ripening. Tomato seeds purchased from several bazaars and markets were screened for the presence of genetic modification by targeting NptII kanamycin resistance, Nos terminator, and 35S promoter gene regions which are the most commonly transformed gene regions in transgenic plants, and then ripening-delayed tomato seeds were tried to be identified in this study. F type truncated-PG gene and Sam-k gene were selected as the indicator of genetically modified ripening delayed tomatoes. DNAs of 25 seed samples were isolated by CTAB method and examined with several primer pairs, and

the primer sets that provided consistent results were selected to conduct routine testing by PCR analysis of the samples.

In screening analysis *via* conventional PCR, amplifications 4 samples were amplified with 35S, Nos and NptII primer sets. Among other samples, 3 of them were amplified with 35S and Nos primer sets and 2 of them were amplified only with 35S primer set. The amplification was observed with Nos, NptII and Sam-k primers in one sample and this sample was identified as 35 1 N, since the sequence result of the PCR product amplified with Sam-k primers showed high homology with the Samase gene of T3 Coliphage. F type truncated- PG gene was not observed in any of the samples.

Although this study demonstrates the presence of commonly used gene regions in genetically modified tomatoes, further analysis of the genetically modified ripening delayed tomato seeds *via* construct specific or event specific PCR techniques is needed for confirmation.

**Keywords:** GMO detection, tomato seeds, delayed ripening, Sam-k gene, truncated-PG gene.

# ÖZ

## DOMATES TOHUMLARINDA GENETİK MODİFİKASYON TARAMASI VE GENETİĞİ DEĞİŞTİRİLMİŞ RAF ÖMRÜ UZATILMIŞ DOMATES TOHUMLARININ TANIMLANMASI

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Domates, böceğe karşı direnç ya da olgunlaşmayı geciktirmek gibi nitelikler kazandırmak amacı ile genetik olarak değiştirilmiştir. Bu çalışmada, pazarlardan ve marketlerden toplanan 25 domates tohumu, transgenik bitkilerde yaygın olarak kullanılan NptII Kanamisin direnç, Nos terminatör ve 35S promotör gen bölgeleri hedeflenerek genetik modifikasyonun tespiti için taranmış ve sonra olgunlaşması geciktirilmiş domates tohumları tanımlanmaya çalışılmıştır. F tipi kesik-PG geni ve Sam-k geni genetiği değiştirilmiş olgunlaşması geciktirilmiş domates tohumlarının göstergesi olarak seçilmiştir. 25 tohumdan CTAB metodu ile

DNA izolasyonu yapılmış, izole edilen DNA örneklerinin değişik primer setleri ile çalışılması sonucu tutarlı sonuç veren primer setleri, bu örneklerin rutin PZR analizleri için kullanılmıştır.

Konvansiyonel PZR ile tarama çalışmalarında 4 örnek, 35S, Nos ve NptII primer setleri yükseltgenmiştir. Diğer örneklerden 3 tanesi 35S ve Nos primerleri ile 2 tanesi ise sadece 35S primer seti ile yükseltgenmiştir. Diğer örneklerden 1 tanesi Nos, NptII ve Sam-k primerleri ile yükseltgenme göstermiş ve Sam-k primeri ile yükseltgenen bu örneğe ait PZR ürününün sekans sonucunun T3 kolifajının Samase geni ile yüksek düzeyde benzerlik göstermesi, örneğin 35 1 N olarak tanımlanmasını sağlamıştır. F tipi kesik PG genine hiçbir örnekte rastlanmamıştır.

Bu çalışma, genetiği değiştirilmiş domateslerde yaygın olarak kullanılan gen bölgelerinin varlığını ortaya koysa da, genetiği değiştirilmiş olgunlaşması geciktirilmiş domates tohumlarının konstrakta özgü ya da konstrakt ile genomik DNA bağlanma noktasına özgü teknikler kullanılarak teyit edilmesi gerekmektedir.

**Anahtar Kelimeler:** GDO tespiti, domates tohumu, raf ömrü uzatılmış, Sam-k geni, kesik PG geni.

To My Father

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## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## LIST OF ABBREVIATIONS

Acc: Aminocyclopropane carboxylate synthase

ACC: 1-aminocyclopropane 1-carboxylic acid

accd: 1-aminocyclopropane 1-carboxylic acid deaminase

A PG: Antisense Poly Galacturonase

µg : Microgram

µl : Microliter

bla gene : (β-lactamase) marker gene

bp : base pair

Bt: Bacillus thuringiensis

CaMV: Cauliflower Mosaic Virus

cDNA: Complementary DNA

CTAB: cetyltrimethylammonium-bromide

Cry proteins: Crystal proteins

DNA: Deoxyribonucleic acid

ds : double stranded

EC: European Commission

ECB: European Corn Borer

EDTA: Ethylenediamineetetraacetic acid

EFSA: European Food Safety Authority

ELISA: Enzyme linked immunosorbent assay

EMBL: European Molecular Biology Laboratory

EPA: Environmental Protection Agency

EtBr: Ethidium Bromide

EU: European Union

FDA: Food and Drug Administration

FSANZ: Food Standards Australia New Zealand

GMO: Genetically Modified Organisms

GM: Genetically Modified

ISAAA: International Service for the Acquisition of Agri-Biotech Applications

JRC: Joint Research Center

Kan: Kanamycin

kb : kilobase

LB medium: Luria Bertoni medium

MAFF: The Ministry of Agriculture, Forestry and Fisheries

MHLW: The Ministry of Health, Labor and Welfare

min : minute

mM : Milimolar

mRNA : Messenger RNA

NCBI: National Centre for Biotechnology Information

ng : Nanogram

Nos: Nopaline synthase

NptII: Neomycin phosphotransferase

PCR : Polymerase Chain Reaction

PEG: Polyethylene glycol

PG: polygalacturonase

pmol : Pico mole

QC-PCR: Quantitative Competitive -Polymerase Chain Reaction

Rpm : Rotation per minute

RT-PCR: Real Time-Polymerase Chain Reaction

SAM: S-adenosylmethionine

Sam-k: S-adenosylmethionine hydrolase encoding gene

USDA: United States Department of Agriculture

UV : Ultra Violet

v/v : volume/volume

w/v : weight/volume

# CHAPTER I

# INTRODUCTION

## 1.1 Modern Biotechnology

For centuries cross-breeding techniques have been used to modify or improve the quality, yield and taste characteristics of food. Those plants and animals with the most desirable characteristics, caused by naturally occurring variations in their genetic makeup, were chosen for food production and for breeding the next generations.

Now, with new technology, it is possible to identify and transfer particular characteristics of living organisms and alter them in a specific and a direct way. By introducing a new segment of genetic material coming from other living organisms whether plant, animal or microbe, the resultant plant or animal is what is called “a genetically modified organism” or GMO (De Leo F. & F. , 2005).

## 1.2 Genetically Modified Organisms

An organism is "genetically modified", if its genetic material has been changed in a way that does not occur under natural conditions through cross-breeding or natural recombination (2001/18/EC Directive). GMO

content of a sample is a percentage of the amount of genetically modified material in the total material amount (Querci *et al.*, 2002).

In individual cases it can be very controversial if an organism has been genetically modified in a way that does not occur "naturally". The fact that cultivated plants scarcely resemble their wild relatives is an example of dramatic, human-induced genetic modifications that would not be defined as "genetically modified" (2001/18/EC Directive).

According to 2001/18/EC, several techniques that lead to genetically modified organisms: 1- Transfer of recombinant DNA that was created outside the organism by laboratory techniques. 2- Certain procedures used for cell fusion. Mutations normally do not create GMOs, not even when these mutations are induced artificially (USDA, 13.01.2007).

## 1.3 Applications of Agricultural Biotechnology

### 1.3.1 Agronomic Traits

Agricultural Biotechnology has being mostly used for improving the protection of agricultural crops (Gachet E., 1999). The application of herbicide resistance is the most common use of genetic engineering in agriculture. The commercially second most important trait conferred to crops by genetic engineering is insect resistance and the third one is insect

resistance/herbicide tolerance (Engel, K.H. *et al.*, 2002, GMO Compass, 21.02. 2007).

The most commonly grown genetically modified crop remains herbicide tolerance soybean, covering 58.6 million hectares in 2006, which makes up over 60 percent of worldwide soybean production. The next most widespread genetically modified crop is GM maize. Next is GM cotton, and rapeseed (GMO Compass, 21.02.2007). GM soybean and maize are mostly introduced resistance to herbicides and increased tolerance to insects and pests (Al-Swailem A.M. *et al.*, 2005).

Protection against viral diseases has been achieved by expressing viral coat proteins or by introducing viral replicase genes. Resistance to fungi is conferred by GM-induced biosynthesis of phytoalexins. Various strategies ranging from expression of antibacterial enzymes to engineered detoxification have been described to confer resistance to bacteria (Engel, K.H. *et al.*, 2002).

Additional strategies to improve crop productivity especially in developing countries are based on increasing crop tolerance to abiotic stresses such as drought resistance, effects of metals, salinity as evoked by the rough environmental conditions (Engel, K.H. *et al.*, 2002).

### 1.3.2 Quality Traits

#### 1.3.2.1 Sensory Properties

Delayed ripening is one of the quality traits introduced to plants as in the case of Flavr Savr™ tomato, the first transgenic crop put on the market in US, exhibiting delayed ripening. Unfortunately, this trait was mainly propagated as a means to increase the shelf life of tomatoes. The potentially positive effect on the flavor implied in the commercial name has actually not been exploited (Engel, K.H. *et al*., 2002).

Approaches that are more recent are intended to modify enzyme-catalyzed steps in the biosynthesis of specific flavour and aroma constituents. The genetic engineering of essential oil production in mint is an example. Considering the role of biotechnology in the production of flavours, the use of modified microorganisms will be of an increasing importance (Engel, K.H. *et al*., 2002).

#### 1.3.2.2 Nutritional Properties

Genetic engineering can be applied to modify macronutrients as well as micronutrients in foods. Improving starch biosynthesis, changing starch composition, genetic engineering of lipid metabolism in oil crops, modification of the chain lengths and the degree of saturation of fatty acids such as high laurate canola oil and sunflower seed oil with high oleic acid content, increased baking quality of wheat are some examples for relevant

applications. As for micronutrients, the wide range of isoprenoids found in plants and the integration of metabolic pathways of steroids, carotenoids and retinoids offers the potential to influence the content of these compounds by genetic engineering (Engel, K.H. *et al.*, 2002).

### 1.4 Concerns about Genetically Modified Organisms

In the assessment of genetically engineered organisms, first concern is the environmental effects on birds, mammals, insects, worms, and other organisms, especially in the case of insect or disease resistance traits. Among all GMOs, only GM crops are formally reviewed to assess the potential for transfer of novel traits to wild relatives. When new traits are genetically engineered into a crop, the new plants are evaluated to ensure that they are free from weed characteristics. Where GM crops are grown close-by related plants, the potential for the two plants to exchange traits *via* pollen must be evaluated before release. Crop plants of all kinds can exchange traits with their close wild relatives when they are grown too close.

Secondly, with respect to food safety, there are concerns about toxicity or causing of allergic response of the proteins produced by the new traits introduced to the crops. Tests designed to examine the heat and digestive stability of these proteins, their similarity to known allergenic proteins.

Thirdly, religious concerns are also voiced as some of the reasons for opposing genetic engineering of foods, while some people object to bio-

engineered foods for personal, ethical, cultural, and aesthetic reasons, as well as infringement on consumer choice, and inability to distinguish GM foods from non-GM counterparts. For example, Jews and Muslims will object to grains that contain pig genes, and usually insist on Kosher and Halal foods whose purity can be documented. Vegetarians may similarly object to vegetables and fruits that contain animal genes (Crist, 1996). Some people fear eating plant foods containing human genes (Uzogara, 2000).

Moreover, there is a concern that organic crops might be contaminated through cross breeding of herbicide resistant plants with wild relatives, or through cross pollination with GM crops in neighboring farms, thereby creating 'monster weeds' resistant to natural pesticides normally used by organic farmers (Uzogara, 2000). There is also a fear that pests resistant to Bt toxin will be produced (Koch, 1998).

Finally, animal rights groups strongly oppose any form of cloning or genetic engineering involving animals, or use of animals in research, and have sometimes resorted to vandalizing animal research facilities (Kaiser, 1999).

## 1.5 Development of Agricultural Biotechnology in the World

### 1.5.1 GMO Production

In 2006, genetically modified crops were planted on 102 million hectares (Figure 1.1). The number of farmers growing biotech crops was 10.3 million

from 22 countries in 2006 (James C., ISAAA, 2006). Ninety percent of these farmers are in developing countries like China, India, and the Philippines. As of 2006 more than 40 percent of GM crops are grown in developing countries (GMO Compass, 21.02.2007).

As of 2006, the 22 countries growing biotech crops comprised 11 developing countries and 11 industrial countries; they are, in order of hectarage, USA, Argentina, Brazil, Canada, India, China, Paraguay, South Africa, Uruguay, Philippines, Australia, Romania, Mexico, Spain, Colombia, France, Iran, Honduras, Czech Republic, Portugal, Germany, and Slovakia (Table 1.1). The first eight of these countries grew more than 1 million hectares each (James C., ISAAA, 2006).

Genetically modified crops are grown in six EU Member States as of 2006, Slovakia, Spain, France, Portugal, the Czech Republic, and Germany. Spain is the lead country in Europe planting 60,000 hectares in 2006 (James C., ISAAA, 2006). In Portugal, Germany and France transgenic crops were primarily grown for small-scale field trials (GMO Compass, 21.02.2007). GM crop production has also reached noteworthy levels in Paraguay, South Africa, Uruguay and Australia. Iran and the Czech Republic are also added to the list of countries commercially growing transgenic crops (GMO Compass, 21.02.2007).

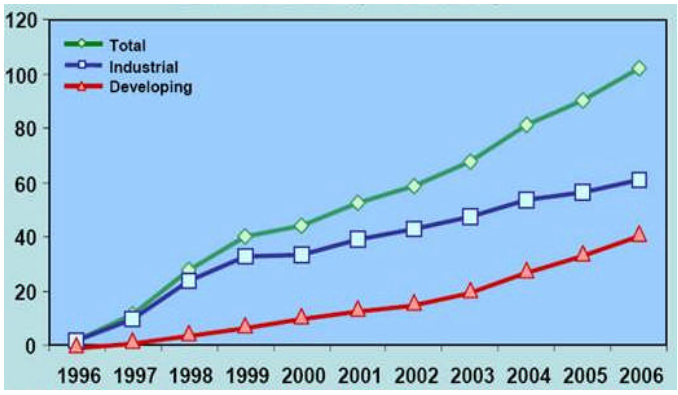


**Figure 1.1 Global Area of GMO Crops**
Million Hectares (1996 to 2006)
**Source:** ISAAA, www.isaaa.org, 20.03.2007

### 1.5.2 GMO Regulation in the World

There are two types of regulatory frameworks in the countries for foods derived from GM crops. GMO Legistlations in some countries, including the European Union (EU) and Australia are 'process-based'. In contrast, other regulatory systems are 'product-based', focusing on the resulting product characteristics and use, and not on the process of genetic modification, as those in the USA and Canada (Konig A. et. al., 2004).

More than 30 countries or regions have issued GMO labeling polices, recently. Differences exist among countries about the detailed requirements of labeling policies. The labeling of GM foods is not compulsory in the USA and Canada. In China, 17 kinds foods derived from five different kinds plants should be labeled, such as tomato seeds, ketchup, soybean milk,

**Table 1.1 Global Area of Biotech Crops in 2006 by Country**

(Million Hectares)

| Country | Area (million hectares) | Rank | Biotech Crops |
|---|---|---|---|
| USA | 54.6 | 1* | Soybean, maize, cotton, canola, squash, papaya, alfalfa |
| Argentina | 18.0 | 2* | Soybean, maize, cotton |
| Brazil | 11.5 | 3* | Soybean, cotton |
| Canada | 6.1 | 4* | Canola, maize, soybean |
| India | 3.8 | 5* | Cotton |
| China | 3.5 | 6* | Cotton |
| Paraguay | 2.0 | 7* | Soybean |
| S8outh Africa | 1.4 | 8* | Maize, soybean, cotton |
| Uruguay | 0.4 | 9* | Soybean, maize |
| Philippines | 0.2 | 10* | Maize |
| 8Australia | 0.2 | 11* | Cotton |
| Romania | 0.1 | 12* | Soybean |
| Mexico | 0.1 | 13* | Cotton, soybean |
| Spain | 0.1 | 14* | Maize |
| Colombia | <0.1 | 15* | Cotton |
| France | <0.1 | 16* | Maize |
| Iran | <0.1 | 17* | Rice |
| Honduras | <0.1 | 18* | Maize |
| Czech Republic | <0.1 | 19* | Maize |
| Portugal | <0.1 | 20* | Maize |
| Germany | <0.1 | 21* | Maize |
| Slovakia | <0.1 | 22* | Maize |

**Source:** Clive James, ISAAA, 2006.

* 14 biotech mega-countries growing 50,000 hectares, or more of biotech crops

soybean oil, maize oil, rapeseed seeds and cotton seeds (L. Yang *et al.*, 2005). The labeling policies of several countries are given in Table 1.2. When two GMO are crossed, e.g. two different approved genetically modified maize cultivars, the resulting hybrid offspring may possess the genetic modifications from both parent cultivars. This phenomenon is called "gene stacking". In the USA this type of hybrid GMO is not regulated, because both parent cultivars are approved. In the EU however, the hybrid is considered to be a new GMO and requires separate approval. None of the analysis methods will be able to identify cases of gene stacking. Instead, cases of gene stacking will give results indistinguishable from the separate detection and identification of each of the parental cultivars in the sample (Holst-Jensen A., 2001).

## Table 1.2 International GMO Labeling Regulations and Thresholds

| Country | Labeling | % Threshold | Scheme * |
|---|---|---|---|
| European Union (25) | Mandatory | 0.9% | GM |
| Norway* | Mandatory | 2% | GM |
| Hungary*** | Mandatory | 2% | GM |
| Russia | Mandatory | 0.9% | GM |
| Australia / New Zealand | Mandatory | 1.0% | GM |
| Brazil | Mandatory | 1.0% | GM |
| China | Mandatory | 1.0% | GM |
| Israel | Mandatory | 0.9% | GM |
| Saudi Arabia | Mandatory | 1.0% | GM |
| Switzerland | Mandatory | 1.0% | GM |
| South Korea | Mandatory | 3.0%[a] | GM |
| Indonesia | Mandatory | 5.0% | GM |
| Taiwan | Mandatory | 5.0% | GM |
| Thailand | Mandatory | 5.0% | GM |
| Japan | Mandatory | 5.0%[a] | GM |
| USA | Voluntary | 5.0% | Organic |
| Canada | Voluntary | 5.0% | non-GE or GE |
| South Africa*** | Proposed Voluntary | 1.0% | non-GM |
| Philippines | Voluntary | N/A | N/A |

**Source:** Viljoen C.D, (2005), Norway** : (Hardegger *et al.*, 1999)
Hungary** : (Meyer R., 1999), South Africa**** : (De Leo F&F., 2002)

### 1.5.2.1 European Union

The European Union's new regulation on food and feed labeling is process based and the strictest in the world. International rules for the labeling of GM foods vary considerably between nations (De Leo F&F., 2005).

EU directives formulate the regulatory standards for all storage and handling of genetically modified organisms. GMO legislation was revised to strengthen the existing requirements for risk assessment and the decision-making process in 2001. The revised Directive 2001/18/EC on the deliberate release of genetically modified organisms introduces mandatory labeling and traceability requirements. The regulations are process-based (A. Konig *et al.*, 2004). The EU legislation on GMOs establishes the conditions under which a party may develop, use or market a GMO or a food product derived from GMOs. GMOs and food products derived from GMOs placed on the market must also comply with labeling and traceability requirements (EU Food Safety, 12.01.2007).

Although the threshold level is 0.9 for labeling, 0, 5% tolerance has been adopted for all those GMOs, for which the risk assessment has been finalized, but final approval for authorization in Europe has not yet been granted, so not yet authorized but that has a favorable assessment from an EU scientific committee or EFSA and zero tolerance has been adopted for GMOs for which the risk assessment is ongoing and those for which authorization is not applied for (De Leo F&F., 2005).

#### 1.5.2.2 USA

The USA regulatory framework is a vertical, product-based regulatory framework for GM crops and derived foods. Three principal regulatory agencies conduct science-based assessments of risks to human health and the environment: The United States Department of Agriculture (USDA), the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA). Labeling is only mandated for foods that present a health risk to subgroups of the population, such as allergenic foods; the FDA does not mandate process-based labeling informing consumers for instance on a food's content of genetically modified organisms (A. Konig *et al*., 2004).

#### 1.5.2.3 Canada

In Canada all plants with novel traits are regulated, regardless of whether a plant with novel traits was produced by conventional breeding, mutagenesis, or recombinant DNA techniques. Foods derived from GM crops are considered novel foods. The Canadian Biotechnology Advisory Committee reviewed the Canadian regulations of GM foods; its recommendations include that research be carried out in order to monitor for hypothetical long-term health effects (A. Konig *et al*., 2004).

## Table1. 3 Placing on the Market of GM Crops in EU

(**Source:** http://gmoinfo.jrc.it/, 05.03.2007)

| Name of the Product | Company | Country | Trait | Authorization |
|---|---|---|---|---|
| Florigene Moonaqua™ | Florigene Limited | Netherlands | | Pending |
| Dianthus caryophyllus | Florigene Limited | Netherlands | modified flower colour | Pending |
| Maize NK603 × MON 810 | Monsanto | Spain | Glyphosate Herbicide Tolerant | Pending |
| Bt11 maize | Syngenta Seeds | France | Insect resistant | Pending |
| 1507 Maize | Dow AgroSciences Mycogen SeedsPioneer Hi-Bred | Spain | Lepidopteran resistant and glufosinate tolerant | Pending |
| Oilseed rape Ms8xRf3 | Bayer BioScience | Belgium | | Pending |
| Potato variety EH92-527-1 | Amylogene HB | Sweden | modified starch content | Pending |
| maize NK603 × MON 810 | Monsanto | United Kingdom | Cry1Ab Corn borer protection Glyphosate Herbicide Tolerance | Pending |
| Maize MON 863 and maize hybrid MON 863 x MON 810 | Monsanto | Germany | Cry1Ab Cornborer protection Corn Rootworm ProtectionGlyphosate Herbicide Tolerance | Autorized |
| 1507 Maize | Mycogen SeedsPioneer Hi-Bred | Netherlands | Lepidopteran resistant and glufosinate tolerant | Autorized |
| Roundup Ready oilseed rape, event GT73 | Monsanto | Netherlands | glyphosate tolerant | Autorized |
| Roundup Ready Maize, event NK603 | Monsanto | Spain | glyphosate tolerant | Autorized |

#### 1.5.2.4 Japan

In Japan, the Ministry of Agriculture, Food, and Fisheries (MAFF) and the Ministry of Health, Labor, and Welfare (MHLW) administer the regulation of food safety of GMOs, including GM crops and other foods and food additives that contain organisms or have been obtained through recombinant DNA techniques. The food safety assessment of genetically modified organisms is mandatory (A. Konig *et al.*, 2004).

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#### 1.5.2.5 Australia and New Zealand

In Australia and New Zealand, the Food Standards Australia New Zealand (FSANZ) has regulatory oversight over food safety, including the safety of foods derived from genetically modified organisms (A. Konig *et al.*, 2004).

#### 1.5.2.6 Turkey

Ministry of Agriculture and Rural Affairs- General Directorate of Protection and Control is the competent authority for receiving the applications for import, export of GMOs. The field trials have been carrying on by the Agricultural and Research Institute under the Ministry of Agriculture and Rural Affairs. (EU-TR Screening Report, 04.02.2007).

Specific legislation concerning GMOs, except experimental release, has not been available, yet. However, UN Cartagena Biosafety Protocol has been ratified by Grand National Assembly of Turkey in 2003. The Circular on "Field Trials of Transgenic Culture Crops" determines the procedure and principles of field trials of genetically modified plants intended to agricultural production and it applies to all genetically modified plants whether imported or locally developed. Technical study on biosafety law has been completed (TR-EU Screening Report, 2006).

### 1.6 Tomato

Tomato (*Solanum lycopersicum*) botanically this vegetable is a fruit, is a vine-like herb of the nightshade family (Solanaceae) that also includes potatoes, peppers and eggplants (Jaccaud et al., 2003). With a 24 diploid chromosomes, tomato is a climacteric fruit, showing a sharp increase in ethylene production at the onset of ripening (Xie. Y. *et al*., 2006). The tomato is both an important crop and an invaluable plant model. The availability of vast genetic information and rich plant resources put the tomato in the front of attempts to evaluate it (Levin I. *et al*., 2004).

### 1.6.1 GM Tomatoes

GM tomatoes have been approved for commercialization in many countries (Table 1.4) since the first GM tomato Flavr Savr was permitted for planting in 1994 (Yang L. *et al*., 2005). By using the recombinant DNA technology, genetically modified tomatoes with variable improvements such as delayed ripening, increased β-carotene, salt tolerance, virus resistance and insect resistance have been produced (Bestwick R.K. *et al*., 1994).

Seven GM tomato varieties have been authorized for commercialization in many countries. These are 35 1 N from Agritope Inc (USA), 8338 and 5345 from Monsanto (USA), 1345-4 from DNA Plant Technology Corp (USA), FLAVR SAVR from Calgene Inc. (USA), B, Da, F from Zeneca Seeds, and Huafan No 1 from Huazhong Agriculture University (China) (L. Yang *et al*., 2005).

Among these seven GM varieties, three kinds of novel agronomic traits were introduced. The first is delayed softening which was developed by an additional polygalacturonase (PG) gene expressed in Flavr Savr and B, Da, F. The second is delayed ripening which was developed by introduction of 1-aminocyclopropane- 1-carboxylic acid deaminase (accd) gene in event 8338, or the S-adenosylmethionine hydrolase (Sam-k) gene in event 35 1 N, or anti-sense EFE gene in Huafan No 1, or a truncated aminocyclopropane cyclase synthase (ACC) gene in 1345-4, or antisense PG gene in events ICI9 and ICI13. The third is insect resistance which was developed by introduction of one cry1Ac gene in event 5435 (L. Yang *et al*., 2005).

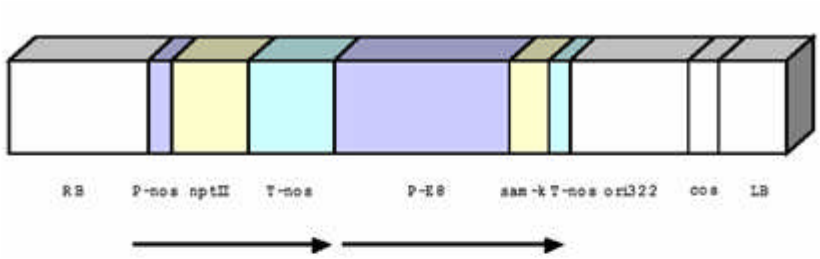
## Table 1.4 Approved GM Tomatoes

| Event Name | Brand Name | Trait | Pro-Moter | Nos | nptII | Event |
|---|---|---|---|---|---|---|
| 1345-4 | Endless Summer | Ripening Delayed | 35S | + | + | ACC synthase gene in sense orientation |
| 35 1 N | | Ripening Delayed | E8 | + | + | Sam-k gene |
| 5345 | | Insect Resistance | 35S | + | + | cry1Ac gene |
| 8338 | | Ripening Delayed | 35S | + | + | Accd gene |
| B, Da, F | Vegadura, Vegaspeso | Ripening Delayed | 35S | + | + | Antisense and sense PG gene |
| Flavr Savr | Flavr Savr, MacGregor's | Delayed Softening | 35S | - | + | Antisense PG gene |
| ICI9, ICI13 | | Increased shelf life, dela yed softening | No map information | | | Antisense PG gene |
| Japan tomato 1 | | Virus Resistance | No map information | | | coat protein Tobacco Mosaic Virus |
| No 4-7 | | Virus Resistance | No map information | | | Satelite RNA |
| 117,106, 1204, 1208 | | Virus Resistance | No map information | | | coat protein Cucumber Mosaic Virus |
| 405,707 | | Virus Resistance | No map information | | | coat protein - Cucumber Mosaic Virus |
| China tomato 1 | | Virus Resistance | No map information | | | coat protein - Cucumber Mosaic Virus |
| China tomato 2 | | Increased shelf life, delayed Softening | No map information | | | Unknown |

#### 1.6.1.1 Ripening Delayed Tomatoes

Calgene Inc. developed one of the earliest approved transgenic crop, which was the Flavr-Savr tomato, a delayed softening variety. Delayed softening tomatoes produced by inserting an additional copy of the polygalacturonase (PG) encoding gene in the anti-sense orientation (A PG) in order to reduce expression of the endogenous PG gene and thus reduce pectin degradation (Bruderer *et al*., 2003).

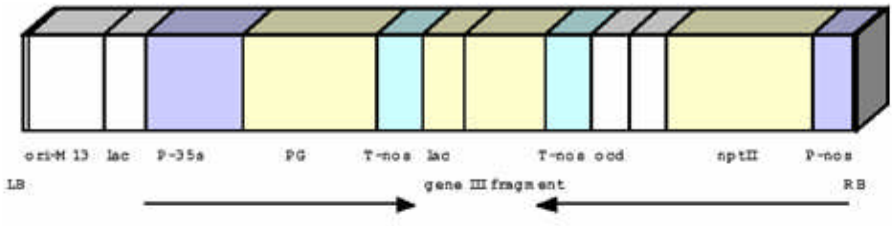
Agritope Inc. developed 35 1 N, another ripening delayed variety (Bruderer *et al*., 2003). Sam-k gene from bacteriophage T3 that encoding the enzyme S adenosylmethionine hydrolase (SAMase) was utilized to generate transgenic tomato plants that produce fruit with a reduced capacity to synthesize ethylene. The phenotype in 35 1 N is characterized by fruit in which ripening on the vine is delayed while ripening off the vine is suspended. However, tomato fruits expressing SAMase ripen normally when exposed to exogenous ethylene (Bestwick *et al*., 1994). The total amount of ethylene produced from these tomatoes was reduced by 80%. The time required for the fruit to develop their final ripened state was approximately two-fold longer, the level of lycopene production was reduced, and the fruit demonstrated increased firmness and a delay in senescence for as long as three months after harvest (Bestwick at al., 1994). The SAM-hydrolase protein is rapidly degraded by heat and gastric conditions and is not toxic or does not cause an allergic reaction (Efendi D., 2001).




Sequence-Details:

| 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 1.2 T-DNA region of construct pAG-5420 RBP used in line 35 1 N**
**Source:** (Bruderer *et al*., 2003)




Sequence-Details:

| Abbreviation | Element-Name | Size [KB] |
|---|---|---|
| LB | Left border | 0.025 |
| ori-M13 | ori-M13 | 0.406 |
| lac | beta-galactosidase | 0.23 |
| P-35s | P-35s | 0.529 |
| PG | polygalacturonase | 0.731 |
| T-nos | T-nos | 0.247 |
| lac | beta-galactosidase | 0.23 |
| gene III fragment | gene III fragment | - |
| T-nos | T-nos | 0.247 |
| ocd fragment | ornithine cyclodeaminase fragment | 0.209 |
| Space | Space | 0.2 |
| nptII | neomycin phosphotransferase | 0.8 |
| P-nos | P-nos | 0.227 |
| RB | Right Border | 0.02 |

**Figure 1.3 T-DNA region in the construct pJR16s used in Line F**
**Source:** (Bruderer et. al, 2003)

#### 1.6.1.2 Stress Tolerance

Many crop plants, including tomatoes, are killed by high salt levels in soil and irrigation water. Zhang and Blumwald, (2001) have developed a tomato plant that is able to tolerate high levels of salt and that holds the salt in its leaves, so the fruit will not taste salty. The GM tomato can grow and produce fruit in irrigation water that is 50 times saltier than normal.

Gupta C. and is friends (2006) introduced a boiling stable protein (bspA) gene isolated from aspen into tomato to screen transgenic plants for enhanced resistance to drought/osmotic stress.

#### 1.6.1.3 Increased Lycopene Content

Tomato is a major food crop and the principal source of the carotenoid lycopene in Western diets (Fraser P.D. *et al*., 2006). Lycopene is a carotenoid that has strong antioxidant properties. Antioxidants prevent oxygen radicals from causing damage in cells. Carotenoids aid in preventing early blindness in children, preventing cancer, enhancing cardiovascular health, and slowing aging. Fraser P. D. and his friends (2002) elevated the level of lycopene content of tomato by 2–3 folds via genetic manipulation of carotenoid biosynthesis using the fruit-specific expression of a bacterial phytoene synthase.

#### 1.6.1.4 Insect Resistant Tomato

Tomatoes are subject to damage from many insects, nematodes and fungal, viral, bacterial pathogens. Tomato engineered with the insecticidal Cry1Ab gene(s) from B. thuringiensis has been reported to provide protection to the plants against tomato fruit worm (Fischhoff *et al*., 1987; Vaeck *et al*., 1987) Insect resistant tomato line 5345 was developed to express the insecticidal protein, Cry1Ac, encoded by the cry1Ac gene from the soil bacterium *Bacillus thuringiensis* subsp. kurstaki strain HD73 (Bruderer & Leitner, 2003).

#### 1.6.1.5 Virus Resistance

The economic losses for tomatoes due to CMV infections in many countries have been so profound that tomato production was abandoned in some areas. While the tomatoes are edible, they are not marketable. There are distortions and reductions in size. Fuchs and his friends (1996) have genetically engineered the resistance by introducing a segment of the viral genome of the cucumber mosaic virus gene into the tomato. The gene resists a plant disease that severely threatens tomatoes.

## 1.7 GMO Detection Methods

Since GMOs are the result of genetic modifications, the most direct detection methods are those that target the genetic modification itself, i.e. the modified DNA (M.Miraglia *et al*., 2004).

The majority of the methods developed for detection of GMO and GMO-derivatives focus on detecting DNA due to following reasons: 1-DNA can be purified and multiplied in billions of copies in just a few hours with PCR technique. Multiplication of RNA and proteins is a more complicated and slow process. 2-DNA is a very stable molecule, while RNA is unstable. The stability of a protein varies and depends on the type of protein. 3-There is normally a linear correlation between the quantity of GMO and DNA if the genetically modified DNA is nuclear, but not if it is extranuclear. However, there is usually no such correlation between the quantity of GMO and protein/RNA. 4-The genetic modification itself is done at the DNA level. At present, the genetically modified DNA is nuclear in all commercialized GMO (Entransfood, 05.02.2007).

### 1.7.1 Protein Based Detection Methods

Protein based detection methods are available for some GMOs, in particular these methods targeting the product resulting from the genetic modification. These methods, however, are unable to detect a genetic modification if the modified gene is inactive in the cells from which an

analytical sample is derived, and they can not be used to distinguish between GMOs modified to produce the same protein, e.g. authorized and unauthorized (M.Miraglia *et al*., 2004). In addition the processing of the material is another significant problem. Antibodies recognize three-dimensional (protein) structures. If a material is heat-treated or hydrolyzed, the structures change often dramatically and are no longer recognized by the antibody, leading to a false-negative result (Pooping B., 2006).

ELISA methods offer a high degree of automation and a high throughput of samples. However, the content of the newly exposed proteins may not be evenly distributed in the whole plant. For instance, in maize the highest values were mostly observed in leaves and not in the grain (Anklam E. *et al*., 2001).

A variation on ELISA, using strips rather than microtiter wells, led to development of lateral flow strip technology (Farid E.A., 2002). They offer a semi-quantitative test of considerable practical value for testing in the field with simple laboratory set-ups (Anklam E. *et al*., 2001). The lateral flow format gives results in 5–10 min, is economical, more amenable to point-of-sale application, and is suitable as an initial screening method early in the food chain. Commercially available lateral flow strips are currently limited to few biotechnology-derived protein-producing GM products, but strips that can simultaneously detect multiple proteins are being developed (Farid E.A., 2002).

Western Blot method is a highly specific method that provides qualitative results suitable for determining whether a sample contains the target

protein below or above a predetermined threshold level, and is particularly useful for the analysis of insoluble protein. This method, however, is considered more suited to research applications than to routine testing (Farid E.A., 2002).

### 1.7.2 RNA Based Detection Methods

These methods rely on specific binding between the RNA molecule and a synthetic RNA or DNA molecule (primer). The primer must be complementary to the nucleotide sequence at the start of the RNA molecule. The specific primers needed for the procedure can not be developed without prior knowledge of the composition of the RNA molecule to be detected. (Entransfood, 05.02.2007).

### 1.7.3 DNA Based GMO Detection Methods

Although several techniques are available, two are commonly used: Southern blot and PCR analyses (Farid E.A, 2002). At present, the most commonly used DNA-based methods involve amplification of a specific DNA with the PCR technique (M.Miraglia *et al*., 2004).

### 1.7.3.1 Southern Blot

The method involves fixing isolated sample DNA onto nitrocellulose or nylon membranes, probing with double-stranded (ds)-labelled nucleic acid probes specific to the GMO, and detecting hybridization radiographically, fluoremetrically or by chemiluminescence. Because only one probe is used in this method, and no amplification is carried out, southern blotting is considered less sensitive than PCR (Farid E.A, 2002).

### 1.7.3.2 Polymerase Chain Reaction

A conventional PCR reaction is typically consisted of separating of the strands of a double-stranded DNA (denaturation), annealing the primer to its target sequence (annealing), and synthesizing the target's complementary strand (extension). The specificity of the amplification is achieved by using primers that match the ends of the target sequence perfectly. (Garcia-Canas V. *et al*., 2004).

Major limitations for PCR-based detection of DNA derived from GMOs are access to information about applicable PCR primers and access to DNA suitable for reliable analysis. Moreover, grinding, heating, acid treatment and other processing rapidly degrades DNA, and refining can lead to efficient removal of DNA. As a consequence, many products contain little GMO-derived DNA, and this DNA is often of low quality. Even with access

to suitable primers, a reliable analytical result may therefore not be achievable due to template DNA restrictions (Holst-Jansen A. *et al*., 2003).

Also, production of PCR products should proceed exponentially. However, in practice it reaches a plateau between 30 and 40 cycles because certain reaction components become limiting (Ahmed F.E., 1995). A number of factors have been presumed to contribute to this plateau: (1) utilization of substrates (dNTPs or primers); (2) thermal inactivation and limiting concentration of DNA polymerase;(3) inhibition of enzyme activity by increasing pyrophosphate concentration; (4) reannealing of specific product at concentrations above 1038 M; (5) reduction in the denaturation efficiency per cycle and (6) destruction of product due to Taq DNA polymerase 5P-3P exonuclease activity (Kainz P., 2000).

***a. False Positives and Carry Over***

Two main sources of false positive results in GMO detection are, unspecific amplification of DNA fragments, and the amplification of PCR products resulting from previous analyses (carry over) (Garcia-Canas V. *et al*., 2004). Another weakness of these screening methods is to differentiate between food products containing GMOs and nonmodified products infected or contaminated with other plants carrying the target sequence naturally (Hübner P., Wurz A. *et al*., 1999). By using confirmation methods in the first case, and by physical and enzymatic contention methods in the second case, these problems can be handled. Despite of these limitations, detection

methods based on sequences frequently found in GM plants are almost the only alternative to detect nondeclared and noncharacterized GMOs (Garcia-Canas V. *et al*., 2004).

***b. Confirmation of Results***

Different methods can be used to confirm the PCR results: (1) specific cleavage of the amplified product by restriction endonuclease digestion ; (2) hybridization with a DNA probe specific for the target sequence; (3) direct sequencing of the PCR product; and (4) nested PCR, in which two sets of primer pairs bind specifically to the amplified target sequence (Farid E.A, 2002).

**1.7. 3. 3 Real-Time PCR**

Many specific PCR-systems for different GMOs have been described, but only a few of them are quantitative. Quantitative PCR-systems are generally based either on competitive coamplification of a known amount of competitor DNA with the target DNA or on real-time PCR (Pauli U. *et al*., 2000).

Real-time PCR is the most commonly used technology for quantification of the GM crop content. The amount of product synthesized during the PCR is measured in real-time by detection of the fluorescence signal produced as a

result of the amplification. It has been shown empirically that the concentration of DNA in real-time PCR reaction is proportional to PCR cycle number during the exponential phase of PCR. Therefore, if the number of cycles it takes for a sample to reach the same point in its exponential growth curve is known, its precise initial DNA (then GMO) content can be determined. Real-time PCR also allows for detection of low copy DNA number. Several real time PCR systems also permit differentiation between specific and nonspecific PCR products (such as primer dimer) by the probe hybridization or by melt curve analysis of PCR products, because non-specific products tend to melt at a much lower temperature than do the longer specific products (De Leo F&F., 2005).

### *a. RT-PCR Hybridization Probes*

Several types of hybridization probes are available that will emit fluorescent light corresponding to the amount of synthesized DNA such as, DNA-binding dye SYBR Green I, hydrolysis probes (TaqMan), hybridization probes or fluorescence resonance energy transfer (FRET) probes and molecular beacons (Ahmed F.E., 2000).

#### *1. SYBR Green I*

The intercalating dye SYBR Green is less toxic and more specific and sensitive than ethidium bromide (from 10 to 25 times) and exhibits fluorescence enhancement upon binding to the ds amplification product (F.

Weighardt, 2007). It offers an inexpensive and sequence unspecific alternative. The fluorescence signal produced directly correlates with the accumulation of PCR product at each cycle, thus providing a quantification of the amount of template DNA in the reaction (Hernandez M. *et al*., 2003). A limitation is represented by its non-specific DNA recognition mode. Nonspecific PCR products and primer dimers are also quantified. Melting curve analysis can be done to overcome this problem (F. Weighardt, 2007).

***2. Hydrolysis Probes (TaqMan Principle)***

The TaqMan chemistry is one of the most commonly used for GMO detection and is based on the simultaneous addition of two primers and a specific probe that yields fluorescence emission upon DNA synthesis (Hernandez M., Pla H. *et al*., 2003). Real time PCR with TaqMan probes has been shown to be extremely accurate and less labor intensive than quantitative competitive PCR giving a high level of precision and a marked improvement of the range of quantification (Terzi V., Ferrari B. *et al*., 2003). Well designed TaqMan probes can be used for multiplex assays; however these are expensive to synthesize (RT-PCR Basics, 22.02.2007).

*3. Uniprimere*

The Amplifluore Universal Amplification and Detection System (Intergen Co., Purchase, NY, USA) is based on a universal hairpin primer (Uniprimere). It anneals to the Z sequence appended to one of the genespecific oligonucleotides and produces fluorescence upon incorporation into the amplification product (Hernandez M. *et al*., 2003).

## 1.8 Types of PCR-based Assays

### 1.8.1 Screening PCR

The promoter and terminator elements used to transform most of the currently approved genetically modified plants are the Cauliflower Mosaic Virus promoter (P-35S) and the *A. tumefaciens* Nos terminator (T-Nos). Although, other promoters and terminators have also been used, almost all GM plants contain at least one copy of the P-35S, T-35S and/or the T-Nos as a part of the gene construct integrated in its genome (Holst-Jensen A., 2001).

The 35S promoter effectively puts its downstream gene outside virtually any regulatory control by the host genome and expresses the gene at approximately two to three orders of magnitude higher, thus allowing a strong positive selection (Hajdukiewicz *et al.* 1994, Ouwerkerk *et al.,* 2001).

The most frequently used terminator in approved GM crops is Nos terminator, isolated from the nopaline synthase gene of *A. tumefaciens*. It is found in 37 products out of 66 surveyed transgenic crops, 62 of them contained at least one genetic sequence that was derived from CMV *A. tumefaciens* or two organisms (Bruderer et. al, 2003).

NptII gene originating from the E.coli transposon Tn5, encodes an aminoglycoside phosphotransferase conferring neomycin and kanamycin resistance and is frequently used as a marker gene in the construction of transgenic plants. In a survey of 30 recombinant food and crop plants targeted for commercialization before the year 2000, 21 contained an NptII gene cloned downstream of a eukaryotic promoter (J. de Vries, W. Wackernagel, 1997).

Targeting the P-35S, T-35S, T-Nos, *bla* or nptII, have wide applications for screening for genetically modified material. However, these screening methods cannot be used to identify the GMO, since the presence of one of the screening targets does not necessarily imply the presence of GMO-derived DNA. The source of P-35S or T-35S could be naturally occurring CaMV, and it is generally believed that Agrobacterium or other soil bacteria containing one or more of the targets are present in soil. It should be noted, though, that the natural prevalence of the targets found in GMOs has not been carefully assessed. An additional source of uncertainty may be presence of cloning vector DNA in the DNA polymerase, for example Ampli-Taq (Applied Biosystems) contains amplifiable bla DNA (Holst-Jensen A. *et al*., 2003).

### 1.8.2 Gene Specific Targets

The gene of interest may also be of a natural origin, but is often slightly modified, for example by truncation or altered codon usage. Furthermore, the choice of available genes is much greater than the choice of available promoters and terminators. Consequently, PCR methods targeting the gene of interest are more specific than screening methods. Normally a positive signal with identification methods implies that GM-derived DNA is present, and in many cases it will even be possible to identify from which GMO the DNA is derived (Holst-Jensen A. *et al*., 2003).

### 1.8.3 Construct-specific Methods

Methods of target junctions between adjacent elements of the gene construct, for example between the promoter and the gene of interest. With these methods a positive signal will only appear in the presence of GM-derived material, and even more often than with identification methods will it be possible to identify the GM source of the DNA. However, the full gene construct may have been transformed into more than one GMO, or may be used in future transformations (Holst-Jensen A. *et al*., 2003).

### 1.8.4 Event-specific Methods

The only unique signature of a transformation event (within the limitation of present day technology) is the junction at the integration locus between the recipient genome and the inserted DNA. This junction is the target of event-specific methods (Holst-Jensen A. *et al*., 2003).

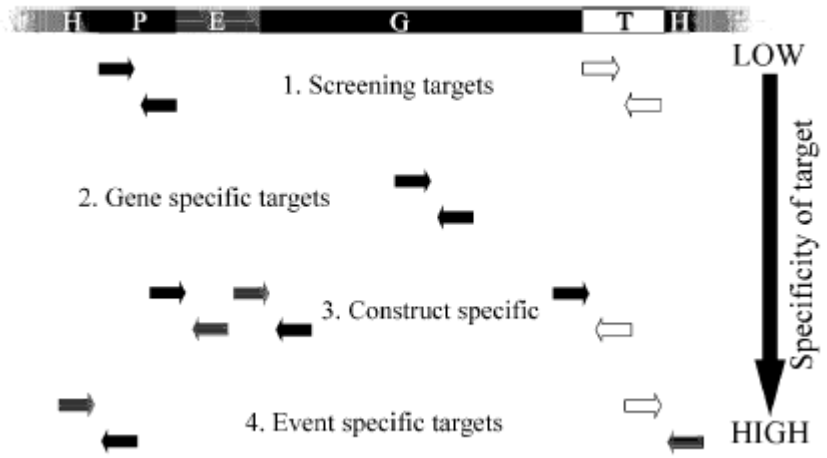



**Figure 1. 4 Summary of four types of PCR-Based assays showing increasing specificity** (from top to bottom). H host genomic DNA, P promoter element, E enhancer element, G gene of interest, T = terminator. The gene construct is composed of P–T and has been inserted into H Source: Holst-Jensen A. et.al., (2003).

## 1.9 Tomatoes Transformed with Sam-k

SAM hydrolase originates from bacteriophage T3 and encodes S-adenosyl-methionine hydrolase (AdoMetase or SAMase, EC 3.3.1.2) (Bestwick *at al.*, 1991).SAMase is normally not present in plant tissues (Patent Storm, 10.03.2007). SAMase gene of Bacteriophage T3 is responsible for overcoming host restriction (Studier F. W, Movva N.R, 1976).

The Sam-k transgene is a version of SAM hydrolase modified in the 5′ region. The only impact of expressing SAM hydrolase in ripening fruit would be reduction of ethylene biosynthesis through the reduction of the SAM pool. As the pool of SAM is depleted by the action of SAM hydrolase, neither ACC nor ethylene is produced (Efendi D., 2003). The ethylene biosynthesis pathway is indicated below:

Methionine → AdoMet (SAM) → ACC → Ethylene

AdoMet (SAM) = S-adenosylmethionine

ACC = 1-aminocyclopropane-1-carboxylic acid

Stable integration of the Sam-k transgene into the tomato genome slows the rate of ripening. As SAMase gene expression is induced by its own promoter E8 at the breaker and orange stage of ripening, the capacity of the tomatoes to produce ethylene is correspondingly reduced (Efendi D., 2003).

SAMase protein is ubiquitous in the digestive tract of human beings due to its association with Coliphage T3 infections of resident E. coli suggesting

that background exposures to this protein are ongoing. Moreover, according to Agritope's statement neither the mode of action of SAMase, nor the by-products of the reaction catalyzed by SAMase, raises any safety concern. (Biotechnology Consultation, 22.02.2007).

### 1.9.1 Other Plants Transformed with Sam-k

#### 1.9.1.1 Avocado

Efendi D. (2003), transformed avocado cultures with Sam-k using an *A. tumefaciens*-mediated method to block ethylene biosynthesis to extend on-tree storage and shelf life of avocado fruit (Efendi D., 2003).

#### 1.9.1.1 Cantaloupe

Cantaloupe lines designated as A and B which was engineered for delayed ripening with Sam-k were also approved in US (Bruderer *et al*., 2003). Agritope used the *A. tumefaciens* T-DNA transformation system to introduce the Sam-k gene with the synthetic combination of the E8 and E4 gene promoters from the tomato and the terminator from the untranslated 3' region of the nopaline synthase gene (Nos) and the kanr to cantaloupe. (Biotechnology Consultation, 22.02.2007).

### 1.10 Tomatoes Transformed with F Type- Truncated PG Gene

PG Gene is derived from a tomato (Solanum lycopersicum Mill. Variety Ailsa Craig) and encodes the enzyme polygalacturonase (PG). Transcription of PG gene fragment results in the inhibition of endogenous PG enzyme. PG enzyme is responsible for the breakdown of pectin molecules in the cell walls of tomato fruit. Pectin is a large polymer consisting of polygalacturonic acid residues. During ripening, the average size of pectin molecules significantly decreases with a coincident increase in soluble polygalacturonic acid molecules. The structure of pectin in tomatoes is a key determinant of tomato fruit texture and of the theological characteristics of processed products. PG catalyses the cleavage of pectin chains by hydrolysis of bonds between adjacent galacturonic acid residues (Bruderer *et al.*, 2003).

Tomato lines Da and F contain the partial PG gene in the sense orientation while line B contains a partal antisense PG gene, essentially a reverse copy (Bruderer *et al.*, 2003). Transgenic line F was developed from the commercial inbred line of processing tomato, TGT7, by the introduction of a truncated PG gene resulting in "downregulation" of the endogenous PG gene. The new hybrids ripen normally but experience less pectin breakdown and, therefore, have increased thickness and consistency that benefits all stages of harvesting and processing. The inbred line F tomato was created by Agrobacterium-mediated transformation in which the transfer-DNA (T-DNA) contained a 3'-truncated open reading frame corresponding to the sequence of the 5'- terminal 731 nucleotides from the

PG gene from the Alisa Craig variety of tomato (Health Canada, 01.03.2007).

For lines Da and F reduced PG expression may be due to coordinate suppression of transcription of both the endogenous gene and the introduced truncated gene. In the case of line B, the mechanism of action is likely linked to the hybridization of antisense and sense messenger-RNA (mRNA) transcripts, resulting in a decreased amount of free positive sense mRNA available for protein translation.(Agbios GM Database, 01.04.2007).

### 1.11 Objectives of This Study

The objective of this study was first to screen 25 different tomato seeds purchased from markets and bazaars for genetic modification by Polymerase Chain Reaction and secondly to identify the screened samples if they are modified with Sam-k and F type truncated PG genes resulting in delayed ripening. Screening was performed by targeting 35S, Nos and nptII regions which are the most common genetic elements used in transformation of plants. In the identification studies of ripening-delayed tomato seeds, Sam-k gene and F-type truncated PG gene were tried to be detected.

Seed DNAs were isolated by CTAB DNA extraction method and extracted DNA samples were amplified with “screening” and “identification” primers. Real time PCR was also performed for comparing the results of Nos region to the conventional PCR result. The sequence analysis and

restriction enzyme digestion were performed for further verification purposes with some of the PCR products.

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Seed Samples

Tomato seeds were randomly purchased from different outdoor markets and supermarkets of Turkey, USA and the Netherlands (Table 2.1). As positive controls, genetically modified tomato seeds which contain 35S promoter, Nos terminator and NptII resistance gene were kindly obtained from Max Planck Research Institute in Germany. *Hygrophyla difformis* containing 35S and NptII regions were kindly supplied from the Ankara University Agricultural Engineering Faculty, Agricultural Plant Protection Department. Besides, plasmids p1320 containing 35S and Nos regions, p2300 containing NptII region designed by the Company Cambia (Canberra, Australia) also obtained from Genetic Laboratory in METU Food Engineering Department. As negative control, tomato seeds obtained from Kapadokya Region were used.

## 2.2 DNA Isolation

### 2.2.1 Surface Sterilization

After seeds were washed for 1 min with 70% ethanol, sterilization method previously designed by N. K. Koç, H. Yetişir *et al.*, (2007) was slightly modified in a way that, seeds were soaked for 15 min in a 5% sodium

hypoclorite solution followed by four washes of sterile distilled water to prevent potential bacterial contamination originating from the soil.

### 2.2.2 DNA Isolation from Seed Samples

DNA extraction from tomato seeds was performed by modified CTAB DNA isolation procedure (Doyle and Doyle, 1990) in such a way that chloroform washing step was done two more times.

100 mg of seed sample was homogenized by liquid Nitrogen in a sterile mortar and homogeneous sample was transferred into a sterile 1.5 mL microcentrifuge tube, 300 µL of sterile deionized water and 500 µL of CTAB-buffer, pH 8.0 (20 g/L CTAB (Applichem), 1.4 M NaCl (Merck), 0.1 M Tris-HCl (Sigma), 20 mM $Na_2$EDTA (Sigma)) were added to microcentrifuge tube and mixed with a loop after each addition. 20 µL Proteinase K (20 mg/mL, MBI Fermentas) was added, mixed and placed at 65°C for over night. After incubation 20 µl RNase A (10 mg/mL, MBI Fermentas) was added, mixed and again kept at 65°C for 5-10 min. After this second incubation, the samples were centrifuged (Hettich Zentrifugen Mikro 12-24) for 10 min at about 16,000xg and the supernatant was transferred to a microcentrifuge tube containing 500 µL chloroform(Applichem), mixed for 30 sec and again centrifuged for 10 min at about 16,000xg until phase separation occurs. 500 µL of upper layer was transferred into a new microcentrifuge tube containing 500 µL chloroform.

## Table 2.1 Seed Samples

| Sample ID | Produced in | Type |
|---|---|---|
| S1 | Konya | Riogrande |
| S2 | Ankara 1 | Riogrande |
| S3 | Balıkesir 1 | Falcon |
| S4 | Adana | SC2121 |
| S5 | Ankara 2 | Unknown (cherry) |
| S6 | Bursa 1 | M-1 F1 |
| S7 | Ankara 3 | Unknown |
| S8 | USA 1 | Beefsteak |
| S9 | USA 2 | Brandwine Red |
| S10 | Ankara 4 | Unknown |
| S11 | Netherland | Unknown |
| S12 | USA 3 | Better Boy |
| S13 | Mersin | Unknown |
| S14 | Ankara 4 | Urbana |
| S15 | Ankara 5 | Unknown |
| S16 | İstanbul 1 | 100 F1 (cherry) |
| S17 | Bursa 2 | H2274 |
| S18 | İstanbul 2 | ACE 55 |
| S19 | Balıkesir 2 | Gülpembe |
| S20 | Kilis | Unknown |
| S21 | Giresun | Unknown |
| S22 | Ankara 6 | Urbana |
| S23 | Ankara 7 | Riogrande |
| S24 | İstanbul 3 | Invictus |
| S25 | Çanakkale | Unknown |

(Applichem) mixed and centrifuged for 5 min at 16,000 xg. This step was repeated for 2 more times. Than, the upper layer was transferred to a microcentrifuge tube and 2 volumes of CTAB precipitation solution pH 8.0 (5 g/L CTAB (Applichem), 0.04 M NaCl (Merck)) was added and mixed by pipetting. After incubation for 60 min at room temperature, samples were centrifuged for 5 min at 16,000 xg, the supernatant was discarded and the precipitate was dissolved in 350 μL NaCl (1.2 M, (Merck)). 350 μL chloroform (Applichem) was added and mixed for 30 s, centrifuged for 10 min at 16,000 xg until phase separation occurs, the upper layer was transferred to a new microcentrifuge tube, 0.6 volumes of isopropanol was added and mixed. After a centrifugation for 10 min at 16,000 xg, the supernatant was discarded. 500 μL of 70% ethanol solution was added and mixed carefully, centrifuged for 10 min at 16,000 xg. The supernatant was again discarded, the pellet was dried and DNA was re-dissolved in 40 μL sterile deionized water.

### 2.2.3 Concentration Determination

The absorbance values of DNA samples were measured at 260 nm and 280 nm in Nanodrop ND-1000 spectrophotometer in Refgen (METU Technopark, Ankara).

## 2.3 Oligonucleotide Primers

PatGc / PatGd primer set which has been previously designed by Jaccaud *et al.* (2003) and producing a fragment 124 bp in length, was used to detect Patatin gene.

Two different primer sets producing 411 bp and 459 bp amplicon were used for NptII detection. The NptII-1/NptII-2 primer set producing 411 bp amplicon was used in a previous study of CORESTA for tobacco, and the Kan F/Kan R primer set giving a 459 bp amplicon was designed by Sönmezalp Z. (2004).

Validated primer sets were used for the detection of 35S promoter and NOS terminator. P35S-afuI/P35S-ar1 primer set produces an amplicon of 207 bp in length and Nos1/Nos3 primers gives a 180 bp long fragment (Bonfini L., 2007).

The control and screening reaction was performed with the primer pair PG34L/PG34 R, which amplifies part of the F type PG-gene (not transgenic: 383 base pairs; transgenic: 383 + 180 base pairs) The primer pair PG34L/t-NOS is specific of the genetic modification giving a 350 bp amplicon (Bonfini L., 2007).

Three different primer sets were used to detect Sam-k gene which were Sammp F/Sammp R, Sam5'Rev/ Sam3'Rev, SAMf3/ SAMf5. The Sammp F/Sammp R primer set was previously designed by Efendi D., (2003). Other primers were used by the advice of Dr. Efendi D. (Bogor Agricultural University, Bogor, Indenosia) and checked with the sequence in NCBI

GenBank accession no. AJ318471.1. The primers were expected to produce an amplicon of 395 bp, 310 bp and 217 bp in length respectively.

All primers used in this study were presented in Table 2.2. Primers were synthesized by GENSUTEK (Ankara).

**2.4 Conventional PCR Amplification**

Amplification reactions were performed with 20 ng sample DNA in 30 μL volume using Biorad MJ-Mini and Techne-Progene thermocyclers. Final concentrations of the reaction components for the detection of Patatin gene, 35S promoter, Nos terminator, and NptII genes were as follows: 1X PCR Buffer (Fermentas), $MgCl_2$ (1.5 mM for Patatin, 35S, and 3,6 mM for NptII, Nos, Sam-k and PG) (Fermentas), 0.2 mM dNTP (Fermentas), forward and reverse primers (20 pmol for Patatin and PG, 30 pmol for 35S, and Nos, 60 pmol for NptII, Sam-k,) and 1 unit Taq DNA polymerase (Fermentas) for 35S, Sam-k and Nos and NptII and 2 unit for F type truncated PG gene. The rest of the reaction-mix was completed with PCR Grade Water (Dr. Zeydanlı A.Ş., METU Technopark, Ankara). The PCR conditions of each primer set were presented in Table 2.3, 2.4, 2.5, 2.6, and 2.7.

**Table 2.2 Primer Sets Used in This Study**

| | | | | | |
|---|---|---|---|---|---|
| **Control** | **Patatin** | PatGc-F CTCATTTAggCACTggCACT<br>PatGd-R gTAAgAACTTgCTgCACTAgTC | 124 | X03932 | Jaccaud *et al.,* (2003) |
| **Screening** | **35S** | 35S-1-f gCT CCT ACA AAT gCC ATC A<br>35S-2-r gAT AgT ggg ATT gTg CgT Cg | 479 + 195 | AF078810 | Bonfini L., (2007) |
| | | P35S-af1u CCTACAAATgCCATCATTgCg<br>P35S-ar1 gggTCTTGCgAAggATAgTg | 207 | AF078810 | Bonfini L., (2007) |
| | | 35S-cf3/35S-cr4R<br>TCCTCTCCAAATGAAATGAACTTCC<br>35S-cf3/35S-cr4F CCACGTCTTCAAAGCAAGTGG | 118 | AF078810 | Bonfini L., (2007) |
| | **Nos** | NOS1-F gAATCC TGT TgC Cgg TCT Tg<br>NOS 3-R TT TCCTAg TTT gCg CgC TA | 180 | U12540 | Bonfini L., (2007) |
| | **NptII** | NPTII-F1gCCCTgAATgAACTgCAggACgAg gC<br>NPTII -R2 gCA ggCATCgCCATg ggTCAC gAC gA | 411 | DQ449900 | CORESTA Task Force GM TobaccoDetection Methods |
| | | Kan-F TTgCTCCTgCCgAgAAAg<br>Kan-RgAAggCgATAgAAggCgA | 459 | AF274974 | Sönmezalp Z., (2004) |
| **Identification** | **Sam-k** | sammp3 CgC TTCCg TTC TAA CCT CT<br>sammp5 gggACCgA ACT CAT CAA TA | 395 | X04791 | Efendi D., (2001) |
| | | SAM5'R<br>gTTTCCgCTTTCCgTTCTAACCTCTgC<br>SAM3'R<br>CCCgTCgATACCTTTAgCgTACACCA | 310 | X04791 | Personal communication with Efendi D., (23.11.2006) |
| | | Sam5 f ggCTCCgTTgAgTCAACCgA<br>Sam3 F ACCCGTCGATACCTTTAGCG | 217 | X04791 | Personal communication with Efendi D., (23.11.2006) |
| | **PG** | PG34L ggATCCTTAgAAgCATCTAgT<br>PG34R CgTTggTgCATCCCTgCATgg | 180 (380) | X05656 | Bonfini L., (2007) |
| | | PG34L ggATCCTTAgAAgCATCTAgT<br>T-NOS CATCgCAAgACCggCAAC Ag | 350 bp | X05656 | Bonfini L., (2007) |

**Table 2.3 PCR Conditions for Patatin**

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

**Table 2.4 PCR Conditions for P-35S and T-Nos**

| | Temperature | Time |
|---|---|---|
| Initial Denaturation | 95°C | 3 min |
| Denaturation | 95°C | 36 sec |
| Annealing | 54°C | 72 sec |
| Extension | 72°C | 84 sec |
| Number of cycles | 40 | |
| Final Extension | 72°C | 3 min |
| | 4°C | - |

**Table 2.5 PCR Conditions for NptII**

| | Temperature | Time |
|---|---|---|
| Initial Denaturation | 95°C | 5 min |
| Denaturation | 95°C | 30 sec |
| Annealing | 60°C | 40 sec |
| Extension | 72°C | 40 sec |
| Number of cycles | 40 | |
| Final Extension | 72°C | 7 min |
| | 4°C | - |

**Table 2.6 PCR Conditions for PG**

(Same conditions for both control and identification PCR)

| | Temperature | Time |
|---|---|---|
| Initial Denaturation | 94°C | 10 min |
| Denaturation | 94°C | 30 sec |
| Annealing | 60°C | 1 min |
| Extension | 72°C | 1 min |
| Number of cycles | 40 | |
| Final Extension | 72°C | 6 min |
| | 4°C | - |

**Table 2.7 PCR Conditions for Sam-k**

| | Temperature | Time |
|---|---|---|
| Initial Denaturation | 94°C | 3 min |
| Denaturation | 94°C | 30 sec |
| Annealing | 60°C | 30 sec |
| Extension | 72°C | 1 min |
| Number of cycles | 35 | |
| Final Extension | 72°C | 5 min |
| | 4°C | - |

## 2.5 Agarose Gel Electrophoresis

PCR products were analyzed for *NptII*, PG and Sam-k gene on 1.5% and for Patatin, 35S promoter, Nos terminator on 2.0% agarose (Prona Basica Le) gels prepared and electrophorased in 1X TAE Buffer ( Fermentas). 100 bp DNA Ladders (Fermentas) was used as DNA marker. All gels were electrophoresed at 80V (BioRad) for 1 hour and stained with ethidium bromide for 20 min. PCR products were visualized under UV light on BioRad UV Transilluminator.

## 2.6 Real-Time PCR Amplification

Real-time PCR assays were carried out on Applied Biosystems ABI 7500 Real Time PCR System in a final volume of 25 μL. Fluorescence was monitored at the end of the elongation phase. The real-time PCR reactions contained the following components: 17 μL 35S and Nos Sure Food (Congen Biotech., Germany) reaction mix or inhibition control mix and 1 μL FDE, 0.1 Ml Taq Polymerase. The reporter dye was FAM, and quencher dye was TAMRA.

The Real-Time Kit was purchased from GenBio (Ankara). Real-time PCR reactions were carried according to the the program in Table 2.8.

## 2.7 Confirmation of PCR Results

35S Primer specific PCR products were digested with PdmI (Xmn1) (Fermentas) restriction enzyme. Other targeted genes were sequenced in İontek (İstanbul). The sequence results were reversed to their complementary strands by ChromasPro Version 1.34 Software programme. Then, the sequences were searched in the NCBI Genebank Database using blast algorithm for exact and nearly matches of short strands. RT PCR was also conducted with the Nos specific PCR products from the conventional PCR for further confirmation.

**Table 2.8 Real-Time PCR conditions for T-Nos**

| | Temperature | Time |
|---|---|---|
| Initial Denaturation | 95°C | 5 min |
| Denaturation | 95°C | 10 sec |
| Annealing | 60°C | 15 sec |
| Extension | 65°C | 30 sec |
| Number of cycles | 45 | |

## 2.8 Plasmid Isolation

### 2.8.1 Transformation of Competent Cells

0.5 µL of isolated plasmid DNA (obtained from Food Engineering Genetic Laboratory) was mixed with 50 µL TE Buffer (pH: 8.0). 300 µL competent cells (obtained from Food Engineering Genetic Laboratory) were mixed with diluted plasmid and kept on ice for 30 min.

The mixture was transferred into a 42 $^0$C water bath for 90 second followed by 2 min on ice. Then, 1 ml LB medium preheated before to 42 $^0$C was added and the mixture was incubated in water bath at 37 $^0$C for 1 hour. 150 µL aliquots were spread on LB agar plates containing 50 µg/µL kanamycin and incubated overnight at 37 $^0$C.

### 2.8.2 Isolation of Plasmid DNA

5 mL LB Broth each of which contained 2,5 mL including kanamycin at a concentration of 50 μg/mL was prepared in 15 mL tubes. *E.coli* XL1 Blue MRF' cells containing plasmid were cultivated in these LB Broth overnight at 37 $^0$C. The cells were centrifuged at 6000 rpm for 8 min. The supernatant was discarded. The collected cells were resuspended in 200 μL of solution 1 (Appendix B) room temperature for 15 min. Then, 200 μL of solution 2 (Appendix B) was added and mixed gently for 7-8 times, incubated 5 min on ice. After that, solution 3 (Appendix B) was added gently mixed for 7-8 times and inoculated on ice for 15 min. The tubes were centrifugated at 13 000 rpm at 4 $^0$C for 10 min. The supernatant was transferred to sterile eppendorf. 2 volume of cold absolute ethanol was added to each tube and incubated at –20$^0$C for 1 hour. The tubes were centrifuged at 13000 rpm at 4 $^0$C for 10 min and supernatant was discharged. The collected cells were resuspended in 200 μL NE buffer (Appendix B) on ice for 1 hour. After the tubes were centrifuged at 4 $^0$C for 15 min the supernatant was transferred to another eppendorf tube. 400 μL absolute cold ethanol was added and incubated at –20 $^0$C for 30 min. Supernatant was discarded after centrifugation at 4 $^0$C for 10 min. The collected DNA was air dried at room temperature overnight, and redissolved in 15 μL of sterile double distilled water. Both plasmid DNAs were used in the optimization of PCR conditions since the positive control tomato DNA was limited in quantity.

# CHAPTER III

# RESULTS AND DISCUSSION

## 3.1 DNA Isolation

In the beginning of the study Sure Food DNA Isolation Kits (Congen Biotech.) and CTAB DNA extraction method were compared to determine the better procedure for DNA isolation. The spectrometer results showed that the absorbance values were not high enough to use kit-isolated DNA in PCR. Moreover the concentrations of DNAs were lower when isolation kit was used. So, in the rest of the study extraction was carried out using CTAB method. The CTAB and kit isolated DNAs were given in Figure 3.1 and Figure 3.2 for making a visual comparison between the concentrations of DNAs. Prior to starting extraction, the seeds were surface sterilized in order to inhibit possible contamination from soil.

Since absorbance values showed that DNAs were highly contaminated by proteins when chloroform washing was performed for two times, CTAB method was enriched by adding two more chloroform washing steps. DNA concentrations and obtained A260/ A280 values were summarized in Table 3.1. The reason for differences in concentration values was probably due to handling during sample homogenization with liquid Nitrogen.

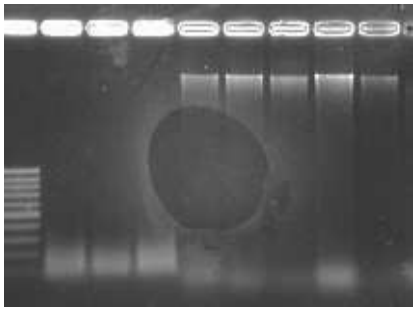


**Figure 3.1 DNAs isolated with Sure Food Kit**

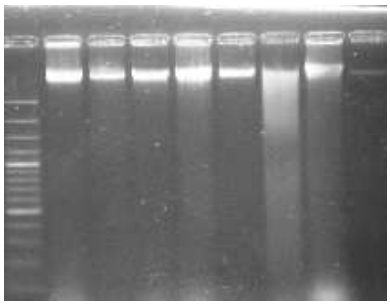


**Figure 3. 2 DNAs isolated with CTAB Method**

### 3.2 PCR Amplifications

Six different conventional PCR systems were performed for each sample with several primer sets. The first PCR was for evaluating the amplification capacity of the seed DNAs. The three PCR systems were developed for the detection of screening targets commonly used in genetically modified crops. The other two PCR systems were performed to identify the delayed ripening

genetically modified tomatoes by targeting F type truncated PG gene, and Sam-k gene. RT PCR systems were also carried out for the verification of Nos positive results from conventional PCR. Diluted plasmid DNAs were used throughout the optimization of PCR conditions.

### 3.2.1 Patatin Specific Control PCR

The PCR using the primers Pat-F/Pat-R determines if amplifiable tomato DNA is present in the sample. All DNAs extracted from collected tomato seed samples and from positive/negative tomato seeds were tested with this PCR system. All seed samples showed 124 bp amplicon, indicating their amplification capacity for further detection analysis. All amplified DNA samples were shown in Figure 3.3.

## Table 3.1 Concentration of Samples

| Sample ID | Produced in | Conc ng/μL | A260 | A280 | 260/280 |
|---|---|---|---|---|---|
| S1 | Konya | 235.86 | 4.717 | 2.857 | 1.65 |
| S2 | Ankara 1 | 800.02 | 16 | 9.301 | 1.72 |
| S3 | Balıkesir 1 | 392.07 | 7.841 | 4.616 | 1.70 |
| S4 | Adana | 437.70 | 8.754 | 4.977 | 1.76 |
| S5 | Ankara 2 | 497.45 | 9.949 | 7.106 | 1.40 |
| S6 | Bursa 1 | 632.43 | 12.649 | 6.824 | 1.85 |
| S7 | Ankara 3 | 392.07 | 7.841 | 4.616 | 1.70 |
| S8 | USA 1 | 173.89 | 3.478 | 1.868 | 1.86 |
| S9 | USA 2 | 361.63 | 7.233 | 4.247 | 1.70 |
| S10 | Ankara 4 | 173.05 | 3.461 | 1.890 | 1.83 |
| S11 | Netherland | 187.56 | 3.751 | 2.516 | 1.50 |
| S12 | USA 3 | 166.43 | 3.329 | 1.758 | 1.89 |
| S13 | Mersin | 134.39 | 2.688 | 1.717 | 1.57 |
| S14 | Ankara 4 | 173.89 | 3.478 | 1.868 | 1.86 |
| S15 | Ankara 5 | 482.36 | 9.647 | 5.592 | 1.73 |
| S16 | İstanbul 1 | 238.67 | 4.773 | 2.445 | 1.95 |
| S17 | Bursa 2 | 587.17 | 11.744 | 6.09 | 1.93 |
| S18 | İstanbul 2 | 484.54 | 9.691 | 4.97 | 1.95 |
| S19 | Balıkesir 2 | 482.36 | 9.647 | 5.592 | 1.73 |
| S20 | Kilis | 193.36 | 3.867 | 2.215 | 1.75 |
| S21 | Giresun | 103.3 | 2.066 | 1.347 | 1.53 |
| S22 | Ankara 6 | 632.43 | 12.649 | 6.824 | 1.85 |
| S23 | Ankara 7 | 630.44 | 12.609 | 6.399 | 1.97 |
| S24 | İstanbul 3 | 126.12 | 2.522 | 1.556 | 1.62 |
| S25 | Çanakkale | 121.37 | 2.427 | 1.8122 | 1.34 |

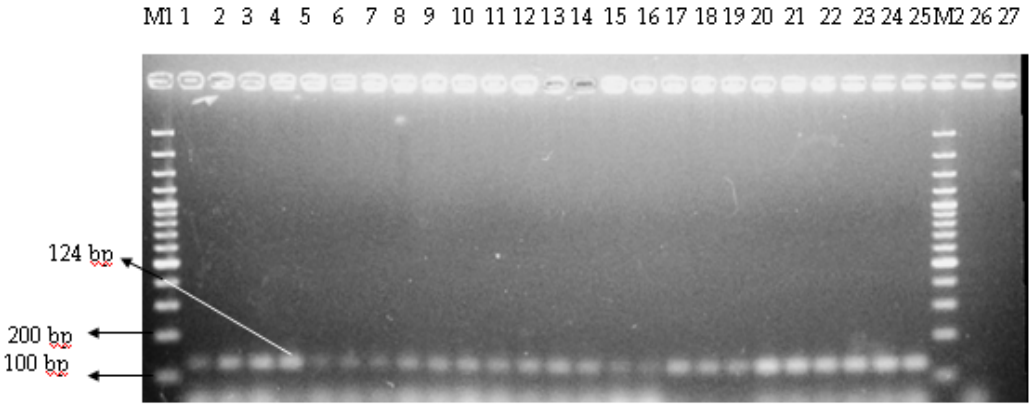



**Figure 3.3 Patatin specific PCR results**: **Line M1 and M2:** 100 bp DNA ladder, **Line 1-25:** Samples, **Line 26:** Maize DNA (does not contain patatin gene), **Line 27:** no-template PCR.

### 3.2.2 Screening PCR Systems

The antibiotic resistance genes such as *NptII, bla* and regulatory elements such as the Nos terminator, the 35S promoter and the 35S terminator have been used for general screening of genetically modified crops. However, the presence of one of the screening targets does not necessarily imply the presence of GM-derived DNA. The source of P-35S or T-35S could be naturally occurring CaMV, and it is generally believed that *Agrobacterium* or other soil bacteria containing one or more of the targets are present in soil (Holst-Jensen A. *et al.*, 2003). So, it should be noted that the detection of one of the targets is not enough to identify the plant as GMO. The seeds were surface-sterilized (N. K. Koç, H. Yetişir *et al.*, 2007) to prevent potential bacterial contamination originating from the soil before the DNA extraction.

Moreover, recently, scientists have begun to improve crops by using organism specific promoter and terminator regions, such as tomato-fruit specific E8 promoter in tomato. Therefore, it should also be notified that the absence of these targets does not necessarily imply that the DNA is not GM-derived.

#### 3.2.2.1 35S Promoter Spesific PCR System

Many of the approved transgenic crops contain a copy of the constitutive 35S promoter (P-35S) from the CaMV or one of the derivatives of this promoter like the enhanced and duplicated 35S promoter regions. The P-35S has been widely used in the screening detection methods. At total 56% of genetically modified crops contains CaMV 35S promoter which provides constitutive expression of inserted gene of interest (Bruderer *et al.,* 2003). A comparison of P-35S sequences available from public sources shows that they are not identical and there are different sequence mutants of P-35S fragments in different GM crops (Bruderer *et al.,* 2003).

Among 25 tomato seed samples 9 of them produced 207 bp were fragment with P35S-afuI/P35S-ar1 primer set *via* conventional PCR (Figure 3.4). NEB Cutter 2.0 Software Programme was used to find the appropriate restriction enzyme for the reference 35S gene with NCBI accession no. V00141. PdmI (Xmn1) restriction enzyme, giving 112 and 93 bp bands was selected to digest the PCR product for confirmation (Xmn1 cut site is indicated in Appendix

D). The 112 and 93 bp long fragments after digestion were shown in Figure 3.5, confirming that 207 bp fragment is 35S promoter gene region.

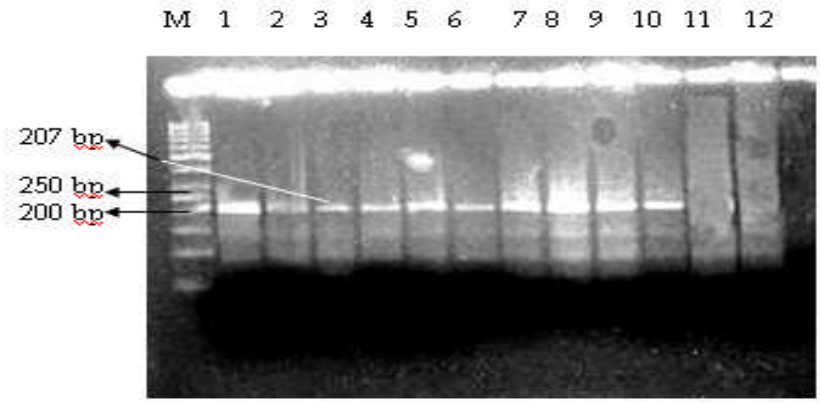



**Figure 3.4 35S Promoter specific PCR results: Line M:** 50 bp marker, **Line 1:** + Control (tomato DNA), **Line 2:** S24, **Line 3:** S17, **Line 4:** S18, **Line 5:** S19, **Line 6:** S20, **Line 7:** S22, **Line 8:** S4, **Line 9:** S21, **Line 10:** S25, **Line 11:** - Control (tomato DNA), **Line 12, 13:** No-template PCR.

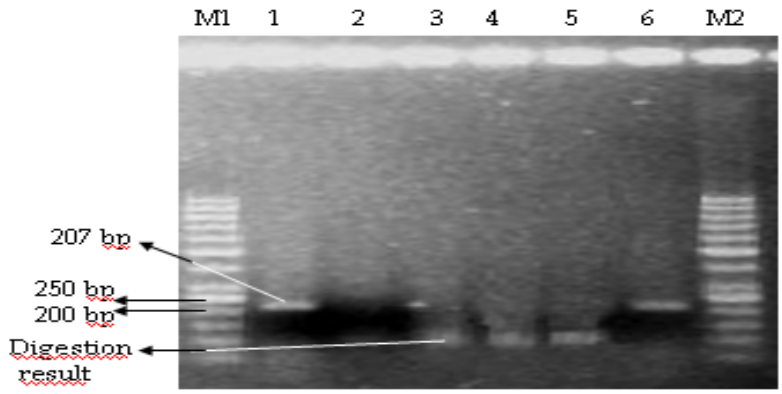



**Figure 3.5 Digestion with Restriction Enzyme (Xmn1): Line M1, M2:** 50 bp marker, **Line 1, 6:** 35S specific PCR product, **Line 2-5: Digested** 35S specific PCR product**.**

### 3.2.2.2 Nos Terminator Specific PCR System

The Nos terminator isolated from the Nopaline synthase gene of *A. tumefaciens* is commonly used in transgenic crops for the termination of transcript of trait genes. Among all approved genetically modified crops 37% of them contain *A. tumefaciens* Nos terminator region (Bruderer *et al.,* 2003).

The validated primer set Nos-1/Nos-3 allows the amplification of a 180 bp fragment. In this study, 8 of 25 tomato seed samples produced amplicons with Nos-1/Nos-3 *via* conventional PCR (Figure 3.6). The sequenced Nos specific PCR product were searched for alignment to the *A. tumefaciens* Nos terminator gene region using blast algorithm in NCBI Database. Nos PCR product showed a close homology to Nos gene regions of several vectors. The one with the closest homology was shown in Table 3.2.

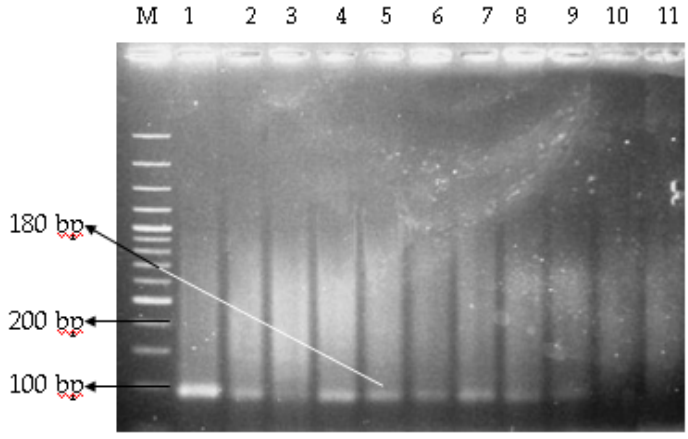



**Figure 3.6 Nos Terminator specific PCR results:** Line M: 100 bp marker, Line 1: + Control (tomato DNA), Line 2: S3, Line 3: S4, Line 4: S17, Line 5: S18, Line 6: S19, Line 7: S20, Line 8: S22, Line 9: S24, Line 10: - Control, Line 11: No template PCR.

**Table 3.2 Sequences Producing Significant Alignments with Nos Specific PCR Product**

| Accession | Description | Max score | Total score | Quality coverage | E value | Max. ident |
|---|---|---|---|---|---|---|
| EF546437.1 | Binary vector pGFPGUSplus, complete sequence | 153 | 306 | 71% | 2e-34 | 94% |

**Alignment of Nos Specific PCR Product with Nos region of Binary vector pGFPGUSplus**

gb|EF546437.1| Binary vector pGFPGUSplus, complete sequence
Length=13701

Score = 153 bits (77), Expect = 2e-34
Identities = 95/101 (94%), Gaps = 0/101 (0%)
Strand=Plus/Plus

```
Nos_samp  41 AATGCATGACGTTCTTTATCCGATGGGCTTTTATGATTAGAGTCCCGCAATTATACCTTT 100
             ||||||||||||| |||||  |||||| |||||||||||||||||||||||||||| |||
Nos     2195 AATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTT 2254

Nos_samp 101 AATACGCGATAGAAAACCAAATATAGCGCGCAAACTAGGAT  141
             ||||||||||||||||| ||||||||||||||||||||||
Nos     2255 AATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGAT  2295
```

### 3.2.2.3 NptII Gene Specific PCR System

NptII gene is used as a marker gene in the construction of transgenic plants, the expression of this gene confers resistance to the antibiotic kanamycin. The purpose of inserting the NptII gene into crops with any other transgene is to use it as a marker gene. NptII originates from the E.coli transposon 5. Nearly all approved genetically modified tomato lines including 1345-4, 35 1 N, 5345, 8338, B-Da-F and Flavr Savr contains NptII kanamycin resistance gene (Bruderer *et al*., 2003).

Although the three regions; 35S promoter, Nos terminator, and NptII are the most widely used screening targets, kanamycin resistance gene provides the most general detection for tomato among those regions. It gives the opportunity to screen different GM tomatoes with different modified characteristics like delayed ripening and insect resistance. Therefore, the tomato samples were tested for the presence of NptII gene by means of the primer pair Kan-F/Kan-R which generates an amplicon of 411 bp in length. 5 samples out of 25 produced the expected 411 bp amplification signal (Figure 3.7).

The sequenced NptII PCR product was searched in NCBI GeneBank Database using blast algorithm and homology with Tn5 tronsposon was observed (Table 3.3). The mismatches were probably due to the poor quality of the PCR product.

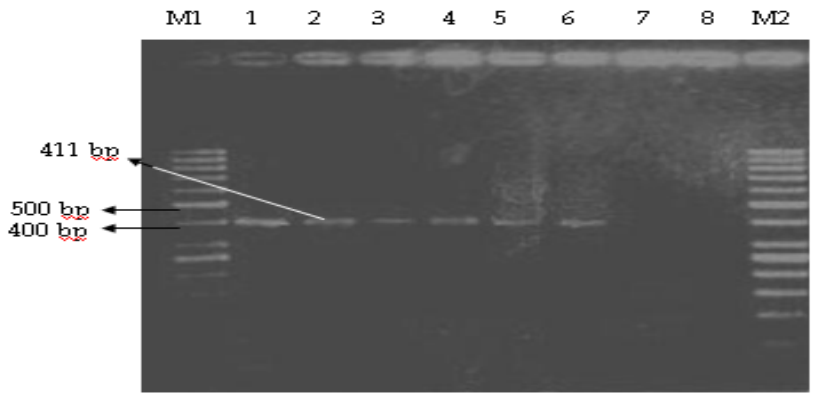



**Figure 3. 7 NptII specific PCR results**: Line M1 and M2: 50 bp DNA ladder, Line 1: + Control, Line 2: S3, Line 3: S17, Line 4: S18, Line 5: S19, Line 6: S20, Line 7: - Control, Line 8: No-template PCR.

**Table 3.3 Sequences Producing Significant Alignments with NptII Specific PCR Product**

| Accession | Description | Max score | Total score | Query coverage | E value | Max. ident |
|---|---|---|---|---|---|---|
| DQ449900.1 | Uncultured bacterium clone K003 transposon Tn5 neomycin phosphotransferase (nptII) gene, partial cds | 48.1 | 48.1 | 33% | 0.009 | 86% |

**Alignment of NptII Specific PCR Product with Uncultured bacterium clone K003 transposon Tn5 neomycin phosphotransferase (nptII) gene, partial cds**

>DQ449900..1| Uncultured bacterium clone K003 transposon Tn5 neomycin phosphotransferase (nptII) gene, partial cds
Length=728

Score = 48 bits (24), Expect = 0.018
Identities = 45/52 (86%), Gaps = 0/52 (0%)
Strand=Plus/Minus

```
Kan_sample   178  GGTTGAAAGGGGCGGGTAACCGGTTCAAGGGTTTGCAGCCGCAATCCATC 229
                  ||||||| ||||| |||| |||| ||||| |||||||||||| ||||||
tn5-neom     397  GGTTGAATGGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCATTCCATC 346
```

### 3.2.3 Identification PCR Systems for Detecting Genetically Modified Ripening Delayed Tomatoes

#### 3.2.3.1 F-Type Truncated PG Gene

The tomato lines Da and F from Zeneca, have been genetically engineered for suppressed polygalacturonase enzyme activity. Da and F contain the partial PG gene in the sense orientation. (Bruderer *et al.*, 2003). Reduced PG

expression may be due to coordinate suppression of transcription of both the endogenous gene and the introduced truncated gene (GM Database, 21.03.2007).

The method for detecting F-type truncated PG gene consists of two parts. The first step serves as a control reaction of isolated DNA and enables the screening of genetic modification of the tomato. The control and screening reaction is performed with the primer pair PG34L/PG34 R, which amplifies part of the PG gene (not transgenic: 383 base pairs; transgenic: 383 + 180 base pairs). In the second part of the investigation another PCR is carried out which covers adjacent sequences between the cDNA of the PG gene and Nos terminator. The primer pair PG34L/t-NOS (350 base pairs) is specific of the genetic modification (Verlag B., 1999).

In order to identify delayed ripening GM tomato, it was aimed to detect F-type truncated PG gene first. For this purpose, 2 different above mentioned primer pairs were designed. All of the previously screened samples resulted single band with the first primer set; emphasizing that the transformed line was not F-type truncated PG gene (Figure 3.8).

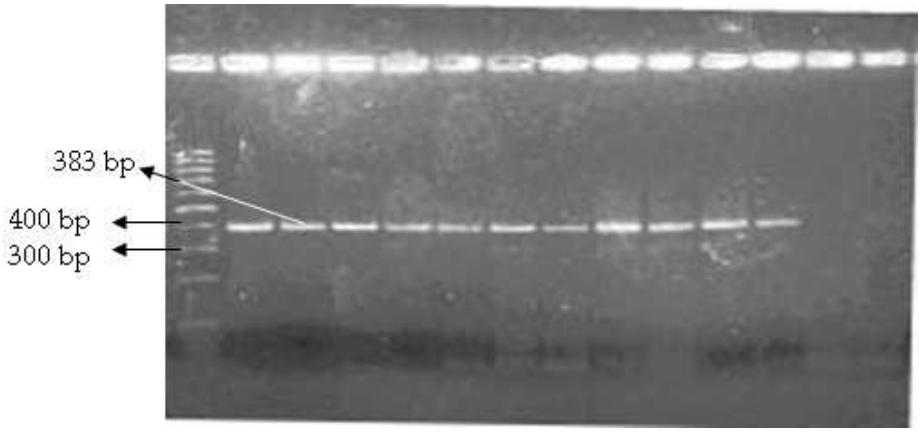



**Figure 3. 8 F Type Truncated PG Gene specific PCR results: Line M:** 100 bp DNA Lader, **Line 1:** S3, **Line 2:** S4, **Line 3:** S17**, Line 4:** S18**, Line 5:** S19, **Line 6:** S20, **Line 7:** S22, **Line 8:** S24, **Line 9:** S13, **Line 10:** - Control, **Line 11:** S14**, Line 12:** *Hygrophyla differmis* DNA, **Line 13:** no-template PCR.

If two bands would had been observed, then the amplification could had been carried out with the second primer set, PG34L/t-NOS, which is specific of the genetic modification and gives a band of 350 bp.

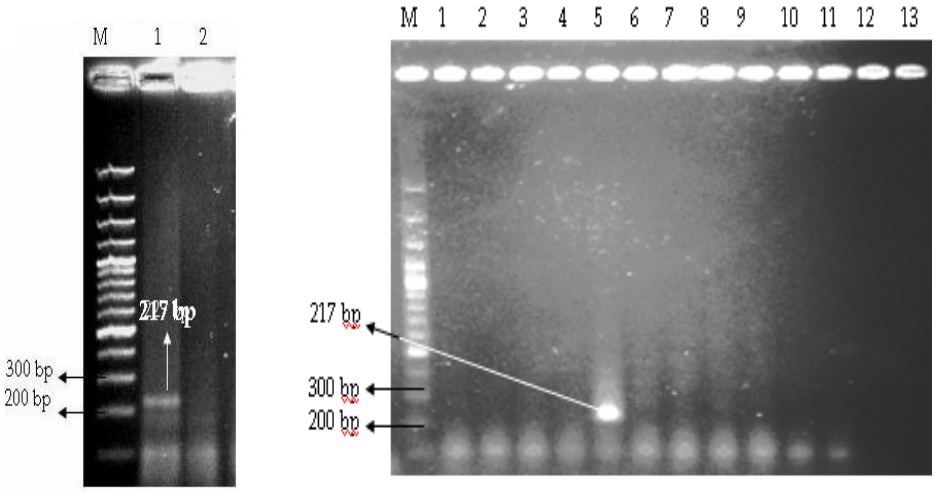
### 3.2.3.2 Sam-k Gene

Tomato 35 1 N from Agritope has been genetically engineered to delay fruit ripening in such a way that the Sam-k gene encoding the enzyme S-adenosylmethionine hydrolase has been introduced in the tomato genome. The enzyme alters the ethylene biosynthestic pathway and delays ripening of the tomato on the vine. Tomato 35 1 N ripens normally when exposed to exogenous ethylene.

In the second part of the identification study, event 35 1 N was selected for futher identification of delayed ripening GM tomato seeds. For this purpose,

three different primer sets were used to detect Sam-k gene which are Sammp F/Sammp R, Sam5'Rev/ Sam3'Rev, SAMf3/SAMf5.

The primer pair Sam5 f/Sam3 f gave a 217 bp amplicon only in sample 3 out of 25 samples (Figure 3.9). In order to confirm the product of Sam5 f/Sam3 f primer set, 30mL of the PCR product with 5 mmol of each primer was sent for automatic sequencing to Iontek (Istanbul/Turkey). The sequenced PCR product were searched in NCBI GeneBank Database using blast algorithm for short and nearly exact matches and high homology with Coliphage T3 S-adenosyl-L-methionine hydrolase (Table 3.4) was obtained.




**A** **B**

**Figure 3.9 Sam-k specific PCR results:** A; Line M: 100 bp DNA ladder, Line 1: S3, Line 2: No-template. B; Line M: 100 bp DNA ladder, Line 1: S4, Line 2: S17, Line 3: S18, Line 4: S20, Line 5: S3, Line 6: S19, Line 7: S22, Line 8: S24, Line 9: No-template PCR.

**Table 3. 4 Sequences Producing Significant Alignments with Sam-k Specific PCR Product**

| Accession | Description | Max Score | Total Score | Query Coverage | E value | Max ident. |
|---|---|---|---|---|---|---|
| AJ251805.1 | Bacteriophage phiYeO3-12 complete genome | 246 | 246 | 100% | 2e-62 | 95% |
| AJ318471.1 | Bacteriophage T3 complete genome, strain Luria | 222 | 222 | 100% | 3e-55 | 93% |
| **X04791.1** | **Coliphage T3 S-adenosyl-L-methionine hydrolase (AdoMetase, E.C. 3.3.1.2)** | **222** | **222** | **100%** | **3e-55** | **93%** |

**Alignment of Sam Specific PCR Product with Coliphage T3 S-adenosyl-L-methionine hydrolase (AdoMetase, E.C. 3.3.1.2)**

>emb|X04791.1|PODOT3AD Coliphage T3 S-adenosyl-L-methionine hydrolase (AdoMetase, E.C. 3.3.1.2)
Length=628
Score = 222 bits (112), Expect = 3e-55
Identities = 146/156 (93%), Gaps = 1/156 (0%)
Strand=Plus/Minus

```
Sam_samp  1   CGCAAGCTTGCTCCC-CTCATTAAAAGCTAAGCGTGCAACATTTAGTGCCTGCGCCTTGT  59
              ||||| ||||||||| ||||||| |||||| |||||||||||| |||||||||||| ||||
Samase  387   CGCAATCTTGCTCCCACTCATTACAAGCTAGGCGTGCAACATTGAGTGCCTGCGCTTTGT  328

Sam_samp  60  CCTTGCAGCGTACACGAACCGTTTTTTCCTCAGTCGGTGCGCTTGCGATTGCCTCACGAT  119
              |||||||||||||||||||| |||||||||||||| |||||||||| ||||||||||||||
Samase  327   CCTTGCAGCGTACACGAACAGTTTTTTCCTCAGTTGGTGCGCTTGAGATTGCCTCACGAT  268


Sam_samp120   AGCACCCGGTCAAATCGGTTGACTCTACGGAGCCAT  155
              |||||||||||||||||||||||| ||||||||||
Samase  267   AGCACCCGGTCAAATCGGTTGACTCAACGGAGCCAT  232
```

### 3.3 Interpretation of Results

The control of extracted DNA from samples and positive/negative control tomato seeds were performed by patatin gene specific PCR. All DNAs from seed samples and positive/negative control tomato seeds produced the expected amplicons, which indicated the presence and amplification capacity of the DNA samples.

Among 25 DNA samples, 15 of them did not produce any amplicon with any of the screening primers used in this study. These tomato seeds are either not genetically modified or other marker, promoter or terminator regions were used in transgenic tomato lines instead of nptII, 35S and Nos, regions. It was known that in recent studies, scientists begin to improve crops by using their own promoter and terminator regions. Besides, in recent studies marker genes are removed after transformation of the gene.

The amplification of Sam-k gene could not be achieved except for one primer set. The main reason was the difficulties in the optimization of PCR conditions without having a positive control DNA for Sam-k gene. The positive control tomatoes for Sam-k gene was kindly requested from Agritope. However, they could not provide us with the positive control tomato. One more reason for the defectiveness of primer sets other than Sam5 f/Sam3 f primer pair might have been due to some insertions or deletions within the primer binding sites.

In screening studies, samples 4, 17, 18, 19, 20, 21, 22, 24 and 25 produced amplicons with 35S primer set and, 3, 4, 17, 18, 19, 20, 22 and 24 produced

amplicons with Nos primer set. Furthermore, samples 3, 17, 18, 19 and 20 also produced amplicons in NptII specific PCR (Table 3.6). In the identification PCR studies, any amplification signal was not observed from F-type truncated PG gene, however sample 3 produced positive signal with Sam-k primer set. The PCR results are summarized in Table 3.7. S17, S18, S19, and S20 with 35S, Nos and nptII regions could be either containing PG gene in antisense orientation or containing accd gene or acc gene in sense orientation among other approved ripening delayed tomatoes (Table 3.5). Moreover, S4, S22 and S24 with 35S and Nos regions could be containing PG gene in the antisense orientation as in the case of Flavr Savr. S25 with 35S region could not have been categorized according to the known transgenic maps. All these samples might belong to other group of genetically modified tomatoes, such as insect resistance. As mentioned by Bruderer *et al.,* (2003), although genetically modified tomato lines with variable improved properties were approved in different parts of the world, there are several approved transgenic tomato lines with no genetic map information. In order to evaluate our positive results whether they improved to provide delayed ripening or not, sequence information is needed.

In summary, among 25 samples, identification was achieved only for S3, and it showed the characteristics of 35 1 N, with its NptII, Nos terminator and Sam-k gene regions. The lack of 35S promoter in this sample verified our results since E8 Promoter was used in 35 1 N instead of 35S promoter (Table 3.5).

**Table 3.5 Known Genetic Maps for Ripening Delayed Tomatoes**

| Event name | Brand name | Trait | Promoter | Terminator | Marker | Event |
|---|---|---|---|---|---|---|
| 1345-74 | Endless Summer | Ripening Delayed | 35S | Nos | NptII | ACC Synthesase gene in sense orientation |
| 35 1 N | | Ripening Delayed | E8 | Nos | NptII | Sam-k gene |
| 8338 | | Ripening Delayed | 35S | Nos | NptII | Accd gene |
| B, Da,F | Vegadura, Vegaspeso | Ripening Delayed | 35S | Nos | NptII | Antisense and sense PG gene |
| Flavr Savr | Flavr Savr, MacGregor' | Delayed Softening | 35S | - | NptII | Antisense PG gene |

**Table 3.6 Summary of the Results**

| | Screening | | | | Identification | |
|---|---|---|---|---|---|---|
| | RT PCR | Conventional PCR | | | Conventional PCR | |
| | NOS | 35S | NOS | NPTII | PG | Sam-k |
| **Sample** | | | | | | |
| S1 | | - | - | - | - | - |
| S2 | | - | - | - | - | - |
| S3 | + | - | + | + | - | + |
| S4 | + | + | + | - | - | - |
| S5 | | - | - | - | - | - |
| S6 | | - | - | - | - | - |
| S7 | | - | - | - | - | - |
| S8 | | - | - | - | - | - |
| S9 | | - | - | - | - | - |
| S10 | | - | - | - | - | - |
| S11 | | - | - | - | - | - |
| S12 | | - | - | - | - | - |
| S13 | | - | - | - | - | - |
| S14 | | - | - | - | - | - |
| S15 | | - | - | - | - | - |
| S16 | | - | - | - | - | - |
| S17 | + | + | + | + | - | - |
| S18 | + | + | + | + | - | - |
| S19 | + | + | + | + | - | - |
| S20 | + | + | + | + | - | - |
| S21 | | + | - | - | - | - |
| S22 | + | + | + | - | - | - |
| S23 | | - | - | - | - | - |
| S24 | + | + | + | - | - | - |
| S25 | | + | - | - | - | - |
| +Control | + | + | + | + | - | - |
| - Control | - | - | - | - | - | - |
| P2300 | | - | - | + | - | - |
| P1320 | | + | + | - | - | - |

# CHAPTER 4

# CONCLUSION

The application of modern biotechnology to food and plants is currently the focus of intense public and political debate with particular reference to the issue of food safety. This situation has led to the completion of regulatory framework for GM products in most countries, mostly centered on the requirements of traceability and labeling. It is really necessary to detect and evaluate the presence of GM foods in Turkey food markets to contribute to the building up such a regulatory framework.

In this study, screening of 25 tomato seed samples for genetic modification was attempted. The identification of the screened tomato seeds for the presence of ripening delayed trait was achieved among the screened samples. Also, other approved ripening delayed events, F event from Zeneca and event 35 1 N from Agritope were targeted. Event 35 1 N had an approval for food use in USA, and event F had an approval for food use in Canada since 1996.

In order to detect ripening delayed GM tomatoes, three screening targets and two identification target genes were used by designing primer sets that were specific to 35S promoter, Nos terminator, NptII kanamycin resistance gene, F-type truncated PG and Sam-k genes.

In conclusion NptII, 35S, Nos and Sam-k genes were detected. In screening analysis via conventional PCR, amplifications with 35S, Nos and NptII primer sets were observed in 4 samples. Among other samples, 3 of them were amplified with 35S and Nos primers and 2 of them were amplified only with 35S primers. The amplification was observed with Nos, NptII and Sam-k primers in 1 sample. The sequence result of the PCR product amplified with Sam5 f/Sam3 f primer set showed high homology with the S-adenosyl-L-methionine hydrolase gene of T3 Coliphage. Since in the event 35 I N from Agritope, tomato is positive for NptII, Nos and Sam-k gene regions, this sample could be identified as 35 1 N. The lack of 35S promoter verified our result in identification of Sample 3, since E8 promoter was used in this event as promoter region. F type truncated- PG gene was not present in any of the samples. Nos specific conventional PCR results were also verified by RT-PCR.

Although, the surface sterilization of seeds was performed to eliminate the risk of possible contamination from soil before conducting DNA isolation, there were always possible risks of contamination from any source. However, finding more than one of the most commonly screening genetic elements after surface sterilization diminished our concerns about the contamination problem.

In conclusion, these obtained data showed the possible existence of tomatoes in GM food market. However, these results should also be validated by the help of other laboratories. Further verification could be achieved by using more specific detection applications such as Construct Specific and Event Specific Methods.

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# APPENDICES

## APPENDIX A

## Chemicals and Suppliers

| CHEMICALS | SUPPLIERS |
|---|---|
| Ficol 400 | Sigma |
| Agarose | Applichem |
| Bromphenol Blue | Sigma |
| Chloroform:Isoamylalcohol | Applichem |
| CTAB | Applichem |
| EDTA | Sigma |
| Ethanol | Delta Kimya |
| Ethidium Bromide | Sigma |
| HCl | Applichem |
| Hydrogen Peroxide | Sigma |
| Isopropanol | Delta Kimya |
| NaAc | Applichem |
| NaCl | Merck |
| NaOH | Merck |
| PCR Grade Water | Dr. Zeydanlı A.Ş. |
| Tris | Sigma |

## APPENDIX B

## Buffers and Solutıons

### 1.Solutions for DNA isolation

### 1.1 . Hexadecyltrimethyl-Ammonium Bromide (cTAB) Buffer

cTAB 20g/l
NaCl 1.4 M
Tris HCl 100 mM
EDTA 20 mM
Bring the last volume to 1 liter with dH2O. Adjust the pH to 8 and autoclave ($121^0$C, 15 min).

### 1.2. CTAB Precipitation Buffer

CTAB 5g/l
NaCl 0.04 M
Bring the last volume to 1 liter with dH2O. Adjust the pH to 8 and autoclave (121 oC, 15
min).

### 1.3. 1.2 M NaCl

NaCl 70g
$dH_2O$ 1 liter
Dissolve in 1000 ml dH2O and autoclave ($121^0$C, 15 min).

### 1.4. Washing Buffer

$dH_2O$ 300ml
Pure EtOH 700ml

## 2. Buffers for Electrophoresis

### 2.1 . Electrophoresis Buffer 50X TAE (Tris-Acedic acid–EDTA)

2M Tris
1M Acedic Acid
100 mM $Na_2EDTA$
48. 44g, 11.8g and 7.45g respectively for 200 ml solution were dissolved in $dH_2O$
and pH was adjusted to 8.0. The solution was diluted 50 times before using.

### 2.2 Loading Buffer for Agarose Gel Electrophoresis

0.25 % Bromophenol Blue
0.25 % Xylene cyanol
15 % Ficol 400
40% (w/v) Sucrose
Dissolved in $dH_2O$

### 2.3 Ethidium Bromide Solution

10 mg/mL EtBr was dissolved in $dH_2O$

### 2.4 Solutions for Plasmid Isolation

**Solution 1 (Resuspension Buffer)**
50 mM Tris-Cl, pH = 8.0
10 mM EDTA
100 μg/ml RNase A

**Solution 2 (Lysis Buffer)**
200 mM NAOH
1 % SDS

**Ne Buffer (Neutralization Buffer)**
3.0 M Potassium acetate, pH = 5.5

## APPENDIX C

### Enzymes, Markers and Reagents

| ENZYMES, MARKERS AND REAGENTS | SUPPLIER |
|---|---|
| DNA Taq Polymerase | MBI Fermentas |
| Ribonuclease A | MBI Fermentas |
| Proteinase K | MBI Fermentas |
| DNA ladder | MBI Fermentas |
| dNTPs | MBI Fermentas |
| Xmn1 | MBI Fermentas |

# APPENDIX D

## Annealing Sıtes of Prımer Sets

### 1- Annealing sites of PatGc-PatGd primer set

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

2761 tgttgttgct ***ctcattaggc actggcact***a cttcagagtt tgataaaaca tatacagcag aagagacagc taaatggggt actgcacgat ggatgttagt tatacagaaa at***gactagtg cagcaagttc ttac***atgact gattattacc tttctactgc ttttcaagct cttgattcac2941

### 2- Annealing sites of P35S-afuI/P35S-ar1 primer set

Annealing sites of **P35S-afuI/P35S-ar1** primer set were illustrated on Cauliflower Mosaic Virus Genomic sequence with NCBI accession no. V00141.

7141 tgcccagcta tctgtcactt tattgtgaag atagtggaaa aggaaggtgg ct ***cctacaaa tgccatcatt gcg*** ataaagg aaaggccatc gttgaagatg cctctgccga cagtggtcccaaagatggac ccccacccac gaggagcatc gtggaaaaa g aaga cgttcc aaccacgtcttcaaagcaag

**Xmn1 cut side**

tggattgatg tgatatctcc actgacgtaa gggatgacgc acaatcc***cactatccttcgc aagaccc*** ttc ctctatataa ggaagttcat ttcatttgga gaggacacgc 7441

**3- Annealing sites of Nos-1/Nos-3 primer set**

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

1561 tgatccccga tcgttcaaac atttggcaat aaagtttctt
*aagatt**gaat cctgttgccg gtcttg***cgat gattatcata taatttctgt
tgaattacgt taagcatgta ataattaacatgtaatgcat gacgttattt
atgagatggg tttttatgat tagagtcccg caattataca
tttaatacgc gatagaaaac aaaata***tagc gcgcaaacta gga***taaatta
tcgcgcgcgg 1801

**4. Annealing sites of Kan F/Kan R primer set**

Annealing sites of Kan F/Kan R primer set was illustrated on Uncultured bacterium clone K003 transposon Tn5 neomycin phosphotransferase (nptII) gene sequence with NCBI accession DQ449900.

151ctgtccggt***g ccctgaatgaactgcaggac*** gaggcagcgc
ggctatcgtg gctggccacg acgggcgttc cttgcgcagc tgtgctcgac
gttgtcactg aagcgggaag ggactggctg ctattgggcg
aagtgccggg gcaggatctc ctgtcatctc accttgctcc tgccgagaaa
gtacccatca tggctgatgc aatgcggcgg ctgcatacgc ttgatccggc
tacctgccca ttcaaccacc aagcgaaaca tcgcatcgag cgagcacgta
ctcggatgga agccggtctt gtcgatcagg atgatctgga cgaagagcat
caggggctcg cgccagccga actgttcgcc aggctcaagg cgcgcatgcc
cgacggcgag ***gatctcgtcg tgacccatgg cgatgcctgc*** ttgccgaata
tcatggtgga aaatggccgc 601

**5- Annealing sites of Sam 5 F/Sam 3 F primer set**

Annealing sites of Sam 5 F/Sam 3 F primer set was illustrated on Coliphage T3 S-adenosyl-L-methionine hydrolase (Adometase, E.C.. 3.3.1.2.) gene sequence from the source Enterobacteria phage T3 with NCBI accession X04791.

181 tgagcagaca ccgccacatg gtaagcactt tacgtgccgc accgggtctt tat ***ggctccg*** ***ttgagtcaac cga***tttgacc gggtgctatc gtgaggcaat ctcaagcgca ccaactgagg aaaaaactgt tcgtgtacgc tgcaaggaca aagcgcaggc actcaatgtt gcacgcctag cttgtaatga gtgggagcaa gattgcgtac tggtatacaa atcacagact cacacggctg ***cgctaaaggt atcgacgggt*** ataaggctga acgtctgccg ggtagtttcc 481

**6- Annealing sites of PG34L/PG34R primer set**

Annealing sites of PG34L/PG34R primer set were illustrated on Tomato mRNA for polygalactronase (PG, EC 3.2.1.15) sequence from the source Solanum lycopersicum with NCBI accession X05656.

421 cttttcaggt ccatgcagat cttctatttc agtaaagatt ***tttggatcct tagaagcatctagt***aaaatt tcagactaca aagatagaag gctttggatt gcttttgata gtgttcaaaa tttagttgtt ggaggaggag gaactatcaa tggcaatgga caagtatggt ggccaagttc ttgcaaaata ataaatcac tg ***ccatgcag ggatgcacca acg***gccttaa ccttctggaa 661

# APPENDIX E

## Nos Specific RT-PCR Results

**File:** nos-RT PCR
**Print Date:** Monday, May 21, 2007 08:01:07
**User:** msil
**Plate Type:** Absolute Quantification
**PCR Volume:** 20 µL

**Document Information**

**Operator:** ABI 7500
**Run Date:** Saturday, April 21, 2007 22:22:04
**Last Modified:** Monday, May 21, 2007 07:55:54
**Instrument Type:** Applied Biosystems 7500 Real-Time PCR System

**Comments**

**Comments:**
SDS v1.3.1

**Thermal Profile**

| Stage | Temperature | Time | Repeat | Ramp Rate | Auto Increment |
|---|---|---|---|---|---|
| 1 | 95.0 °C | 5:00 | 1 | Auto | |
| 2 | 95.0 °C | 0:10 | 45 | Auto | |
| | 60.0 °C | 0:15 | | Auto | |
| | 65.0 °C | 0:32 | | Auto | |
| 3 | 4.0 °C | 1:00 | 1 | Auto | |

**9600 Emulation Mode**
**Data Collection: Stage 2, Step 3**

**Analysis Methods:**

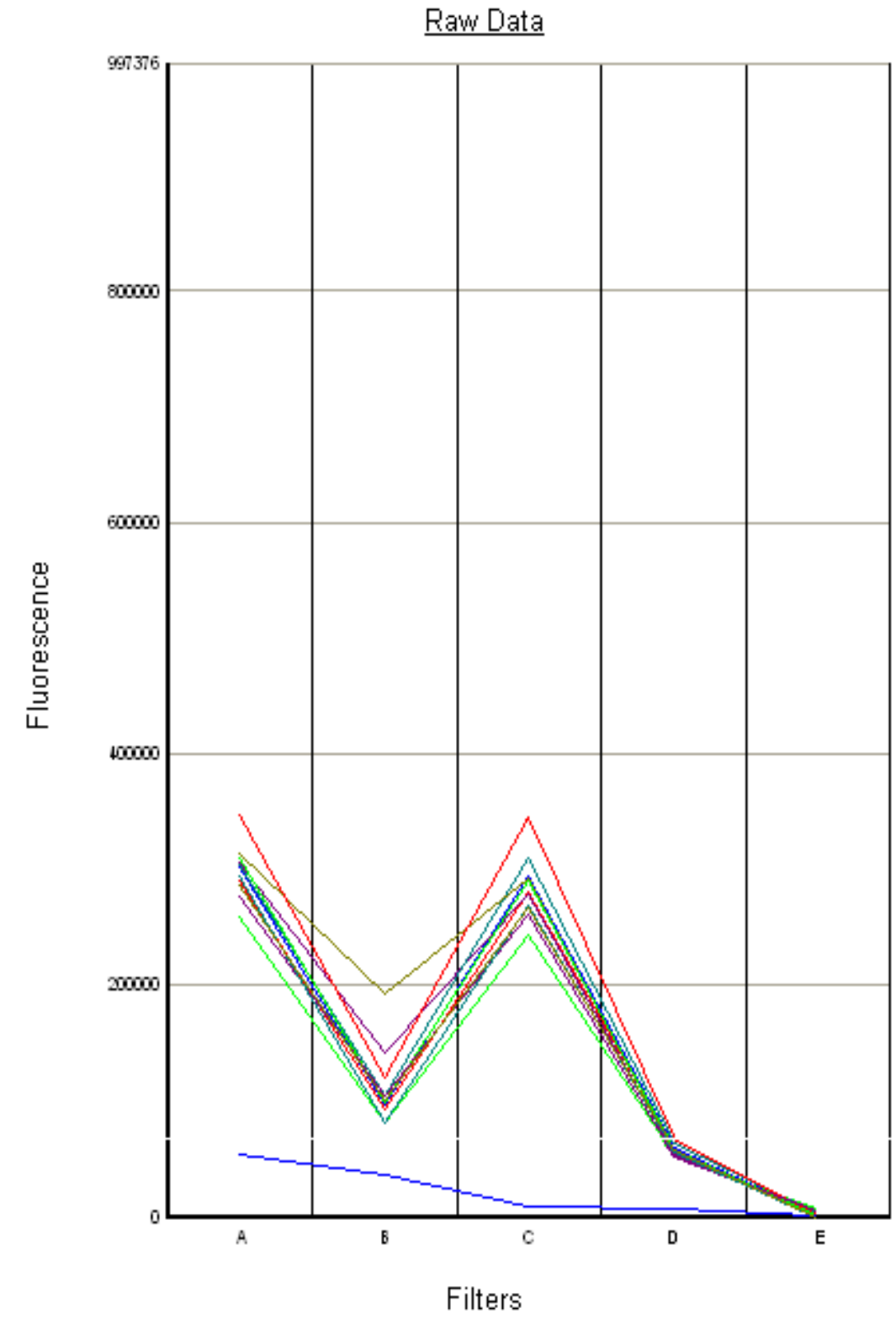
Delta Rn

**Detector Information**

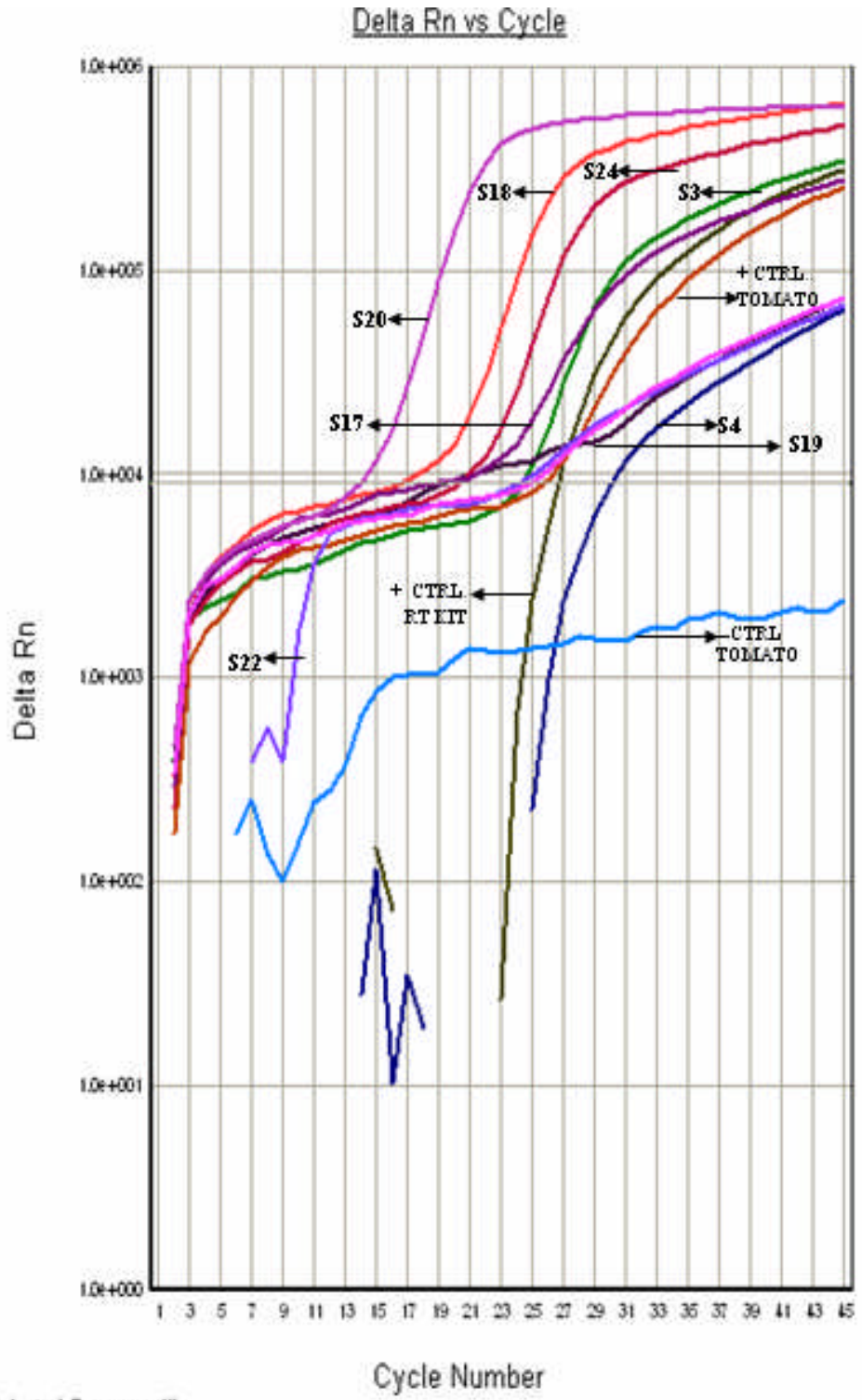
| Detector Name | Reporter | Quencher | Threshold | Baseline Start | Baseline End |
|---|---|---|---|---|---|

NOS FAM TAMRA <9008.031250> Auto Auto

| Well | Sample Name | Detector | Task | Ct | StdDev Ct | Qty | Mean Qty | StdDev Qty | Filtered | Tm |
|---|---|---|---|---|---|---|---|---|---|---|
| A5 | S3 | NOS | Unknown | 24.28 | | | | | | |
| A6 | S4 | NOS | Unknown | 30.09 | | | | | | |
| B5 | S19 | NOS | Unknown | 19.34 | | | | | | |
| B6 | +RT KIT CRL. | NOS | Unknown | 26.61 | | | | | | |
| C5 | S24 | NOS | Unknown | 20.26 | | | | | | |
| C6 | +TOM. CTRL. | NOS | Unknown | 25.69 | | | | | | |
| D5 | S18 | NOS | Unknown | 16.57 | | | | | | |
| E5 | S17 | NOS | Unknown | 18.92 | | | | | | |
| E7 | - TOM. CTRL. | NOS | Unknown | Undet. | | | | | | |
| F5 | S20 | NOS | Unknown | 13.95 | | | | | | |
| G5 | S22 | NOS | Unknown | 24.15 | | | | | | |
| H5 | BT11 | NOS | Unknown | 25.00 | | | | | | |




Reading: 1
Well(s): A5-A6,B5-B6,C5-C6,D5,E5,E7,F5,G5,H5




Selected Detector: All
Well(s): A5-A6,B5-B6,C5-C6,D5,E5,E7,F5,G5,H5