EXTENSION OF FLOWER LONGEVITY IN TRANSGENIC PLANTS VIA ANTISENSE BLOCKAGE OF ETHYLENE BIOSYNTHESIS

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ABSTRACT

EXTENSION OF FLOWER LONGEVITY IN TRANSGENIC PLANTS VIA ANTISENSE BLOCKAGE OF ETHYLENE BISYNTHESIS

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Ethylene (C_2H_4) is a very simple molecule, a gas, and has numerous effects on the growth, development and storage life of many fruits, vegetables and ornamental crops. In higher plants, ethylene is produced from L-methionine in essentially all tissues and ACC Synthase and ACC Oxidase are the two key enzymes in the biosynthesis of ethylene.

The objective of the present study was to transform tobacco (*Nicotiana tabacum* L. cv. Samsun) plant with partial sequence of torenia *acc oxidase* gene in antisense and sense orientations via *Agrobacterium*-mediated gene transfer system, and to analyze its effect on ethylene production in transgenic plants.

Six antisense and seven sense T_0 putative transgenic lines were obtained and were further analyzed with several assays. Leaf disc assay and chlorophenol red assay under selection (75 mg/L kanamycin) revealed positive results compared to the non-transformed plant. T_1 generations were obtained from all putative transgenic lines. PCR analysis and Northern Blot Hybridization results confirmed the transgenic nature of T_1 progeny. Furthermore, ethylene amount produced by flowers were measured with gas chromatography, which resulted in an average of 77% reduction in S7 line and 72% reduction in A1 line compared with the control flowers. These results indicated that, transgenic tobacco plants carrying torenia *acc oxidase* transgene both in antisense and sense orientations showed reduced ethylene production thus a possibility of flower life extension.

Keywords: ACC Oxidase, Ethylene, *Nicotiana tabacum*, antisense gene, sense gene, flower longevity

ÖZ

TRANSGENİK BİTKİLERDE ETİLEN BİYOSENTEZİNİN TERS-YÖNLÜ GEN TRANSFERİ İLE ENGELLENMESİ VE ÇİÇEK ÖMRÜNÜN UZATILMASI

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Etilen (C_2H_4) gazı basit moleküler yapısına rağmen meyve, sebze ve süs bitkilerinin büyümesi, gelişmesi ve raf ömrü üzerine sayısız etkilere sahiptir. Etilen yüksek yapılı bitkilerdeki tüm dokularda L-metiyonin'den sentezlenir ve biyosentezindeki iki anahtar enzim ACC Sentaz ve ACC Oksidaz dir.

Bu çalışmanın amacı torenia *acc oksidaz* geninin bir kısmını ters-yönlü ve anlamlı yönde tütün (*Nicotiana tabacum* L. cv. Samsun) bitkisine *Agrobacterium* yolu ile aktarmak ve transgenik bitkilerin etilen üretimindeki etkilerini analiz etmektir.

Altı ters-yönde ve yedi anlamlı yönde gen taşıyan T_0 aday transgenik bitkileri elde edilmiş ve bu bitkiler çeşitli testlerle analiz edilmişlerdir. Yabani ve transgenik bitkilerde seçici ortamda (75 mg/L) uygulanan yaprak diski testi ve klorofenol kırmızısı testleri, transgenik bitkilerin yabani bitkiye göre pozitif sonuçlar verdiğini ortaya koymuştur. Tüm aday transgenik bitkilerden T_1 dölleri elde edilmiştir. PCR analizi ve Northern Blot Hibridizasyonu da T_1 döllerinin transgenik yapısını desteklemiştir. Ayrıca, yabani ve transgenik bitkilerin çiçeklerinin ürettiği etilen miktarları gaz kromatografisi yardımı ile ölçülmüş, bu da yabani çiçeklerle karşılaştırıldığında S7 transgenik hattındaki etilen üretiminin %77 A1 transgenik hattındaki etilen üretiminin %72 oranında azaldığını göstermiştir. Bu sonuçlar, torenia *acc oksidaz* genini ters-yönde ve anlamlı yönde taşıyan transgenik tütün bitkilerinin daha az etilen ürettiklerini ve bunun da çiçek ömrünün uzatılmasına olanak sağlayabileceğini göstermektedir.

Anahtar Kelimeler: ACC Oxidaz, Etilen, *Nicotiana tabacum*, ters-yönlü gen, anlamlı gen, çiçek ömrü

To my family for all of their support dedicated to me

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TABLE OF CONTENTS

PLAGIARISM PAGE…….. iii
ABSTRACT……. iv
ÖZ……. vi
DEDICATION..……... viii
ACKNOWLEDGEMENTS…..…….. ix
TABLE OF CONTENTS…...……... xi
LIST OF TABLES..……. xv
LIST OF FIGURES..…… xvi
LIST OF ABREVIATIONS..……... xviii
CHAPTER
I. INTRODUCTION..……..…… 1
1.1. Genetic Engineering for Crop Improvement.…..…….……. 1
1.2. Gene Transfer Techniques Used to Manipulate Plants.…… 2
1.3. Transgenic Plants and Their Application in Agriculture..... 4
1.4. *Agrobacterium*-Mediated Gene Transfer Technique..... 6
1.5. Ethylene (C_2H_4)..... 8
1.5.1. Biochemical Synthesis of Ethylene.……..... 9
1.5.2. Functions of Ethylene..... 12
1.5.3. Ethylene Perception and Signal Transduction..... 14
1.5.4. Engineering of Plants against Ripening and Senescence..... 18
1.5.4.1. Control of Fruit Ripening and Flower Senescence via Exogenous Application of Chemicals..... 18
1.5.4.2. Down Regulation of Ethylene Production via Antisense and Sense RNA Inhibition of ACC Oxidase or ACC Synthase… 20

1.6. Aim of the Study..……. 24
II. MATERIALS AND METHODS..…….. 25
2.1. Materials..…….. 25
2.1.1. Bacterial Strains..……. 25
2.1.2. Plasmids..……. 25
2.1.3. Culture Conditions..……. 26
2.1.4. Plant Material and Plant Tissue Culture Media..……. 26
2.1.5. Other Chemicals and Materials..…….. 26
2.2. Methods..…….. 27
2.2.1. Bacterial Growth..…… 27
2.2.2. Transformation Studies with Bacterial Cells..... 27
2.2.2.1. Mobilization of Vectors to *E. coli*..…….. 28
2.2.2.1.1. Competent *E. coli* Preparation..…….… 28
2.2.2.1.2. Transformation of *E. coli*…….. 28
2.2.2.2. Plasmid Isolation..…… 29
2.2.2.2.1. Mini Scale Plasmid Isolation …… 29
2.2.2.2.2. PCR Analysis.....…… 30
2.2.2.3. Mobilization of Vectors to *Agrobacterium tumefaciens*..…… 32
2.2.3. Maintenance of Plant Material..……..... 33
2.2.3.1. Surface Sterilization of Tobacco Seeds..…….. 33
2.2.3.2. Micropropagation of Tobacco Plants..……..……... 33
2.2.4. Leaf Disc Transformation of *Nicotiana tabacum*..…….…..... 33
2.2.5. Analysis of Putative Transgenic Plants..……... 35
2.2.5.1. Leaf Disc Assay..... 35
2.2.5.2. Color Viability Test…….……..... 35
2.2.5.3. Analysis of Putative Transgenic Plants via PCR..... 36
2.2.5.4. Expression Analysis of *acc oxidase*…….…..... 38
2.2.5.4.1. Total RNA Isolation…..…….……. 38
2.2.5.4.2. Preparing RNA for Northern Blot..……. 39
2.2.5.4.3. Northern Blotting..…….. 40
2.2.5.4.4. Probe Labelling, Hybridization and Detection..…….. 40

2.2.5.5. Mendelian Inheritance Pattern.……..…… 42
2.2.5.6. Ethylene Measurement of T_1 Lines..... 42
III. RESULTS AND DISCUSSION…...……. 44
3.1 Transformation studies.……. 44
3.1.1. Transformation of *Agrobacterium* cells via Triparental Mating..... 44
3.1.2. Regeneration and Selection of T_0 and T_1 Transgenic Plants..... 47
3.1.2.1. Callus Induction ……..… 49
3.1.2.2. Shoot and Root Development.…… 51
3.1.2.3. Development in Soil.……..……. 53
3.2. Analysis of T_o Putative Transgenic Tobacco Plants..……... 56
3.2.1. Leaf Disc Assay…….. 56
3.2.2. Color Viability Test.……. 57
3.2.3. PCR Analysis.…….. 59
3.3. Analysis of T_1 Transgenic Tobacco Plants...……... 60
3.3.1. PCR Analysis.…….. 61
3.3.2. Expression Analysis of *acc oxidase* in T_1 Transgenic Plants..... 63
3.3.3. Mendelian Inheritance Pattern...…….. 65
3.3.4. Ethylene Measurements of T_1 Transgenic Tobacco Flowers..... 70
3.3.5. Overall Evaluation of Transgenic Lines according to the Tested Parameters 72
IV. CONCLUSION. ……...……. 75
REFERENCES…..……... 77
APPENDİCES..…… 87
A. STRUCTURE OF BINARY VECTORS pAO-A AND pAO-S..... 87
B. PRIMERS…… 88
C. TORENIA FOURNIERI mRNA for ACC OXIDASE, PARTIAL CDS.... 89
D. NICOTIANA TABACUM mRNA for ACC OXIDASE, COMPLETE CDS..... 90
E. BACTERIAL CULTURE MEDIA..…….. 91
F. TBE, TAE AND TE SOLUTIONS ...…….. 93
G. PLASMID ISOLATION SOLUTIONS…….. 94

H. COMPOSITIONS OF PLANT TISSUE CULTURE MEDIA……... 95
I. CTAB DNA ISOLATION SOLUTIONS……. 96
J. RNA ISOLATION SOLUTIONS…… 97
K. NORTHERN BLOT ANALYSIS SOLUTIONS..... 99

LIST OF TABLES

TABLE

1.1 Plant responses to ethylene.……...……..... 12

2.1 The composition and the purpose of plant tissue culture media.……. 27

2.2 PCR cycling conditions to amplify *nptII* gene.…….. 31

2.3 Optimized conditions of PCR to amplify *nptII*.....**.**..... 31

2.4 PCR cycling conditions to amplify *nptII* and *acc oxidase.*……..…….. 37

2.5 Optimized conditions of PCR to amplify *nptII and acc oxidase*..... 37

3.1 Mendelian Inheritance Analysis of T_1 progeny of tobacco seeds.……... 69

3.2 Overall evaluation of transgenic lines according to the tested parameters……..... 73

LIST OF FIGURES

FIGURE

1.1 Ethylene biosynthetic pathway of higher plants..... 10
1.2 A genetic pathway of ethylene signal transduction..... 17
1.3 The action mechanism of antisense gene..... 21
3.1 Agarose gel results of restriction enzyme digestion of plasmids pAO-A and pAO-S..... 45
3.2 PCR Analysis of n*ptII* gene amplification after triparental mating..... 47
3.3 The appearance of the wild type explants on the fourth week..... 50
3.4 The appearance of the transformed explants on the fourth week..... 50
3.5 Development of S7 line on 75 mg/L kanamycin containing MSC media after transformation..... 51
3.6 The regeneration of the representative putative transgenic S17 line..... 52
3.7 The root development of putative transgenic plantlets in 75 mg/L kanamycin containing MSC media..... 52
3.8 The growth of putative transgenic S6 line in soil..... 53
3.9 The growth of T_0 putative transgenic plants under greenhouse conditions... 54
3.10 The growth of T_1 transgenic plants under greenhouse conditions..... 55
3.11 The appearance of leaf discs after 5 weeks of growth..... 57
3.12 Results of color viability test..... 58
3.13 PCR analysis of pAO-A transformed T_0 putative transgenic plants with *nptII* primers..... 59
3.14 PCR analysis of pAO-S transformed T_0 putative transgenic plants with *nptII* primers..... 60
3.15 PCR analysis of T_1 sense putative transgenic plants using *nptII* primers... 61
3.16 PCR analysis of T_1 antisense putative transgenic plants using *nptII*

primers..... 62
3.17 PCR analysis of T_1 antisense putative transgenic plants using *acc oxidase* primers..... 62
3.18 PCR analysis of T_1 sense putative transgenic plants using *acc oxidase* primers..... 63
3.19 Total RNA isolated from transgenic and wild type plants..... 64
3.20 Northern blot analysis of T_1 antisense transgenic tobacco plants..... 64
3.21 Northern blot analysis of T_1 sense transgenic tobacco plants..... 65
3.22 Photographs of T_1 antisense transgenic plants selected on 75 mg/L kanamycin containing medium..... 66
3.23 Photographs of T_1 sense transgenic plants selected on 75 mg/L kanamycin containing medium..... 67
3.24 Ethylene production from flowers after 24 hours of wounding..... 70
3.25 Percent change of ethylene amount in A1 line and wild type control..... 71
3.26 Percent change of ethylene amount in S7 line and wild type control..... 71
3.27 Average of percent changes of ethylene amount in S7 and A1 lines..... 72

LIST OF ABREVIATIONS

ABREVIATIONS

A	pAO-A transformed tobacco line
S	pAO-S transformed tobacco line
ACC	1-aminocyclopropane-1-carboxylic acid
ACC Oxidase	1-aminocyclopropane-1-carboxylic acid Oxidase
ACC Synthase	1-aminocyclopropane-1-carboxylic acid Synthase
BA	Benzylaminopurine
CTAB	Hexadecyl-trimethyl amonium bromide
DEPC ……...	Diethylpyrocarbonat
LB ……	Luria-Bertani Broth
MOPS……..	3-N-[Morpholino] propane sulfonic acid
MS ……...	Murashige and Skoog
NAA ……	Naphtalen acetic acid
OD ……...	Optical Density
PCR…….	Polymerase Chain Reaction
Ti…….	Tumor inducing
T-DNA…….	Transferred DNA
TAE…….	Tris Acetate EDTA
TBE…….	Tris Borate EDTA
TE ……	Tris EDTA
YEB…….	Yeast Extract Broth

CHAPTER I

INTRODUCTION

1.1.Genetic Engineering for Crop Improvement

Genetic manipulation of plants has been carried out for thousand of years by applying conventional plant breeding methods. Plant breeding principles consist of identifying and selecting desirable traits and combining these into one individual plant. By this way breeders have been able to create new varieties that have desirable traits, such as color and shape. Today, there exists a promising method of developing superior plants: genetic engineering.

Genetic engineering depends on the fact that the same molecule, DNA, makes up the genes of all organisms. This opens the way that, genes from one species can be transferred to another species; either related or unrelated. The process of introducing a gene into an organism via recombinant DNA technology is known as transformation and genetically engineered plant species are called as transgenic plants or genetically modified organisms (GMO).

With this technology, many genetically modified plants have been regenerated during recent years. Overall aim of these studies are; improved product yield and quality, increased resistance to diseases, increased resistance to biotic stress factors such as nematodes, insects and fungus, increased resistance to

various abiotic stresses factors such as drought, flooding, extremes of temperature, high soil contents of salt, heavy metals and improved post harvest qualities such as maintenance of fruit or flower quality during storage.

1.2. Gene Transfer Techniques Used to Manipulate Plants

Developments of the recombinant DNA techniques, plant tissue culture techniques and the design of selection methods for transgenic plant cells enabled us to obtain transgenic plants that carry desired genes.

Generation of a transgenic plant starts with the isolation and manipulation of a useful gene. Next step is the transfer of the gene to a host plant and integration of it into the host plant genome. Fertile plants must be regenerated from selected transformed plants and the gene must be expressed in the host plant in correct tissue at right time and transmitted to the next generation when the host plant reproduces.

There are several techniques for transferring genes artificially into recipient organisms. These techniques can be categorized as direct gene transfer techniques such as; protoplast fusion, electroporation, microprojectile bombardment and microinjection and indirect gene transfer techniques such as; DNA or RNA viruses and *Agrobacterium*-mediated gene transfer techniques.

Direct gene transfer techniques do not rely on biological vectors like plasmids and viruses, and naked DNA is directly used. One example to direct gene transfer is protoplast fusion. Protoplasts are plant cells from which the cell walls have been removed which make it easier to transfer foreign DNA into cells either by treatment with particular chemicals, or by the use of high voltage electric shocks. The chemicals polyethylene glycol (PEG) and polyvinyl alcohol, together with Ca^{+2} and high pH, induce protoplasts to take up naked DNA (Paszkowski *et al.*, 1984).

Electroporation is another direct gene transfer technique, which has been developed for mammalian cell transformation, and now adapted to both monocot and dicot species. The application of strong electrical field and high voltage electrical pulses to solution, containing cells and DNA creates reversible pores in the cell membrane that allows the entry of the genes (Fromm *et al.,* 1985).

In microprojectile bombardment, biolistics or gene gun method, metal slivers are used to deliver the genetic material into the cell. Micro carriers (i.e. metal slivers like tungsten or gold) are much smaller than the diameter of the target cell are coated with genetic material. Micro carriers are carried with a macro carrier or bullet and fired into the target tissue by pressurized gas like helium. A perforated metal plate stops the macro carrier but allows the movement of micro carriers into the living cells. Once in the cell, the genetic material is transported to the nucleus where it is incorporated into the host genome (Klein *et al.,* 1987).

Microinjection is another method and based on the transfer of naked DNA molecules to intact plants or protoplasts by mechanical ways (Crossway *et al.,* 1986). In the microinjection technique the difficulties of callus and plant regeneration can be overwhelmed. The only difficulty is the handling and immobilization of the plants.

The indirect way of introducing genes into plant cells involves the plant DNA and RNA viruses and *Agrobacterium*-mediated gene transfer. Studies showed the ability of DNA and RNA viruses to mediate the expression of foreign genes in plant cells. Advantage of such a system is the ease of gene induction but they offer difficulties in the control, as they are the cause of diseases, and stabilization of transformation. Currently the most widely used technique is *Agrobacterium*-mediated gene transfer technique. It is based on the transfer of foreign DNA by using natural gene transfer capacity of *Agrobacterium* species (Horsch *et al*., 1985). *Agrobacteria* are gram-negative rod-shaped bacteria that are abundant in soil and classified in genus *Rhizobium* and in family *Rhizobiaceae*.

1.3.Transgenic Plants and Their Application in Agriculture

Since the development of transgenic plants in 1970s, many millions of hectares of commercially produced transgenic crops such as, soybean, tobacco, cotton, potato and maize has been grown annually in a number of countries, including the United States (28.7 million hectares in 1999), Canada (4 million hectares), China (0.3 million hectares) and Argentina (6.7 millions hectares) (James *et al.,* 1999).

One of the application areas of transgenic plants is conferring resistance to weeds, which competing with crops decrease the yield. More than a decade ago the first transgenic plant that is resistant to the herbicide Basta® was achieved (De Block *et al*., 1987).

Another area is generation of transgenic plants that are resistant to insects (Dulmage *et al*., 1981). *Bacillus thrungiensis* (Bt) forms spores carrying crystal proteins that are toxic to insects. Insect resistance was achieved in transgenic plants by expressing toxic proteins of *Bacillus thrungiensis*. Apart from *Bacillus thrungiensis* crystal genes, α-amylase inhibitors (Shade *et al*., 1994) or trypsin inhibitors (Altpeter *et al*., 1999) are used to generate insect resistant transgenic plants.

Another strategy is conferring resistance against nematodes that cause an estimated 10% to 25% annual reduction in crop yield. One of the most useful approaches is the introduction of genes whose product weaken or kill nematode feeding structures (Cai *et al.*, 1997)

Furthermore, genes that provide resistance against plant viruses have been successfully introduced into crop plants. By this technology plants gain resistance against many plant viruses such as, potato virus X (Hoekema *et al*., 1989). Expression of chitinase and glucanase genes (Zhu *et al*., 1994; Jack *et al*., 1995)

resulted in transgenic plants that show resistance against bacterial and fungal pathogens.

Abiotic stresses such as; heat, cold, chilling, salt, heavy metals, drought, flooding also affect the crop productivity. Today a large fraction of world's irrigated cropland is so laden with salt that it cannot be used to grow most of the important crops. Similarly many lands are contaminated with heavy metals that are released from factories, exhausts and chimneys. Because of excess irrigation, the topsoil becomes saltier and all of those adverse conditions make agriculture impossible. Genetic modifications have been carried out and transgenic plants have been generated that can tolerate environmental stresses. The *gutD* gene from *Escherichia coli* has been used to generate salt-tolerant transgenic maize plants (Liu *et al*. 1999).

Another application of GMO technology is improving the nutritional quality of plants. Rice is the staple food for a large fraction of the world's human population. Milling rice removes the beta-carotene, which is the precursor of vitamin A. Vitamin A deficiency causes half a million children to become partially or totally blind each year especially in the countries of Southeast Asia. With the introduction of new genes to rice, transgenic rice exhibits an increased production of beta-carotene so may overcome vitamin A deficiency in children (Ye *et al*., 2000). It is also known that iron deficiency causes anemia in pregnant women and children, and about 400 million women come across with iron deficiency and they are more prone to stillborn or underweight children and to mortality at childbirth. Transgenic rice with elevated levels of iron has been produced which prevent those effects (Lucca *et al.,* 2000). These plants contain 2-4 times the levels of iron normally found in non-transgenic rice.

Production of vaccines and pharmaceuticals in transgenic plants is another area. About one-third of medicines used today are derived from plants, one of the most famous examples being aspirin. Currently, there is intensive research in

progress to investigate the potential of GMO technology to increase the yields of active compounds, or to allow their production in other plants that are easier to manage. An anti-cancer antibody has recently been expressed in rice and wheat seeds that recognizes cells of lung, breast and colon cancer and hence could be useful in both diagnosis and therapy in the future (Stoger *et al*., 2000). Also vaccines against Hepatitis B have been produced in potato, which is easier to use (Richter *et al.,* 2000).

Another application area is the modulation of flower properties and creation of flowers with desirable traits such as color, shape, plant architecture, vase life and resistance against pests and insects. Via expression of antisense gerbera *chalcone synthase* gene, anthocyanin synthesis blocked and the color of flowers were changed dramatically (Elomaa *et al.,* 1993). Moreover, flowers with changed inflorescence were produced with a mutation in a single gene (Coen and Nugent, 1994). Also, with the expression of *acc synthase* or *acc oxidase,* the key enzymes of ethylene biosynthesis, increased flower life of flowers were obtained (Savin *et al.,* 1995).

1.4. *Agrobacterium*-Mediated Gene Transfer Technique

One of the best developed systems for DNA transformation in higher plants is *Agrobacterium*-mediated gene transfer system. This system is derived from the tumor inducing mechanism of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. This method is applicable for most dicotyledonous as well as some monocotyledonous and gymnosperm species.

Agrobacterium has a natural ability to transfer DNA into plants: it has been called, as "nature's own genetic engineer". *Agrobacterium tumefaciens* has the exceptional ability to transfer a particular DNA segment from its extrachromosomal component to a plant cell. Further studies provide the evidence for the presence of a large megaplasmid (more than 200 kb), which carries the

virulence in the *Agrobacterium*. The virulence plasmid is called Ti (tumor-inducing) plasmid in *A. tumefaciens* and Ri (root-inducing) plasmid in *A. rhizogenes*. Both Ti and Ri plasmids are large, circular, double stranded DNA molecules that can replicate, independently from bacterial genome.

There are two well-defined regions on the Ti plasmid: transfer DNA (T-DNA) and virulence (*vir*) regions. Molecular analysis show that not all of the plasmid is transferred to the plant but only a part of it and it is called as T-DNA. There are 25-bp direct repeats at the ends of T-DNA, right border and left border, and any DNA between these borders is transferred to the plant cell. T-DNA contains two types of genes. One type is the oncogenes (*tms1, tms2, and tmr*) encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation. Second type is the genes encoding for the synthesis of aminosugars, opines, which are consumed by *A. tumefaciens* as carbon and nitrogen sources. It has been found that none of the T-DNA genes are essential in the transfer of the T-DNA and it can still be transferred in the absence or mutation of the T-DNA (Kung and Wu, 1993). This is a very important property of the bacterium that enables the use of it in gene transfer studies and at this form it is called as "disarmed Ti plasmid".

The 30 kb virulence (*vir*) region is a regulon organized into eight operons that are essential for the T-DNA transfer (*virA, virB, virD and virG*) or for the increasing of transfer efficiency (*virC* and *virE*) and two non-essential operon (*virF and virH*). These vir genes are involved in the processing of the T-DNA from the Ti plasmid and T-DNA transfer from the bacterium to the plant cell (Riva *et al.,* 1998). The third bacterial element necessary for attachment of bacterial cell to the plant cell consist of a number of chromosomal genes such as, *chvA, chvB chvE, cel and pscA*.

The process of gene transfer from *A. tumefaciens* to plant cells composed of several steps: (1) Bacterial colonization, attachment of bacteria to the plant cell

surface. (2) Induction of bacterial virulence system, *vir* genes. The activation of the *vir* system depends on the external factors like temperature and pH such as at temperatures greater than 32^0C vir genes are not expressed. (3) Generation of a T-DNA transfer complex. The activation of *vir* genes leads to the production of single stranded proteins (ss) that prevents the exonucleolytic attack to the single strand ss-T-DNA complex. (4) T-DNA transfer. The ss-T-DNA complex must be translocated through membranes, plant cell wall and cellular spaces with the help of VirD2 protein. (5) Integration of T-DNA into the plant genome. Inside the plant cell, the ss-T-DNA complex is targeted to nucleus crossing the nuclear membrane. Transgene integration in plants transformed by *Agrobacterium* occurs through illegimate recombination (IR). Recent evidence indicated that the VirD2 protein, which is covalently attached to the T-DNA, interacts with the TATA box binding protein suggests a possible mechanism for targeting T-DNA integration into promoter regions (Bako *et al.,* 2003).

1.5. Ethylene (C_2H_4)

Hormones play a central role in regulating plant growth and development. There are five well-characterized hormones, the chemically simplest of which is the gaseous olefin ethylene (C_2H_4). However many aspects of ethylene physiology remain enigmatic. Ethylene is a very simple molecule, a gas, has been used in practice since the ancient Egyptians, when applied to figs stimulate ripening. The ancient Chinese burned incense in closed rooms to enhance the ripening of pears. In 1864, leaks of gas from streetlights were discovered to cause stunting of growth, twisting of plants, and abnormal thickening of stems. In 1901, Dimitry Neljubow showed that the active component was ethylene (Neljubow, 1901). It was discovered in 1917 that ethylene stimulated abscission and plants have the ability to synthesize ethylene (1934). In 1935, it was proposed that ethylene was the plant hormone responsible for fruit ripening. Elucidation of the C_2H_4 biosynthetic pathway by Adams and Yang (1979) and recent molecular biology applications has greatly stimulated the research in this area.

1.5.1. Biochemical Synthesis of Ethylene

Ethylene is produced in all higher plants and is produced from L-methionine in essentially all tissues. Production of ethylene varies with the type of tissue, the plant species, and also the stage of development. The mechanism by which ethylene is produced from methionine is a 3 step process that is shown in Figure 1.1 (Kieber and Ecker, 1993). There are two key enzymes in this process. 1-amino-cyclopropane-1-carboxylic acid synthase (ACC Synthase) catalyzes the conversion of S-adenosyl-L-methionine (SAM) to 1-amino-cyclopropane-1-carboxylic acid (ACC). However 1-amino-cyclopropane-1-carboxylic acid oxidase (ACC Oxidase) catalyzes the conversion of ACC to ethylene.

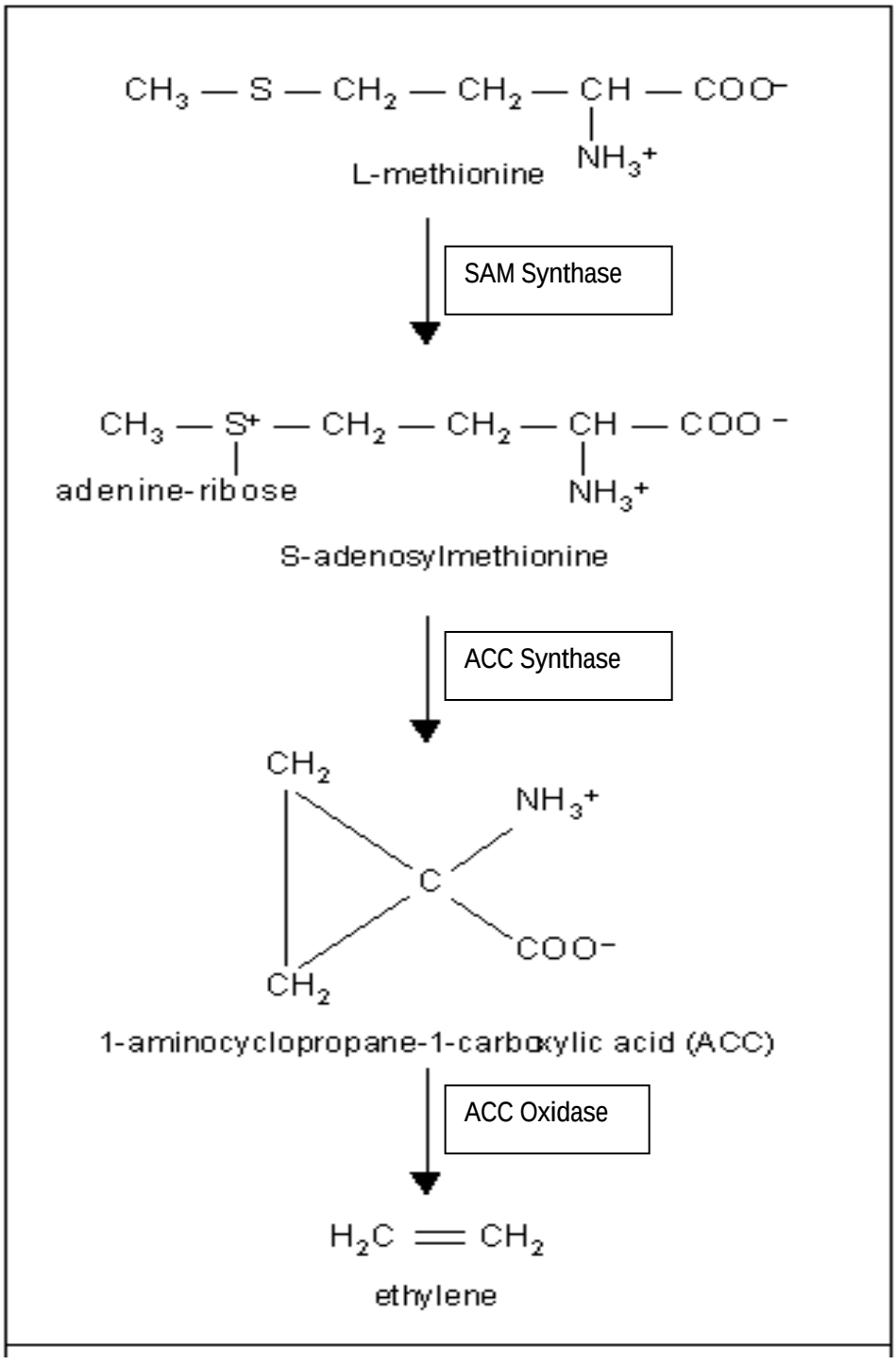

Figure 1.1. Ethylene biosynthetic pathway of higher plants.

ACC Synthase (EC 4.4.1.14) is the rate-limiting enzyme in the ethylene biosynthesis and its activity is highly regulated and closely parallels to the level of ethylene biosynthesis. ACC Synthase has been proven to be quite recalcitrant to biochemical characterization because of its liability and low abundance in plant tissue. A multi-gene family encodes ACC Synthase and these genes are differentially expressed in response to various internal and external stimuli suggesting that different ACC proteins may be functionally distinct, and be produced under different physiological or developmental conditions (Theologis, 1992). ACC Synthase exists in several isoforms. In *Arabidopsis*, there are at least five ACC Synthase genes with different pI values (Kieber *et al.,* 1993).

Analysis of tomato ACC Synthase cDNA sequences revealed that the genes are 50-95% identical to each other (Jiang and Fu, 2000). It was found that differential expression of two ACC Synthase genes in ripening and wound-induced tomato pericarp tissues (Olson *et al.,* 1991). To date, nine ACC Synthase genes have been found in tomato fruit. It was found that among those genes *LE-ACS2* and *LE-ACS4* are greatly induced prior to and during climacteric fruit ripening. It is suggested that ACC Synthase genes other than *LE-ACS2* and *LE-ACS4* are expressed first, and are followed by *LE-ACS2* and *LE-ACS4*, which once induced by ethylene provide autocatalysis of ethylene and trigger climacterium (Olson *et al.,* 1991).

ACC Oxidase (EC 1.14.17.4) is a member of ferrous ion-dependent family of none-heme oxygenase. It requires ferrous ion as a cofactor and utilizes dioxygen as a co-substrate. Unlikely ACC Oxidase uses ascorbate as a co-substrate. It also requires bicarbonate or CO_2 as an activator. Like ACC Synthase it is a member of multi-gene family. The appearance of ACC Oxidase mRNA precedes the ethylene burst and the highest transcript accumulation is coincident with climacterium (Jiang and Fu, 2000). In tomato experiments on LE-ACO1 and LE-ACO3 revealed that genes are coordinately induced by ethylene, causing a very intense autocatalytic ethylene production (Oetiker and Yang, 1995).

1.5.2. Functions of Ethylene

Continued research on the mechanism of synthesis, perception and action in cells suggest that ethylene has numerous effects on the growth, development and storage life of many fruits, vegetables and ornamental crops (Kieber and Ecker, 1993). Plant responses to ethylene are numerous and varied and listed in Table 1.1.

Table 1.1. Plant responses to ethylene (Saltveit, 1998).

Ethylene Stimulates
Synthesis of C_2H_4 in ripening climacteric fruit
Ripening of fruit
Pigment (e.g. anthocyanin) synthesis
Chlorophyll destruction and yellowing
Seed germination
Adventitious root formation
Respiration
Phenylpropanoid metabolism
Flowering of bromeliads
Sex determination in some plants (e.g. cucumber)
Abscission
Senescence
Ethylene Inhibits
Ethylene synthesis in vegetative tissue and non-climacteric fruit
Flower development in most plants
Auxin transport
Shoot and root elongation (growth)

Ethylene's actions on plants can be beneficial or detrimental. Promotion of flowering in pineapple and the acceleration of tomato and melon ripening can be viewed as beneficial. Oldest and perhaps best-known role of ethylene is the ripening of climacteric fruits. Ethylene synthesis is inhibited via negative feedback inhibition by itself in vegetative and immature climacteric or non-climacteric tissues. However, its synthesis is promoted via positive feedback promotion by itself in climacteric tissues (Yang, 1985). Ethylene is effective in plant tissues at part-per-million (ppm, $\mu L\ L^{-1}$) and part-per-billion (ppb, $nL\ L^{-1}$) concentrations. Ethylene promotes germination of seeds from many plant species. In some plants, such as cucumber it affects sex determination (Malepszy and Niemirowicz-Szczytt, 1991). Color development is enhanced through stimulation of pigment synthesis in apples and tomatoes or chlorophyll destruction in banana and citrus and this property of ethylene is commercially used. Ethylene's actions on plants, however, also can be undesirable. For example, ethylene gas is the causative agent in many post harvest disorders of horticultural commodities. It causes premature senescence of many tissues, promotes discoloration (browning), sprouting, yellowing of vegetables, abscission of flowers and fruits, stimulates abortion of flowers, development of russet spotting in lettuce.

However, sometimes the same response is viewed as beneficial for some species whereas deleterious for other. As an example, acceleration of chlorophyll loss can be beneficial for degreening of citrus but deleterious for yellowing of green vegetables or, promotion of ripening can be beneficial for climacteric fruits however deleterious for overly soft fruits or, stimulation of phenyl propanoid metabolism is beneficial for defense against pathogen but deleterious for browning and bitter taste (Saltveit, 1998).

A wide variety of environmental stresses including oxygen deficiency, wounding, pathogen invasion, chilling and drought can lead to ethylene production in plants. Stress induced ethylene elicits adaptive changes in plant development, for example, the formation of air-conducting channels (aerenchyma) in water-

logged terrestrial plants and rapid stem elongation in submerged aquatic plants. Wounding and pathogen invasion may result in ethylene-mediated acceleration of senescence and abscission of infected organs and in the induction of specific defense proteins such as chitinase, glucanases, and hydroxyproline-rich glycoproteins (Bleecker *et al.,* 1988).

In order to produce physiological responses, plants should sense ethylene. Sources of ethylene not only include other plants but also include smoke, exhaust gases, ethylene releasing chemicals (e.g. ethephon), catalytic production of ethylene from ethanol, and ethylene analogues (propylene, carbon monoxide, acetylene and 1-butene) produced by a variety of processes.

1.5.3. Ethylene Perception and Signal Transduction

To understand how ethylene or any other signaling molecule affects a process, control of biosynthesis, transport of signal from source to target, molecular mechanisms underlying perception and removal of signal from target should be taken into consideration. Because of its gaseous nature, its transport both throughout the plant and across the cell membranes and diffusion away from the site of action is under question. In this respect, ethylene is similar to nitric oxide and carbon monoxide, which have been shown to act as signaling molecules in mammalian cells.

Dicotyledonous seedlings grown in the dark undergo dramatic morphological changes in the presence of ethylene. These changes are collectively referred to as the "triple response". The use of triple response screen in *Arabidopsis* has allowed the identification of genes involved in ethylene perception, in control of ethylene biosynthesis or in the propagation of its stimulus (Kieber *et al.,* 1993; Chang *et al.,* 1993). In pea, inhibition of epicotyl and root elongation, radial swelling of epicotyl and root cells and development of horizontal growth habit (loss of normal gravitropizm) were seen whereas in

Arabidopsis inhibition of root and hypocotyls elongation, radial swelling of the hypocotyls and root and exaggeration in the curvature of the apical root were seen. It was suggested that dramatic morphological changes might be a stress-induced adaptation that allows seedlings to penetrate the soil without damaging to the apical meristem (Ecker, 1995). Induction of triple response depends on the plants ability to perceive and respond to ethylene because inhibitors of ethylene perception or biosynthesis and mutations that eliminate all ethylene responses prevent these morphological changes. Because of its high reproducibility and the ease of screening large numbers of individuals at an early stage of development, the triple response phenotype in *Arabidopsis* provides a simple means to identify mutants that either fail to respond to exogenous ethylene or constitutively display the response in the absence of the hormone.

In *Arabidopsis thaliana*, a number of ethylene-response mutants have been isolated, defining several loci in the ethylene signal transduction pathway. Most of the mutants have been ordered into a genetic pathway (Kieber *et al.,* 1993). The first mutant to be described was *etr* (renamed *etr1-1*). The *etr* mutant was suspected to have a defect in ethylene perception, owing to its reduced ethylene binding and its failure to respond to ethylene. It's a dominant ethylene insensitive mutation (Bleecker *et al.,* 1988). Studies showed that ETR1 is an ethylene receptor. It was shown that when mutant *etr* allele was used there was no detectable ethylene binding in yeast cells, indicating that the missense mutation in *etr* (replaces cystein to tyrosine at position 65) blocks ethylene binding, possibly disrupting the binding site (Chang, 1996). Ethylene binding takes place within a membrane, as ethylene gas is 14 times more soluble in lipids than in water. A second gene encoding ethylene receptor was identified called as ERS. ERS consists of an amino terminal domain with 75% identity to ETR, indicating that ERS is a mutant form of ETR and ethylene binds to both of them on plasma membrane (Ecker, 1995). Third gene is CTR1 and it was shown that loss of function of *ctr1* gene causes constitutive activation of the ethylene response pathway in adult plants even in the presence of inhibitors of ethylene biosynthesis

or binding (Kieber *et al.,* 1993). Studies strongly suggest that *ctr1* is a protein kinase and revealed that highest degree of homology (41% identity to the kinase domain) is found with members of the serine-threonine protein kinases in mammals that are mitogen activated protein kinase kinase kinases (MAPKKKs). CTR1 aminoacid sequence suggests that ethylene signaling in plants might involve a similar phosphorylation cascade.

The first step in ethylene response pathway is ethylene perception, which is the binding of the gas to a receptor molecule such as ETR or ESR. Binding of ethylene to a receptor either promotes or inhibits cytoplasmic autophosphorylation of the conserved histidine. In either case, CTR1 is inactivated, because CTR1 is a downstream negative regulator of ethylene response. Downstream of CTR1, there is presumably a phosphorylation cascade leading to altered gene expression (Chang, 1996). The signal transduction pathway of ethylene was first described by Yang and Hoffman (1984) and the pathway is shown in Figure 1.2.

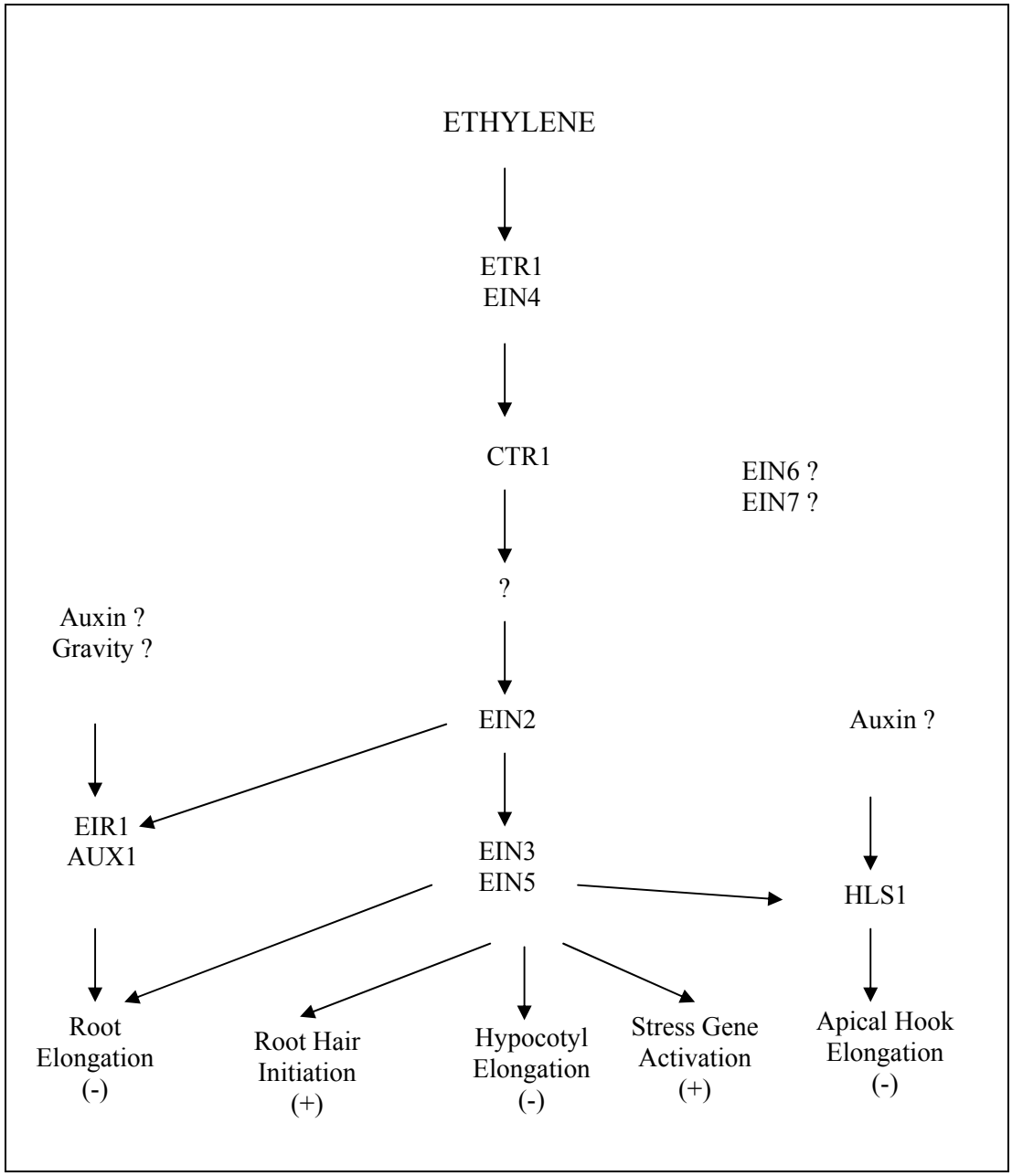

Figure 1.2. A genetic pathway of ethylene signal transduction (Yang and Hoffman, 1984).

Genetic engineering of ETR1, ERS, CTR1 and other ethylene response genes will provide modification of ethylene responses in a variety of plants. As an example transgenic carnation plants were obtained that are expressing *Arabidopsis etr1-1* allele (Bovy *et al.,* 1999). Half of the obtained transgenic carnations showed an increase in vase life of 5.7 days relative to control plants. The transgenic plants did not show the petal inrolling phenotype, typical for ethylene-dependent carnation flower senescence. Instead the petals remained firm throughout the senescence process and slowly became brown on the edges and started to lose color. Also ACC Oxidase mRNA levels were strongly reduced in transgenic plants relative to control plants indicating that in ethylene insensitive *etr1-1* plants, the autocatalytic induction of ethylene biosynthetic genes are blocked, hence senescence process did not initiated.

1.5.4. Engineering of Plants against Ripening and Senescence

In order to increase the post harvest quality of many ornamentals and fruits they have been engineered against ripening. Either exogenous application of some chemicals or expression of foreign genes can be used to extend shelf life of fruits or flowers.

1.5.4.1. Control of Fruit Ripening and Flower Senescence via Exogenous Application of Chemicals

A variety of chemicals can be exogenously applied to the fruits or flowers to postpone the ethylene effects. Silver thiosulfate (STS) is a known inhibitor of ethylene action that is used for this purpose. It has become an essential tool for delay of senescence of flowers and has been applied in the cut flower industry (Reid and Wu, 1992). But STS is a potential environmental hazard and many countries currently prohibit the use of it. At present there are very few alternatives to STS. Some synthetic cyclopropenes have been shown to bind to ethylene receptors and so prevent the physiological action of ethylene. One example is the

use of 1-methycyclopropene (1-MCP), an ethylene inhibitor. It was shown that when applied, at a proper concentration, senescence of parsley leaves were retarded (Ella *et al.,* 2003). It was also found that ACC synthase mRNA could not be detected during storage.

Aminotriazole (AT) is another compound that inhibits the climacteric peak of ethylene production and prolongs the vase life of carnation flowers (Altman and Solomos, 1994). However AT has been classified as a putative carcinogen and therefore it is difficult to use as a cut flower preservative commercially. Aminooxoacetic acid (AOA), a known inhibitor of ACC synthase activity (Van Altvorst and Bovy, 1995) is currently being used in Holland in the cut flower industry, particularly for carnations. Also AOA is rather expensive and carries certain toxicological risks. Boric acid is already in use in preservative solution mixtures and it's a good competitor when price is concerned. It was shown that preservative solutions containing boric acid delay senescence of carnation flowers (Serrano *et al.,* 2001). Boric acid treatments inhibit ethylene production in flowers by lowering ACC Synthase activity and availability of ACC.

Another strategy used to delay ripening is the expression of some of the genes that inhibit ethylene production. One approach to reduce ethylene synthesis in plants is irreversible degradation of the precursor of ethylene, ACC (Klee *et al.,* 1991). Soil bacteria containing an enzyme, ACC deaminase, were identified by their ability to grow on ACC as a sole nitrogen source. Bacterial ACC deaminase gene was cloned and introduced into tomato plants. Visually, transgenic fruits stored at room temperature indicated a significant reduction in softening while control fruits completely become rotten. Degradation of ACC inhibits ethylene synthesis but does not interfere with the fruit's ability to perceive ethylene thus; transgenic fruits when exposed to exogenous ethylene ripen normally.

While ethylene causes premature senescence of both flowers and leaves, it has been shown that cytokinins delay flower and leaf senescence (Long-Fang *et*

al., 2001). This property of cytokinins was used to generate transgenic broccoli over-expressing isopentyl transferase (IPT) gene, the rate-limiting enzyme in cytokinin biosynthesis for post harvest yellowing retardation. Based on chlorophyll retention rate, transformants exhibited the effect of retarding yellowing in detached leaves. Thus, delaying post harvest senescence through endogenous regulation of cytokinins with a transgenic technique appears feasible.

Transforming plants with antisense RNA provides selective inhibition of the gene of interest. A variety of genes were blocked with this technology and one example is the polygalacturonase (PG) gene. Polygalacturonase plays an important role in fruit softening by partially solubilizing pectin from the cell walls. PG antisense gene transferred to tomato plants causes a reduction in PG enzyme activity when the fruit ripen (Smith *et al.,* 1988). Although PG seems successful in the retardation of fruit softening, the most popular genes are ACC Synthase or ACC Oxidase, which codes for the key enzymes in ethylene biosynthesis.

1.5.4.2. Down Regulation of Ethylene Production via Antisense and Sense RNA Inhibition of ACC Oxidase or ACC Synthase

Antisense gene is the nucleic acid sequence complementary to the coding (sense) genetic sequence of the target. It specifically binds to its complementary messanger RNA and blocks the corresponding protein translation. The result is that both antisense and sense gene products are inactivated, and no protein is produced. The hypothetical action mechanism of antisense gene is shown in Figure 1.3. The exact mechanism by which antisense gene brings about a down regulation of sense gene expression is still not clear. However oligonucleotides of as few as 15 bases have been shown to have a down regulatory effect (Bolitho *et al.,* 1997).

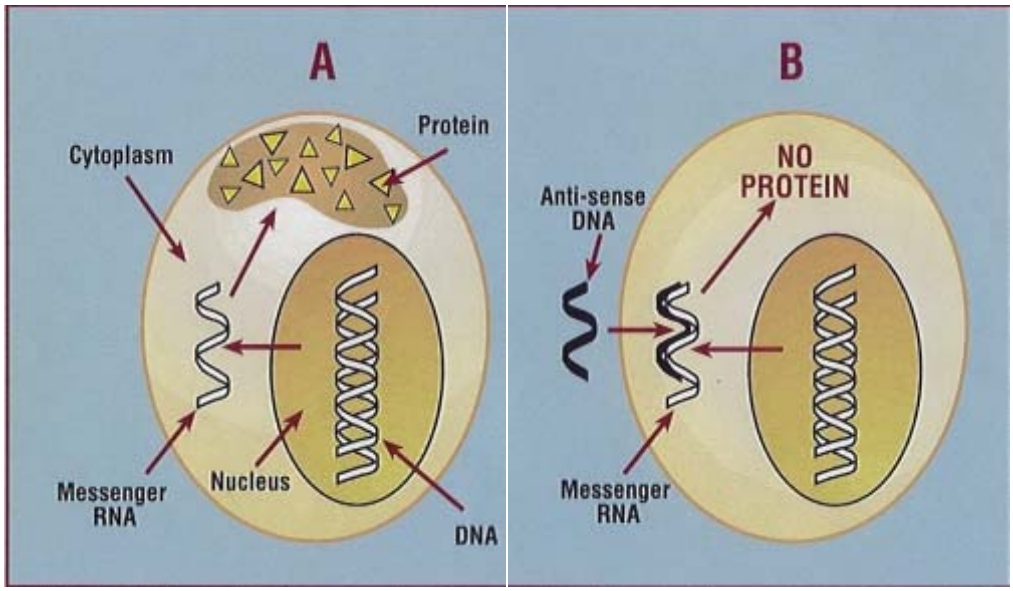

Figure 1.3. The action mechanism of antisense gene. (A) Normal metabolism in plant cell, (B) Effect of antisense gene

Modulating gene expression using antisense technology is rapidly becoming an important approach for achieving targeted alterations in plant biochemical pathways. Commercial applications include alterations of food quality, flower color, flower longevity and fruit ripening.

Concerning food quality, the development of brown discoloration in a wide range of fruit and vegetables is of significant economical importance. Potato was transformed with antisense polyphenol oxidase, which inhibits melanin formation, and at the end there is a reduction in the browning of potato (Bachem e*t al*., 1994). Another example is the accumulation of zeaxanthin, which is the major carotenoid in human retina, in transgenic potato tubers via inactivation of zeaxanthin epoxidase enzyme (Römer *et al.,* 2002).

An important application of antisense technology to the ornamental plant industry is the possibility of changing flower color. The antisense inhibition of

chalcone synthase gene in *Gerbera hybrida* inhibited flower pigmentation (Elomaa *et al.,* 1993) and dihydroflavonol-4-reductase gene inhibition in torenia caused blue color development (Aida *et al.,* 2000).

A major benefit arising from this technology is provision of a longer shelf life of the fruits and flowers. Since ACC Synthase and ACC Oxidase are the key enzymes in ethylene biosynthesis, altering the rate of these enzymes expression via antisense technology may control ripening. Antisense gene transferred fruits and flowers could be stored for longer periods with considerably less damage than is currently sustained. To achieve this, firstly the appropriate *acc synthase* gene needs to be isolated from a particular plant. The correct gene is cloned into a plasmid downstream of a promoter element, in a reverse orientation to the naturally occurring gene. In the next stage, the plasmid needs to be placed into the plant and maintained in a stable way. Because ethylene regulation cannot be selected in a test tube, selectable markers, such as *neomycin phosphotransferase II (npt II*), are used which permits transformed cells to proliferate while non-transformed cells die.

A variety of species have been transformed with antisense ACC Oxidase or antisense ACC Synthase genes. Tomatoes containing an antisense RNA that inhibits the synthesis of one or both of ACC Synthase or ACC Oxidase showed reduced ethylene production (Hamilton *et al.,* 1990; Oeller *et al*., 1991). There is a reduction in ripening rate and ethylene production was inhibited 99.5% as compared with the control fruits in antisense gene transferred tomatoes. Also, the coloration resulting from chlorophyll degradation and carotenoid biosynthesis is inhibited in antisense fruits. Treatment with ethylene accelerates normal ripening and artificially ripened fruits are indistinguishable from naturally ripened fruits with respect to aroma, color, texture and comprehensibility. These results indicates that ethylene controls the climacteric rise of respiration, fruit ripening and biochemical changes like softening, color, aroma development in tomato and if ethylene synthesis is blocked then those effects could be delayed (Oeller *et al.,* 1991; Picton *et al.,* 1993). In another study transgenic cantaloupe melon carrying

melon antisense *acc oxidase* gene exhibited a sharp reduction in ethylene production (Ayub *et al.,* 1996).

Carnation (*Dianthus caryophyllus* L.) has been used for many years as a model system for studying the physiology of flower senescence. The petals exhibit a characteristic inrolling behavior during senescence and it appears to be regulated by ethylene. In the antisense *acc oxidase* gene expressed carnation the level of *acc* oxidase mRNA was suppressed and flower life was extended from 5 to 9 days (Savin *et al.,* 1995). Apple antisense *acc synthase* in antisense orientation was transferred to carnation and 40% reduction in ethylene production was observed (Kiss *et al.,* 2000).

Down-regulation of sense gene expression by homologous, antisense RNA has been successful in a number of plant species. However, the antisense technique would be more powerful if constructs containing cDNAs derived from heterologous species could be used to inhibit expression of genes of interest in a model system. Antisense apple *acc oxidase* mRNA containing tomato plants produces fruits with reduced ethylene production. Some lines show a reduction of ~50% ethylene production and some more than 95%. There is also a delay in color development (Bolitho *et al.,* 1997). It was also shown in broccoli that broccoli senescence could be regulated by tomato antisense *acc oxidase* gene (Henzi *et al.,* 1999).

Moreover, it has been found that transformation of plants with additional copies of expressed genes (sense strategies) can either increase expression or reduce expression through co-suppression. An example to former case is the expression of full-length cDNA of *acc synthase* and *acc oxidase* sense genes in tobacco leading to overexpression of those genes (Knoester *et al.,* 1997). For the later case it was shown that inactivation of endogenous gene can occur when re-introduction of gene in sense orientation (Sijen and Kooter, 2000). The inactivation mechanisms with the sense gene are different from those with

antisense gene but the mechanism of suppression is not yet clear. It is hypothesized that these cDNAs hybridize to complementary mRNAs, which are then degraded by double stranded RNA specific RNases (Stam *et al.,* 1997).

1.6. Aim of the Study

The objective of the present study was to evaluate the potency of the sense and antisense sequences of torenia *acc oxidase* gene expression to block ethylene biosynthesis in transgenic tobacco (*Nicotiana tabacum* cv. Samsun) plants.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strains

E. coli DH5α strain was used as competent cells. *E. coli* strain DH5α harboring the helper plasmid pRK2013 (Ditta *et al*., 1980), was used in the triparental mating experiments. The supervirulent *Agrobacterium tumefaciens* strain EHA105 (Hood, 1993) was used in plant transformation studies.

2.1.2. Plasmids

The plasmids pAO-A and pAO-S (Aida *et al*., 1998) (Appendix A) were constructed by substituting cloned fragment of torenia (*Torenia fournieri* Lind.) 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase gene (~850 bp) into the GUS coding region of a binary vector pBI121 (Clontech, CA) in antisense or sense orientation. Plasmids were donated kindly by Dr. Aida, from National research institute of vegetables, ornamental plants and tea, Ano, Mie, Japon.

2.1.3. Culture Conditions

The medium used for *E. coli* growth was Luria-Bertani Broth (LB) (Appendix E). YEB medium (Appendix E) was used for *A. tumefaciens.* Different combinations of antibiotics were added to the media according to the aim of the use. Cultures were grown with aeration at 37^0C or 28^0C. A liquid aliquot of each bacterial strain was kept at -80^0C in 20% glycerol for longer storage.

2.1.4. Plant Material and Tissue Culture Media

Seeds and leaves of *Nicotiana tabacum* L. cultivar Samsun were used as the explant source. MS (Murashige and Skoog, 1962) basal medium supplemented with sucrose and agar was used in plant tissue culture studies. The composition of the media is presented in Appendix H. The entire medium was dissolved in distilled water; pH of the medium was adjusted to 5.7-5.8 with NaOH and sterilized at 121^0C for 20 minutes. Filter sterilized plant growth regulators and antibiotics (naphtalenaceticacid, benzylaminopurine, cefotaxime, rifampicin, and kanamycin) were added freshly to the sterile medium. The composition and the purpose of the media are given in Table 2.1.

2.1.5. Other Chemicals and Materials

The chemicals used in the preparation of tissue culture media were all commercially available from Sigma, Merck, Oxoid, Fluka and Duchefa. Glassware (autoclavable capped tissue culture jars) was from Sigma and 0.2 μm minipore filters were from Sartorius. The chemicals used in molecular biology studies were from MBI Fermentas and Amersham Biosciences.

Table 2.1. The composition and the purpose of plant tissue culture media

Media	Composition	Purpose of Use
Liquid MS	M5519 + 3% sucrose	Diluting *Agrobacterium* culture before transformation
MSA	M5519 + 3% sucrose + 0.8% plant agar + 1 mg/L BA + 0.1 mg/L NAA	Callus and shoot formation for control leaf discs, co-cultivation for *Agrobacterium* treated leaf discs
MSB	MSA + 75 mg/L Kanamycin + 500 mg/L Cefotaxime	Selection of transformed leaf discs and their regeneration
MSC	MSA for control shoots MSB without plant growth regulators for transgenic shoots	Induction of root formation on regenerated shoots

2.2. Methods

2.2.1. Bacterial Growth

E. coli cultures were grown with vigorous shaking at 37°C in LB medium. *A. tumefaciens* cultures were grown with vigorous shaking at 28°C in YEB medium. When needed 1.5% agar was added to solidify the media. Filter sterilized antibiotics were also added to sterile media for selective growth.

2.2.2. Transformation Studies with Bacterial Cells

All vectors were first transformed to *E. coli* cells and selected on kanamycin containing medium. Transformed *E. coli* cells were named as DH5α::pAO-A and DH5α::pAO-S. Presence of the *nptII* gene was tested by PCR

studies. Also plasmids were isolated and digested with restriction endonucleases. These vectors were transferred to *A. tumefaciens* via triparental mating, selected on kanamycin and rifampicin containing medium and named as EHA105::pAO-A and EHA105::pAO-S, respectively. Presence of *nptII* gene was tested with PCR studies and transformed bacterial cells were used for gene transfer studies to tobacco.

2.2.2.1. Mobilization of Vectors to *E. coli*

2.2.2.1.1. Competent *E. coli* Preparation

Competent *E. coli* cells were prepared according to the procedure of Inoue *et al*., 1990. *E. coli* DH5α cells were inoculated on LB plate by streaking and cultured overnight at 37^0C. About ten to twelve large colonies were collected with a loop and inoculated to 250 mL of SOB medium (Appendix E) in a 1 L flask. The culture was grown with vigorous shaking (200-250 rpm) at 18°C until an OD_{600} value of 0.6. The culture was kept in ice bath for 10 minutes and was precipitated by centrifugation at 2500 g for 10 minutes at 4°C. The pellet was suspended in 80 mL of ice-cold TB (Appendix E) and incubated in ice bath for 10 minutes and centrifuged as explained previously. The pellet was suspended in 20 mL of TB and DMSO (dimethyl sulfoxide) was added with gentle swirling to a final concentration of 7%. The suspension was incubated in ice bath for 10 minutes and competent cells were transferred into eppendorf tubes in 0.1 mL fractions and immediately chilled by immersing in liquid nitrogen. The frozen competent cells could be stored at –80°C for a few months.

2.2.2.1.2. Transformation of *E. coli*

The transformation studies were carried out according to Maniatis *et al*., 1989. Frozen competent *E. coli* cells were thawed in ice bath and 5-20 μL (at least 10 ng) of plasmid were added for each transformation. The eppendorf tubes were

mixed by inversion and were kept in ice bath for 45 minutes. After cold treatment a heat shock at 42^0C for 90 seconds was applied to enhance the entrance of plasmids into the cells. The cells were transferred to ice for another 5 minutes. The cells were suspended in 900 μL of LB and incubated at 37°C for 45 minutes with vigorous shaking. After centrifugation at 3000 rpm for 3 minutes, 900 μL of supernatant was removed and cells were suspended in the remaining 100 μL of solution. At the end transformed bacteria were spread on solid LB plates containing 50 mg/L kanamycin and incubated overnight at 37°C. The plates were stored at 4^0C for further use. From positive colonies molecular analyses were performed.

2.2.2.2. Plasmid Isolation

Not all colonies growing on selective media carries the plasmid bearing the desired gene. In order to verify the presence of gene of interest in transformed *E. coli* cells plasmid isolation and PCR studies were performed.

2.2.2.2.1. Mini Scale Plasmid Isolation

In order to use in further studies the plasmids pAO-A and pAO-S were isolated in pure form. For isolation Mini Scale Plasmid Isolation protocol (Maniatis *et al*., 1989) was used.

A single colony of *E. coli* DH5α::pAO-A or DH5α::pAO-S was taken from the selective agar plate by loop. It was transferred to a 3 mL of LB medium containing 50 mg/L kanamycin. The cultures were grown with vigorous shaking at 120 rpm and 37°C. Overnight grown bacterial cells were divided into eppendorf tubes and centrifuged at 13000 rpm for 30 seconds in microcentrifuge. The supernatant was discarded and the pellet was suspended in 100 μL of SOL I (Appendix G) with vigorous vortexing. Then they were incubated for 5 minutes at room temperature and 200 μL of SOL II (Appendix G) was added into each

eppendorf tube. The tubes were mixed by tapping with finger until obtaining a clear mixture and incubated 5 minutes in ice bath. 150 µL of cold SOL III (Appendix G) was added into the tubes gently, and the tubes were incubated in ice bath for 10 minutes. 450 µl of phenol:chloroform:isoamylalcohol (25:24:1) was added to separate cell debris and chromosomal DNA in different phases. After centrifugation at 13000 rpm for 10 minutes upper DNA phase was collected into a clean eppendorf tube. Plasmid DNA was precipitated with equal volume of cold isopropanol at least for 30 minutes. Overnight incubation may be preferable. The plasmid DNA was obtained in the pellet after centrifugation at 13000 rpm for 10 minutes. Pellet was washed with 70% ethanol, centrifuged at 13000 rpm for 10 minutes and ethanol was removed. The pellet was dried completely at 37°C and dissolved in 50 µL of TE buffer (Appendix F) containing 20 µg/mL RNAse. After the incubation for 30 minutes at 37°C for RNAse activity, plasmid DNA was stored at –20°C until usage. Purity and integrity of DNA was confirmed by spectrophotometric measurement (OD_{260} and OD_{280}) and agarose gel electrophoresis, respectively. The plasmids were checked by restriction enzyme digestion and agarose gel electrophoresis for the presence of gene of interest.

2.2.2.2.2. PCR Analysis

The Polymerase Chain Reaction (PCR) was performed in order to verify the presence of *nptII* gene within the plasmids. For this purpose, the gene was amplified with specific primers (Appendix B). After PCR amplification the products were run on agarose gel and photographed.

PCR was carried out in a total volume of 30 µL. All of the components of PCR were kept in ice before usage. Master mix was prepared in ice and 10 µL of mineral oil was added on top of each eppendorf tube in order to prevent evaporation of contents. Cycling program of PCR amplification and optimized conditions of PCR are given in Table 2.2 and Table 2.3 respectively.

Table 2.2. PCR cycling conditions to amplify *nptII* gene

Initial denaturation	94°C 5min	
Denaturation	94°C 1min	
Annealing	55°C 45sec	35 cycles
Extension	72°C 30sec	
Final extension	72°C 5min	

Table 2.3. Optimized conditions of PCR to amplify *nptII*

dH_2O	22.6 µL
10X Reaction Buffer with $(NH_4)_2SO_4$	3 µL
25 mM $MgCl_2$	1.5 µL
2.5 mM dNTP	0.6 µL
Primer Forward (100 pmol/µL)	0.4 µL
Primer Reverse (100 pmol/µL)	0.4 µL
Taq Polymerase (5 u/µL)	0.5 µL
Template DNA (1 µg)	1 µL
Total Volume	30 µL

DNA molecules obtained throughout this study were visualized on agarose gels. Depending on the size of DNA to be detected 0.6 %, 0.8 % or 1 % agarose gels were used. For this purpose 0.3 g, 0.4 g or 0.5 g of agarose was dissolved in 50 mL of 0.5X TBE or 1X TAE buffer (Appendix F) in microwave oven, respectively. Without boiling when it completely dissolved it was cooled down to 50°C and 5 µL of ethidium bromide (10 mg/mL) was added to gel solution. The comb was placed for well formation and without making bubbles the solution was poured into the electrophoresis tray. When the gel completely solidified the comb was removed carefully. The sample solution to be loaded was prepared by mixing

samples with 6X loading buffer to a final concentration of 1X and loaded into the wells. A molecular size marker like DNA ladder, λ-phage DNA digested with HindIII or PstI was also loaded into a separate well. Then the tank was connected to a power supply and electrophoresis was performed under constant voltage of 60 V for 2-3 hours. The gel was observed under UV light and photographed.

2.2.2.3. Mobilization of Vectors to *Agrobacterium tumefaciens*

Triparental mating method was performed according to the procedure of Van Haute *et al*., 1983. The *Agrobacterium* host, EHA105 was grown with vigorous shaking at 160 rpm and 28°C. The *E. coli* strains, HB101::pRK2013, DH5α::pAO-A, DH5α::pAO-S were grown with vigorous shaking at 120 rpm and 37°C.

The overnight grown cultures of *Agrobacterium* and both *E. coli* strains were taken into sterile cabinet. In one tube DH5α::pAO-A, HB101::pRK2013 and *Agrobacterium* host and in another one DH5α::pAO-S, HB101::pRK2013 and *Agrobacterium* host were mixed (1 mL from each) as mate and only *Agrobacterium* host and helper *E. coli* strain were mixed (1 mL from each) as control. The mixtures were centrifuged at 3000 rpm for 5 minutes and the pellets were suspended in about 200 µL of 10 mM $MgSO_4$ and poured on LB plates. The plates were incubated at 28-30°C for overnight growth.

The grown lawns of bacteria were scraped and suspended in 500-1000 µL of $MgSO_4$. 100-200 µL of mixture was spread on selective LB plates containing 20 mg/L rifampicin and 50 mg/L kanamycin. The plates were incubated at 28°C for overnight. This step was repeated 4-5 times to eliminate the escapes. From one part of the plate, a sample was taken and suspended in $MgSO_4$ and streaked on selective LB plates in order to obtain single colonies.

The transfers of binary vectors into the *Agrobacterium* host were checked with PCR studies and positive colonies were cultured in selective LB medium. Glycerol stocks were prepared for -80^0C. Transformed cells, EHA105::pAO-A and EHA105::pAO-S, were used in plant transformation studies.

2.2.3. Maintenance of Plant Material

2.2.3.1. Surface Sterilization of Tobacco Seeds

The tobacco seeds were taken into eppendorf tube and surface sterilized with 30% (v/v) sodium hypochloride for 20 minutes and rinsed 3 times with sterile distilled water. The seeds were kept in sufficient amount of distilled water and placed on culture medium with the help of micropipette.

2.2.3.2. Micropropagation of Tobacco Plants

The surface sterilized tobacco seeds were germinated on M9274 in a growth chamber at 25±2^{o}C with 16 hours light and 8 hours dark photo- cycle for a month. The meristematic part of the emerging shoots were cut and placed into new jars containing the same medium called subculturing and used in further studies.

2.2.4. Leaf Disc Transformation of *Nicotiana tabacum*

A modified transformation method of Öktem *et al*., 1994, which was first described by Horsch *et al*., 1985 was used in the transformation studies of tobacco leaves.

Young leaves from 20-30 days old tobacco plants were used. Either sterile plants were used or non-sterile plants were first surface sterilized in 1.5% (v/v) Sodium Hypochloride and rinsed 3 times with distilled water then used in plant transformation studies.

Edges, midribs and petioles of the tobacco leaves were removed and the remaining parts were cut into 1-2 cm^2 pieces, which are referred to as "leaf disc" in the text. *A. tumefaciens* cells were grown overnight (OD_{600} = 0.6-0.8) in YEB medium containing appropriate antibiotics at 28^oC and they were diluted 1:10 times with liquid MS medium. Leaf discs were incubated in liquid MS and *Agrobacterium* cell mixture for 10 minutes. As a control, some discs were incubated in liquid MS only. At the end of incubation period, the leaf discs were removed and the excess *Agrobacterium* solution remaining on the leaf discs were blotted on sterile filter paper. Then, the explants were transferred on MSA media and kept at dark for co-cultivation for 3 days. After the co-cultivation period, the explants were washed with liquid MS containing 500 mg/L cefotaxime for 3 hours with vigorous shaking at 120 rpm. The leaf discs were blot dried on sterile filter paper and transferred onto MSB media and kept at normal growth conditions. Plates were refreshed at every two weeks.

Callus formation was observed 2-3 weeks after the experiment. Regenerated shoots were cut from the attachment points to callus and transferred to jars containing MSC media to induce root formation and further development. When roots become stronger, regenerated plants were removed from jars and transferred to soil and transferred to a growth chamber. They were used in further molecular studies. When flowers developed they were covered with nylon bags to prevent cross-pollination. When dried completely seeds of putative transgenic plants were collected.

2.2.5. Analysis of Putative Transgenic Plants

2.2.5.1. Leaf Disc Assay

In this assay both putative transgenic plants and wild type plant were used. This assay was based on the kanamycin resistance of transformed lines. If a plant is successfully transformed at the end of this experiment it can withstand kanamycin in the medium and regenerate whereas wild type plant cannot regenerate.

In order to examine the regeneration potencies of putative transgenic plants and wild type plants on selective medium, leaf discs were prepared. Leaf discs were placed on M9274 medium containing 0.1 mg/L NAA, 1 mg/L BA and 100 mg/L kanamycin. After 3-4 weeks they were photographed.

2.2.5.2. Color Viability Test

This test is also referred to as “chlorophenol red test” and it’s based on the color change of culture media because of pH change. The change of color is visualized by the pH indicator chlorophenol red. This test was performed because it was easy to apply and the results of the test were obtained in 4-5 days.

For this test both putative transgenic tobacco plants and wild type tobacco plant were used. Leaf discs were prepared and placed on MSB media containing 75 mg/L kanamycin and 15 mg/L chlorophenol red. The plates were kept at normal growth conditions and photographed at the first day of the experiment and five days after the experiment.

2.2.5.3. Analysis of Putative Transgenic Plants via PCR

The genomic DNA was isolated from plants with CTAB DNA extraction method of Saghai-Maroof *et al*., 1984 with some modifications. According to this method 2 grams of plant material was ground to a fine powder with a pestle and mortar in liquid nitrogen and transferred to centrifuge tubes. 2X CTAB buffer (Appendix I) was heated to 65^{o}C and 20 mL of 2X CTAB buffer containing 0.2% β-mercaptoethanol was added onto the powder and the centrifuge tubes were incubated at 65^{o}C for at least 45 minutes with occasional shaking.

After incubation period, 20 mL of chloroform:isoamylalcohol (24:1) was added and the mixture was mixed vigorously. They were centrifuged at 3000 rpm for 10 minutes. The upper phase was taken to a new centrifuge tube and 4 mL of preheated 10X CTAB buffer was added. Then 20 mL of chloroform was added and the tubes were shaked and centrifuged at 3000 rpm for 10 minutes. Upper phase was taken to a new centrifuge tube and 20 mL of cold isopropanol was added into the tubes and the tubes were inverted gently to precipitate DNA. The tubes were kept overnight at –20^{o}C and, DNA was collected by centrifugation at 9000 rpm for 15 minutes. The upper phase was taken and the pellet was dried with filter paper. The pellet was washed with 70% ethanol and centrifuged at 9000 rpm for 5 to 10 minutes and dried at 37^{o}C. Finally, the pellet was dissolved in 500 μL of TE buffer (Appendix F) containing 20 mg/mL RNAse. The tubes were incubated at 37^{o}C for 30 minutes for RNAse activity. The purity and concentration of DNA was checked by spectrophotometric measurement and agarose gel electrophoresis. The prepared DNA can be stored at –20^{0}C for further usage.

The isolated DNA from putative transgenic plants and control plant were analyzed with PCR for the presence of *nptII* gene and *acc oxidase* gene. Cycling program of PCR amplification and optimized conditions of PCR for *nptII* and *acc oxidase* genes from isolated DNA samples are given in Table 2.4 and Table 2.5., respectively. According to the DNA concentration of putative transgenic plants the

volume of 1 µg of DNA was determined. Remaining part of the reaction was the same as described in part 2.2.5.2.1. At the end of PCR the products were run visualized on 0.8% agarose gel and they were photographed under UV.

Table 2.4. PCR cycling conditions to amplify *nptII* and *acc oxidase*

	nptII primers	*ccc oxidase* primers
Initial denaturation	94°C 5min	94°C 5min
Denaturation	94°C 1min	94°C 1min
Annealing	55°C 45sec	51°C 1min
Extension	72°C 30sec	72°C 2min
Final extension	72°C 5min	72°C 5min

35 cycles

Table 2.5. Optimized conditions of PCR to amplify *nptII and acc oxidase*

	nptII primers	*acc oxidase* primers
dH_2O	23.6-X µL	23-X µL
10X Reaction Buffer with $(NH_4)_2SO_4$	3 µL	3 µL
25 mM $MgCl_2$	1.5 µL	1.5 µL
2.5 mM dNTP	0.6 µL	0.6 µL
Primer Forward	0.4 µL (100 pmol/µL)	0.7 µL (25 pmol/µL)
Primer Reverse	0.4 µL (100 pmol/µL)	0.7 µL (25 pmol/µL)
Taq Polymerase (5 u/µL)	0.5 µL	0.5 µL
DNA (1 µg)	X µL	X µL
Total Volume	30 µL	30 µL

2.2.5.4. Expression Analysis of *acc oxidase*

Northern Blot Hybridization was used to analyze the expression of *acc oxidase* gene. Northern Blot Hybridization was used to show the *acc oxidase* expression in T_1 transgenic tobacco plants using leaf samples of individual lines of tobacco.

2.2.5.4.1. Total RNA Isolation

Prior to RNA isolation, distilled water, mortars, pestles, eppendorf tubes, tips and other equipments were washed with DEPC-treated water and placed under hood to evaporate the excess DEPC. All the solutions used in the RNA isolation were prepared with DEPC-treated water and autoclaved before use. For 1 L of water 800 µL of DEPC was used.

Grinding step was crucial. Mortar, pestle and eppendorf tubes should be chilled with liquid nitrogen before usage. 0.1 gram of leaf sample was ground in liquid nitrogen to obtain a fine powder. Eppendorf tubes were half filled with powder and transferred into liquid nitrogen and they were never let to be warmed up. 1 mL TRI reagent (Appendix J) was added and the eppendorf tubes were incubated at room temperature for 3 minutes with occasional stirring until all plant material dissolved completely. 200 µL of chloroform was added and the contents were mixed for 15 seconds with vigorous shaking. After centrifugation at 14000 rpm for 5 minutes upper phase was transferred to a clean eppendorf tube and equal volume of isopropanol was added. The tubes were incubated at room temperature for 10 minutes. Then the tubes were centrifuged at 14000 rpm for 15 minutes to precipitate the isolated RNA. The supernatant was discarded and the pellet was washed with 70% ethanol. After centrifugation at 14000 rpm for 5 minutes the pellet was dried at 65^0C for few minutes.

Pellet was dissolved in 300 µL of DEPC-treated water with vortexing and heating at 65^0C. When isolated RNA was dissolved completely 30 µL of DEPC-treated 3 M sodium acetate pH: 5.2 and 150 µL of tris-equilibrated phenol pH: 8.0 (Appendix J) were added and the contents were mixed with occasional shaking for 3 minutes at room temperature. 150 µL of chloroform was added and the tubes were vortexed for 30 seconds and centrifuged at 14000 rpm for 5 min. Upper phase was transferred to a new eppendorf tube and equal volume of isopropanol was added and the tubes were incubated at room temperature for 10 minutes. After centrifugation at 14000 rpm for 15 minutes the supernatant was discarded and the pellet containing RNA was washed with 70% ethanol. Another centrifugation at 14000 rpm for 5 minutes was carried out and the supernatant was discarded. The pellet was dried at 65^0C and dissolved in 50 µL of DEPC-treated water. RNA can be immediately used or kept at -80 C for further use.

2.2.5.4.2. Preparing RNA for Northern Blot

The purity and concentration of RNA samples were checked by spectrophotometric measurement. Optical measurements were performed at 260 nm for nucleic acids and 280 nm for proteins. OD_{260}/OD_{280} should be approximately equals to 2 for pure RNA. After measuring the optical densities the concentration of RNA can be calculated as follows:

µg/mL RNA = 40 x Dilution Factor x OD_{260}

2 µg of isolated RNA was loaded to agarose gel check the concentration and the concentration of RNA samples were set to 30 µg. The RNA extracts containing 30 µg of RNA were vacuum-dried.

2.2.5.4.3. Northern Blotting

Vacuum-dried 30 μg of RNA samples were dissolved in 20 μL of freshly prepared sample buffer (Appendix K). For denaturation, the eppendorf tubes were incubated at 65^0C for 10 minutes. The samples were loaded to 1% agarose gel containing, 1X MOPS (Appendix K) and 6% formaldehyde and electrophoresis was run for 2-3 hours.

A Hybond-N^+ nylon membrane, 3 pieces of 3 MM paper and paper towels (at least 5 cm) having same sizes with the gel were cut. A plastic tray was half filled with transfer buffer (Appendix K) and a platform was established and covered with a wick made from two sheets of 3 MM paper. The papers were saturated with transfer buffer and the gel was placed on the wick platform. The circumferences of the gel were covered with parafilm to prevent the absorption of transfer buffer by paper towels directly.

A Hybond-N^+ nylon membrane was placed on the gel and wetted with transfer buffer. Three sheets of 3 MM paper was placed on the membrane one by one and each of them was wetted with transfer buffer after placement. After each step rolling a glass pipette prevented the formation of air bubbles. Absorbent paper towels were placed on 3 MM papers. Finally a glass plate and a 1 kg weight were placed on the towels and the capillary transfer was allowed to proceed overnight. Next day the transfer apparatus was dismantled and the nucleic acids were fixed to the membrane by incubation at 80^0C for 2 hours.

2.2.5.4.4. Probe Labelling, Hybridization and Detection

The membrane was placed in hybridization tube and pre-hybridized with the use of hybridization buffer (Appendix K) for at least 1 hour at 55^0C in a rolling tube hybridization oven. During pre-hybridization the probe that will be used for

hybridization was prepared. Labeling of the probe was done according to Amersham Direct DNA Labelling System.

The probe concentration was adjusted to 10 ng/μL. 10 μL of crosslinker solution was diluted with 40 μL of water supplied with kit to obtain the working concentration. 10 μL/cm^2 DNA was placed into an eppendorf tube and denatured by heating for 5 minutes in a vigorously boiling water bath. Eppendorf tubes were immediately cooled on ice for 5 minutes and centrifuged to collect the contents at the bottom of the tube. 1 μL/cm^2 of reaction buffer was added to the cooled DNA and the contents were mixed thoroughly but gently. 0.2 μL/cm^2 of labeling reagent was added to the eppendorf tube and the contents were mixed thoroughly but gently. 1 μL/cm^2 of crosslinker working solution was added and the contents were mixed thoroughly. The contents were collected at the bottom of the tube by microcentrifugation. Eppendorf tube was incubated at 37^{0}C for 30 minutes. The prepared probe can be used immediately or kept on ice for up to 2 hours.

The labeled probe was then added to the pre-hybridized membrane and left for hybridization at 55^{0}C overnight in rolling tube hybridization oven. The following day, membrane was first washed with primary wash buffer and then secondary wash buffer. The blots were transferred to preheated primary wash buffer and washed two times at 55^{0}C for 10 minutes with gentle agitation. The blot was transferred to a clean container and washed twice with excess secondary wash buffer working solution at room temperature for 5 minutes. Secondary wash buffer working solution should be used immediately after preparation.

The excess secondary wash buffer working solution was drained from the blot and the blot was transferred on a glass surface. 30-40 μL/cm^2 of detection reagent was pipetted on to the blot and the blot was leaved for 2-5 minutes. The blot was wrapped in a stretch film and put into a film cassette in DNA side up position. A sheet of film was placed on the blot and the cassette was incubated at room temperature for 1 hour. The film was taken and developed.

2.2.5.5. Mendelian Inheritance Pattern

Similar to the leaf disc assay both putative transgenic tobacco seeds and wild type tobacco seeds were used. Tobacco seeds were surface sterilized as explained before and then they were placed on M9274 medium containing 75 mg/L of kanamycin. For each line nearly 100 seeds were used. The plates were incubated in a growth chamber at 25±2^{o}C with 16 hours light and 8 hours dark photo- cycle for six weeks. At the end of six weeks the plates were photographed. Kanamycin resistant lines were used for further studies and kanamycin sensitive lines were named as escape lines. The number of surviving plantlets on kanamycin containing medium was counted after 6 weeks and then Chi-Square Analysis were performed to test the Mendelian Inheritance Pattern.

2.2.5.6. Ethylene Measurement of T_1 Lines

The bioassays of T_1 transgenic plants include measurement of ethylene production from flowers. For this purpose, plants were grown in soil at normal growth conditions.

This assay was composed of two parts. The objective of the first part of the experiment was to determine the incubation period at which the flowers produce maximum amount of ethylene. For this purpose wild type control plants were used. The stigmas of the flowers were crushed on the day of anthesis, and the plants were kept in the growth chamber. After 24 hours the flowers were cut and weighed and put in a 15 mL falcon tubes. The tubes were sealed with parafilm and kept at 25^{0}C. 5 mL of gas sample was withdrawn into a syringe for 2 days with 1-hour intervals. The ethylene concentration was determined with a gas chromatograph (TRACE GC), equipped with a flame ionization detector (Ward *et al*., 1978). From these results 2 hours was found to be the time at which maximum amount of ethylene is being produced. In the second part of the assay two transgenic lines were used, A1 and S7. For A1 line two different flowers and for S7 line five

different flowers were used. The amount of ethylene production from flowers after 2 hours of incubation period were measured with the same protocol as explained for wild type control plants. The percent reductions in ethylene production for transgenic lines were calculated with taking the ethylene production of wild type flowers as 100%.

CHAPTER III

RESULTS AND DISCUSSION

3.1. Transformation studies

In order to see the expression of torenia *acc oxidase* gene in sense and antisense orientations in tobacco (*Nicotiana tabacum* cultivar Samsun), firstly pAO-A and pAO-S binary vectors were transferred into *Agrobacterium tumefaciens* strain EHA105 via triparental mating, secondly successfully transformed *Agrobacterium* cells were used to transform tobacco cells.

3.1.1. Transformation of *Agrobacterium* cells via Triparental Mating

Binary vectors pAO-A and pAO-S were isolated from PCR positive *E. coli* DH5α cells to use in triparental mating studies. To verify the orientation of *acc oxidase* gene within the plasmids, pAO-A was digested with PstI and XbaI restriction enzymes and pAO-S was digested with BamHI restriction enzyme. Agarose gel results of the restriction enzyme digestion of plasmids are shown in Figure 3.1.

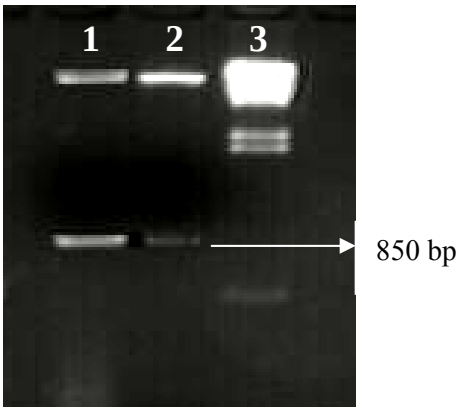

Figure 3.1. Agarose gel results of restriction enzyme digestion of plasmids pAO-A and pAO-S

Lane 1. pAO-A digested with PstI and XbaI

Lane 2. pAO-S digested with BamHI

Lane 3. λ HindIII marker

Both pAO-A and pAO-S plasmids seemed to be containing *acc oxidase* gene as pAO-A and pAO-S plasmid digestions gave band at 850 bp, which is the expected size of torenia *acc oxidase* gene. After proper orientation of *acc oxidase* gene in the plasmids was verified, the plasmids containing the gene were transferred into the *Agrobacterium tumefaciens*. Different protocols are applicable for the mobilization of gene of interest into *Agrobacterium* cells. Electroporation (Shen and Ford, 1989) and triparental mating (Van Haute *et al.,* 1983) are the most widely used techniques for this purpose. In this study, triparental mating was performed to transform *Agrobacterium* cells.

Different from electroporation, which excludes the involvement of other bacteria and integration of gene is reached with the application of high voltage; in triparental mating method *E. coli* was used in transformation studies. In this study *E. coli* strain HB101::pRK2013, *Agrobacterium tumefaciens* strain EHA105 and *E. coli* strains DH5α::pAO-A and DH5α::pAO-S were used. The binary vectors

pAO-A and pAO-S are derivative of pBI121. pBI121 vector carries the *neomycine phosphotransferase-II (nptII) and β-glucorinidase (GUS*) in between the border sequences under the control of nopaline synthase and cauliflower mosaic virus 35S promoters, respectively. Torenia *acc oxidase* gene was inserted into GUS coding region in antisense (pAO-A) and sense (pAO-S) orientations (Aida *et al*., 1998) and the schematic illustrations of the gene cassette in between the border sequences are given in Appendix A.

nptII gene confers resistance to kanamycin and in transformation studies this characteristic was used for selection of transformed cells. The transformed *Agrobacterium* cells survived on rifampicin and kanamycin containing YEB agar plates. Non-transformed *Agrobacterium* cells could not able to survive on those plates because of kanamycin. Similarly *E.coli* cells could not able to survive on those plates because of rifampicin. Only the successfully transformed *Agrobacterium* cells could survive on kanamycin and rifampicin containing plates.

Overnight grown positive colonies from the selective YEB agar plates were used in PCR reaction in which pure plasmid was used as positive control. PCR analysis using *nptII* specific primers (Appendix B) was used to show the presence of the *nptII* gene in other words, T-DNA region of pAO-A and pAO-S in the transformed EHA105 cells. The result of PCR analysis of *nptII* gene amplification after triparental mating is present in Figure 3.2.

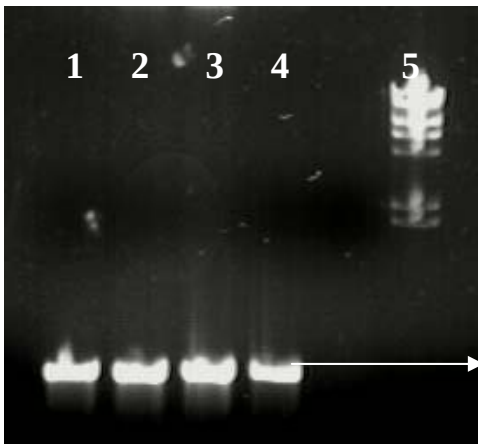

Figure 3.2. PCR Analysis of n*ptII* gene amplification after triparental mating.
Lane 1. EHA105::pAO-A **Lane 4.** pAO-S
Lane 2,3. EHA105::pAO-S **Lane 5.** λ HindIII marker

For PCR one antisense and two sense colonies were chosen. A 717 bp PCR product was present both in plasmid and transformed *Agrobacterium* cells, which is the expected band for *nptII* gene amplification. Besides surviving on selective plates containing rifampicin and kanamycin, presence of PCR products at 717 bp in transformed *Agrobacterium* cells can be explained as *Agrobacterium* cells were successfully transformed with pAO-A and pAO-S and they could be used for plant transformation studies.

3.1.2. Regeneration and Selection of T_0 and T_1 Transgenic Plants

For successful gene transfer to plants three criteria should be followed (Hansen and Wright, 1999). First step is the isolation and cloning of the target gene in a suitable vector. Second step is the choice of a gene transfer technique and last step is the establishment of regeneration protocols. Besides, target tissue should be competent for transformation and regeneration. For tobacco, there are various gene transfer methods, and *Agrobacterium*-mediated transformation is one of the most used techniques. In this study successfully transformed, PCR positive

EHA105 cells, EHA105::pAO-A and EHA105::pAO-S, were used in the *Agrobacterium*-mediated transformation of tobacco.

Ethylene has been shown to cause immature senescence and ripening in fruits, vegetables and flowers, resulting in significant financial losses. In the literature there are several studies concerning the transformation of plants with key enzymes in ethylene biosynthesis, ACC Synthase and ACC Oxidase, to reduce ethylene production from plants. To transfer antisense *acc oxidase* gene from different sources to broccoli (Henzi *et al.,* 1999), carnation (Savin *et al.,* 1995) and tomato (Bolitho *et al.,* 1997), *Agrobacterium*-mediated gene transfer technique was used. Similar result has been reported for antisense acc synthase gene transfer to carnation (Kiss *et al*., 2000) using *Agrobacterium* technique.

Most of the studies in this area based on the transfer of antisense genes but it has been shown that sense gene expression in plants cause similar results with the antisense gene expression (Knoester *et al.*, 1997; Aida *et al.,* 1998). In our experiments we tried to analyze the effect of both sense and antisense expression of torenia *acc oxidase* transgene in tobacco.

The tobacco leaves that are used for transformation were approximately of same age and appearance for transformed and non-transformed plants. In each independent transformation experiment positive and negative control plates and treated plates were present. Positive control means non-transformed leaf discs and they were placed on nonselective MSA media. Callus formation should be seen in positive control leaf discs. Negative control means non-transformed leaf discs and they were placed on selective MSB media. Negative control leaf discs were not able to form callus and necrosis was observed. Transformed leaf discs were placed on selective MSB media and in only transformed ones callus formation was observed. When shoots were emerged they were cut and transferred to MSB media in jars for rooting and then to soil to obtain T_1 progeny. 6 lines of independent putative transgenic plants from pAO-A and 7 lines of independent putative

transgenic plants from pAO-S were obtained. Transgenic plants transformed with pAO-A, named as capital S and with pAO-S named as capital A.

3.1.2.1. Callus Induction

Following transformation, all of the explants were placed on nonselective media for co-cultivation. After 3 days of co-cultivation the explants were washed with 750 mg/L cefotaxime containing liquid MS for the removal of bacterial growth. Then explants were placed on MS media containing 75 mg/L kanamycin. Callus formation was observed after 2-3 weeks.

Kanamycin is an aminoglycoside antibiotic that contains amino sugars bonded by glycosidic linkage. The aminoglycoside antibiotic acts by inhibiting protein synthesis at the level of 30S subunit of the ribosome. *nptII* gene transfers a phosphate group to kanamycin and detoxifies the antibiotic (Madigan *et al.,* 1997). As a result when *nptII* transferred plants were placed on kanamycin medium the gene inactivates the kanamycin in the medium so the plant survives.

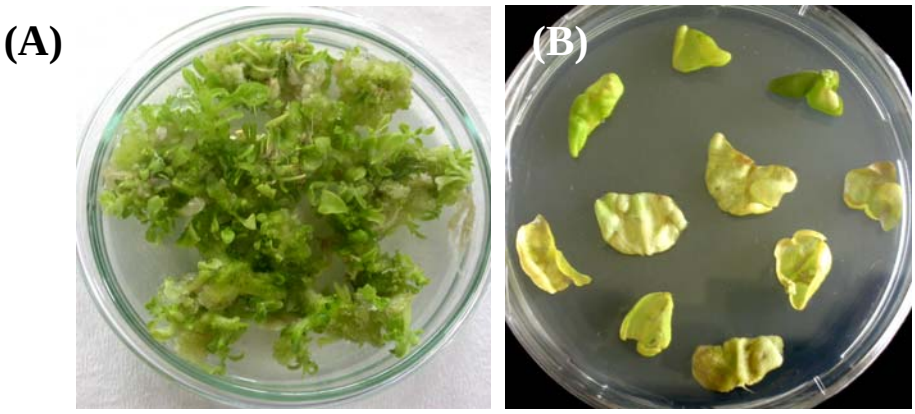

Figure 3.3. The appearance of the wild type explants on the fourth week. **(A)** Positive control plate, callus formation was started. **(B)** Negative control plate, the color of the explants has turned to yellow upon necrosis.

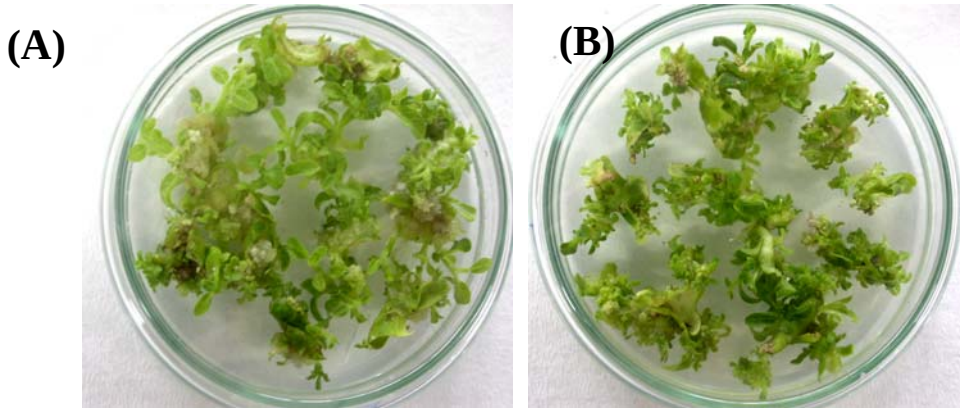

Figure 3.4. The appearance of the transformed explants on the fourth week **(A)** The explants that were treated with EHA105::pAO-A, callus formation was started. **(B)** The explants that were treated with EHA105::pAO-S, callus formation was started.

From Figures 3.3 and Figure 3.4 it can be seen that a high percent of *Agrobacterium*-treated leaf discs were survived on selective medium containing 75 mg/L kanamycin and these gave the first line of evidence that the gene of interest has been transferred to the plant. Under same conditions most of the untreated

explants were died and support the success of transformation. In literature, the reported kanamycin concentration employed for *nptII* gene selection was 50 mg/L (Ghosh *et al.,* 2002) and on average 65% of the explants displayed callus induction in tobacco.

3.1.2.2. Shoot and Root Development

Shoots started to emerge after 3-4 weeks later, which is consistent with Altun, 2001. When shoots emerged, they were cut from their bases and transferred to root inducing media (MSC) containing 75 mg/L kanamycin. Figure 3.5 shows the development of putative transgenic roots in MSC medium. Six lines of independent T_0 putative transgenic plants from pAO-A, named as A1, A2, A4, A8, A9 and A17; and seven lines from pAO-S, named as S2, S6, S7, S8, S17, S19 and S20 were regenerated. Figure 3.5 and Figure 3.6 show the appearance of plantlets in baby jars. Root formation in the presence of 75 mg/L kanamycin can be seen in Figure 3.7. Regeneration ability of transformed callus is another line of evidence that *nptII* gene is present in its genome which confers resistance to kanamycin.

Figure 3.5. Development of S7 line on 75 mg/L kanamycin containing MSC media after transformation. Photograph demonstrates 2 weeks of growth after transferred to rooting media.

Figure 3.6. The regeneration of the representative putative transgenic S17 line. Photograph demonstrates 4 weeks of growth after transferred to rooting media.

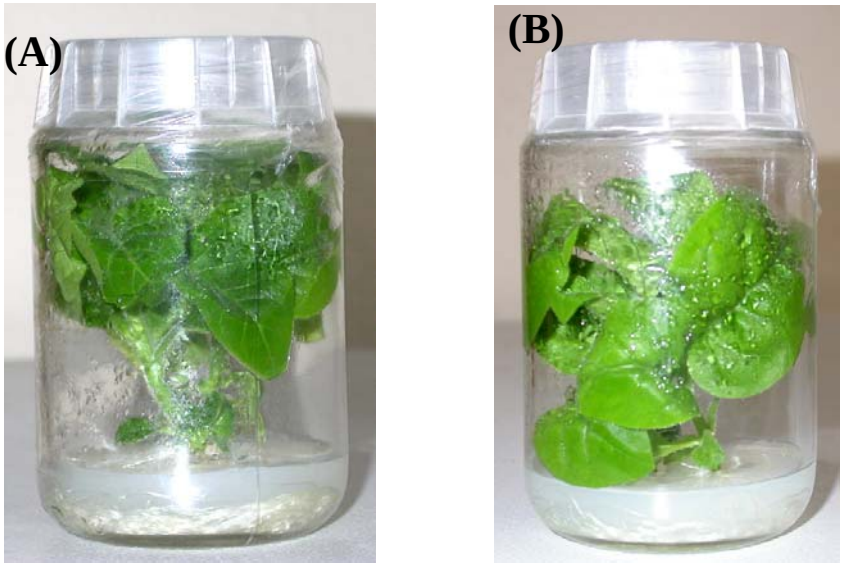

Figure 3.7. The root development of putative transgenic plantlets in 75 mg/L kanamycin containing MSC media. **(A)** A17 and **(B)** S19 lines have been shown in picture. Photograph demonstrates 45 days of growth after transferred to rooting media.

3.1.2.3. Development in Soil

When roots strengthen enough, nearly after a month, the plantlets were transferred to soil. This part of regeneration studies is very crucial as it leads to obtain successive generations and test the stable integration of gene within the genome of the plants.

The plants that were transferred to soil were grown in growth chamber under normal growing conditions and self-pollinated. Covering the flowers with plastic bags during flowering time prevented cross-pollination between the plants. T_1 generation was obtained from all putative transgenic lines.

Figure 3.8. The growth of putative transgenic S6 line in soil. Photograph demonstrates 2 weeks of growth after transfer to soil.

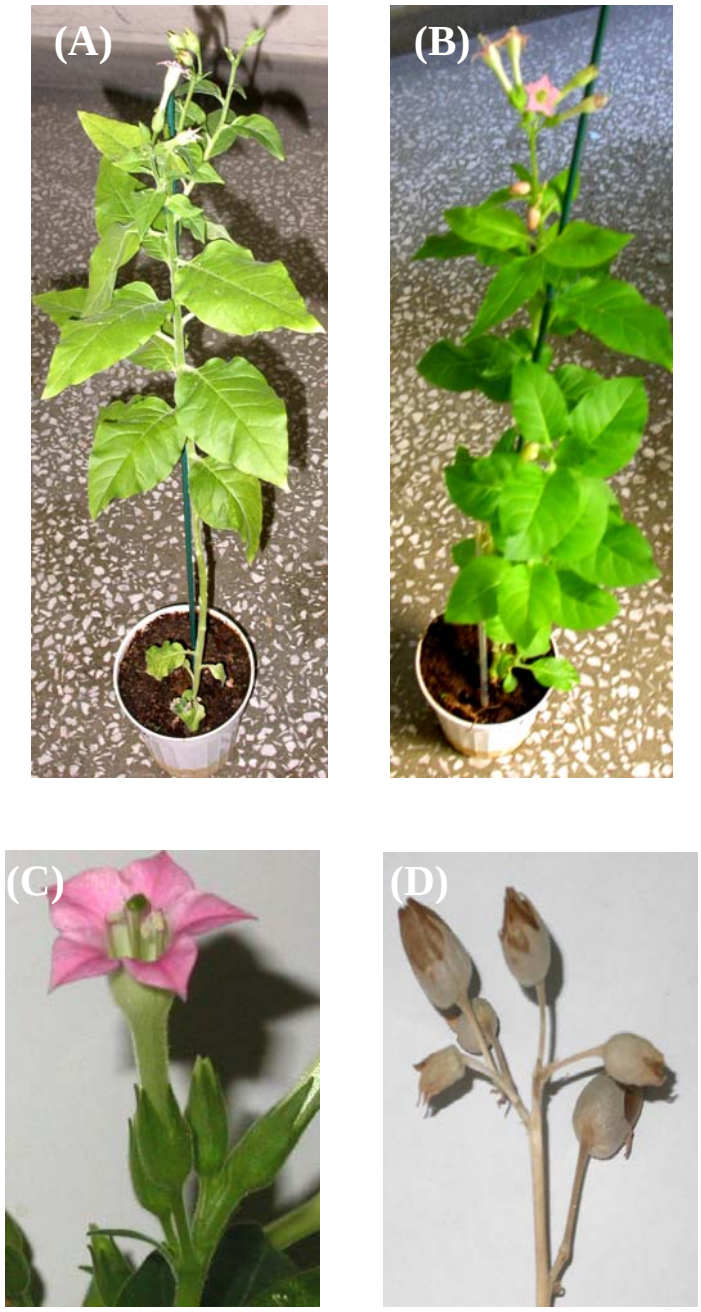

Figure 3.9. The growth of T_0 putative transgenic plants under greenhouse conditions. Putative transgenic lines show no morphological difference when compared with the control. **(A)** Wild type plant after 12 weeks **(B)** Flowering putative transgenic S7 line after 12 weeks **(C)** Flower of putative transgenic S7 line **(D)** Mature capsules of S7 line

T_1 seeds were germinated in 75 mg/L kanamycin containing medium and transferred to selective rooting media. When roots grow efficiently, plants were transferred to soil and grown to maturity in a growth chamber. After flowering and capsule formation T_2 seeds were collected for further analysis. Figure 3.10 shows the regeneration of T_1 transgenic plants.

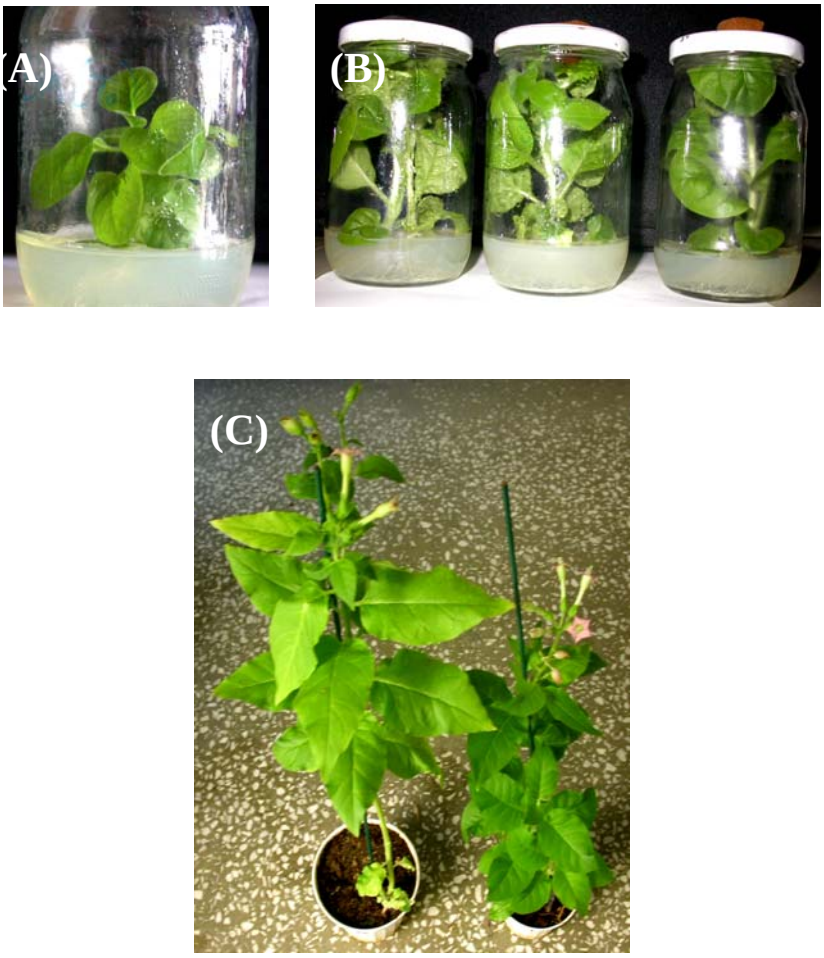

Figure 3.10. The growth of T_1 transgenic plants under greenhouse conditions. **(A)** Growth of control plants in culture medium **(B)** Growth of T_1 transgenic lines in culture medium **(C)** Flowering putative transgenic S7 line after 11 weeks

3.2. Analysis of T_o Putative Transgenic Tobacco Plants

The T_0 putative transgenic plants were analyzed mainly in two different ways: tissue culture tests and molecular analysis. In tissue culture tests leaf discs of the putative transgenic plants were used and they were placed on selective medium to test their regeneration potencies. Also color viability test were performed to confirm the results. Second type of analysis was based on the molecular methods at the DNA level. Totally these assays were performed to choose the successfully transformed lines and minimize the risk of escape plants. The seeds from positive lines were collected and used in further studies.

3.2.1. Leaf Disc Assay

Leaf discs of regenerated putative transgenic lines and wild type plantlets were placed on MSB medium supplemented with 75 mg/L kanamycin and kept under normal growth conditions. This method confirms the viability and callus formation potencies of leaf discs. The transformed leaf discs that have failed to regenerate on selective media were called as escapes and in this assay escape lines separated from regenerated putative transgenic lines.

A high percentage of the leaf discs of all putative transgenic leaf discs placed on selective media were able to form calli and give shoots whereas none of the leaf discs of wild type control plant were able to give regeneration response (Figure 3.11). Together with rooting responses of transformants on MSC media, this data gives evidence indicating that the plantlets that have obtained after transformation were not escapes, but can be considered as transgenics.

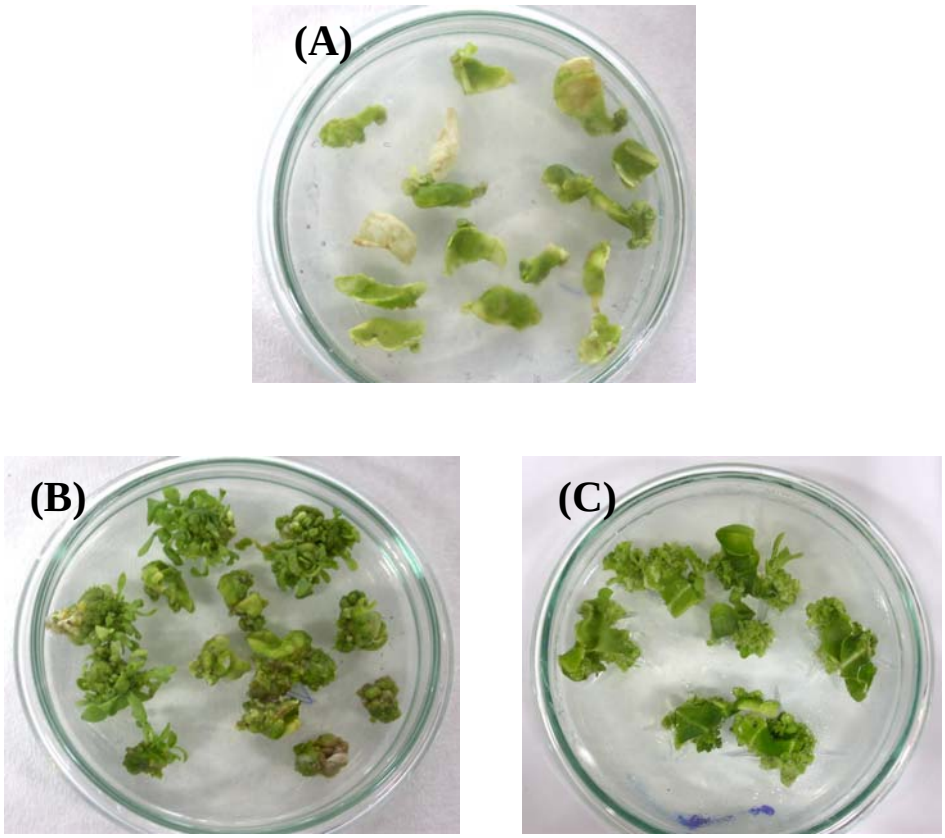

Figure 3.11. The appearance of leaf discs after 5 weeks of growth.
(A) Wild type tobacco **(B)** A9 **(C)** S6

3.2.2. Color Viability Test

The “color viability test” or “chlorophenol red test” gives an easy and quick way of evidence on the resistance behavior of the transformed plants on selective medium. Leaf discs of regenerated putative transgenic lines and wild type plantlets were placed on MSB medium supplemented with 75 mg/L kanamycin and 15 mg/L chlorophenol red and kept under normal growth conditions. Chlorophenol red is a pH indicator and gives red color when pH is in the range of 4.8-6.4 and in acidic pH it gives yellow color. This test is based on the color change of media due to regeneration of leaf discs.

The initial color of the medium at pH 5.7 is red. Leaf discs of putative transgenic plantlets that were able to survive on selective medium acidified the medium and the color changed from red to yellow. Leaf discs of wild type or escape plants were unable to survive on selective media and pH of the medium did not acidified so stayed red. The results of the chlorophenol red test are shown in Figure 3.12.

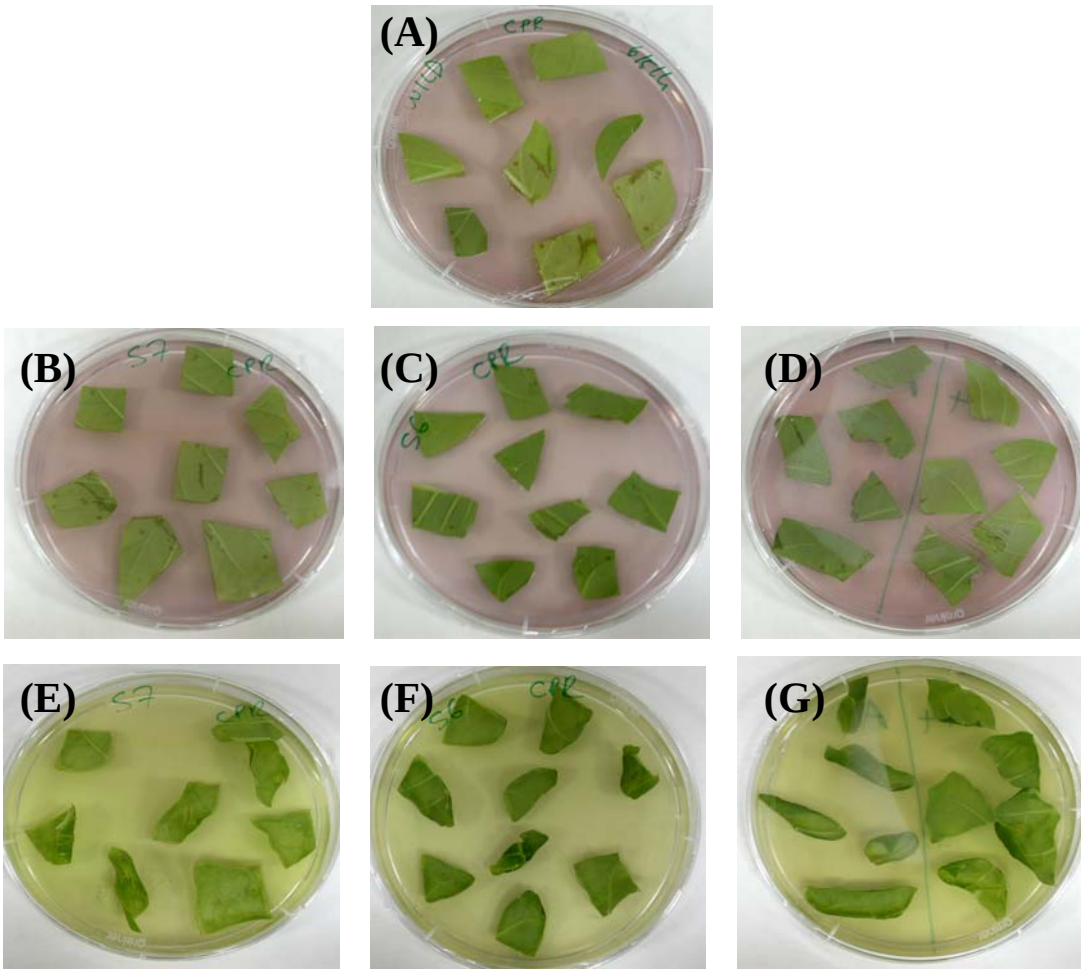

Figure 3.12. Results of the color viability test. **(A)** Wild type control plant on the first day of the experiment. No color change was observed at fifth day **(B)** S7 **(C)** S6 **(D)** A2 B, C, and D were taken on the first day of the experiment **(E)** S7 **(F)** S6 **(G)** A2 E, F and G were taken 5 days after the experiment.

The color of medium that contains leaf discs of all putative transgenic lines were changed from red to yellow while no color change was observed for wild type control plate. This result supported the idea of transgenic nature of transformed lines. Similar results were obtained by Altun, 2001 that demonstrated the color change of transgenic line from red to yellow.

3.2.3. PCR Analysis

PCR analysis was used to verify the transfer of gene of interest to the putative transgenic lines. For this purpose from each putative transgenic line CTAB DNA extraction was carried out. PCR analysis was done with *neomycin phosphotransferase II (nptII)* primers. As a negative control, the genomic DNA of wild type plant was isolated and used in PCR analysis. As a positive control, pure plasmids pAO-A or pAO-S were isolated and used in PCR analysis. The PCR results of pAO-A transferred putative transgenics and pAO-S transferred putative transgenics are shown in Figure 3.13 and Figure 3.14, respectively.

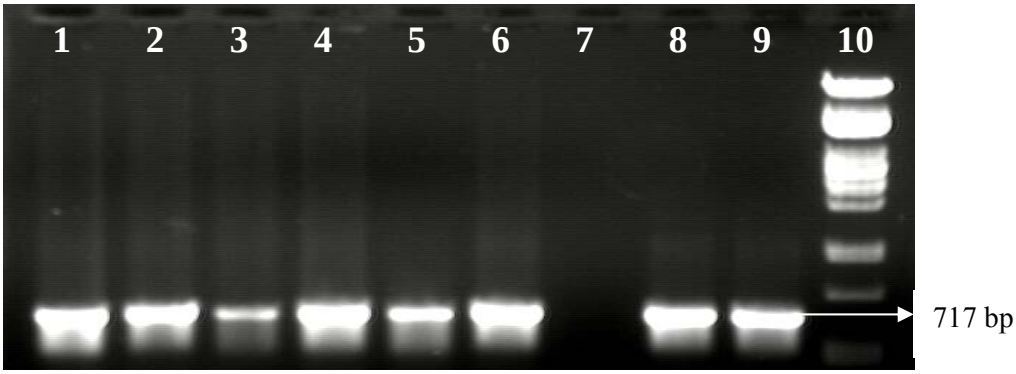

Figure 3.13. PCR analysis of pAO-A transformed T_0 putative transgenic plants with *nptII* primers. The samples used in PCR;
Lane 1. A1 **Lane 2.** A2 **Lane 3.** A4 **Lane 4.** A8 **Lane 5.** A9 **Lane 6.**A17
Lane 7. Wild Type **Lane 8.** pAO-A **Lane 9.** pAO-S **Lane 10.** λ PstI marker

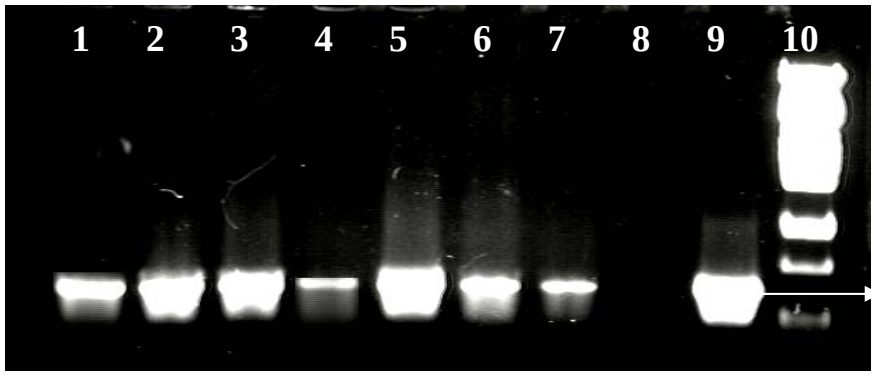

Figure 3.14. PCR analysis of pAO-S transformed T_0 putative transgenic plants with *nptII* primers. The samples used in PCR;
Lane 1. S2 **Lane 2.** S6 **Lane 3.** S7 **Lane 4.** S8 **Lane 5.** S17 **Lane 6.** S19
Lane 7. S20 **Lane 8.** Wild Type **Lane 9.** pAO-S **Lane 10.** λ PstI marker

From the results presented in Figure 3.13 and Figure 3.14, it can be seen that in all T_0 putative transgenic lines a 717 bp fragment was amplified which is the expected size of *nptII* gene amplification. The PCR results indicate the presence of the transferred *nptII* gene in the genome of the putative transgenic plants but not in wild type control plant.

3.3. Analysis of T_1 Transgenic Tobacco Plants

T_0 putative transgenic plants were self-pollinated and when mature capsules were formed they were collected. Those seeds were named as T_1 transgenic line and they were germinated on 75 mg/L kanamycin containing medium and transferred to root induction medium. Then they were transferred to soil and used in further molecular studies. After self-pollination, flowering and capsule formation T_2 seeds were collected.

T_1 transgenic plants were analyzed mainly in two different ways: molecular analysis and bioassays. Molecular analyses were based on the methods at the DNA and RNA level. Extracted DNA was used in PCR studies and RNA was used in Northern Blotting. Secondly bioassays were performed which include measurement of ethylene production from flowers.

3.3.1. PCR Analysis

PCR analysis was used to verify the inheritance of gene of interest in T_1 transgenic lines. PCR analyses of T_1 transgenic plants were carried out as described in section 3.2.3. PCR analyses were performed both with *nptII* and *acc oxidase* gene specific primers (Appendix B). The PCR results using *nptII* specific primers in T_1 sense and T_1 antisense putative transgenic plants genome are shown in Figure 3.15 to Figure 3.16, respectively. The results of the verification of torenia *acc oxidase* gene in T_1 antisense and sense transgenics genome with PCR analysis using *acc oxidase* primers were shown in Figure 3.17 and Figure 3.18, respectively.

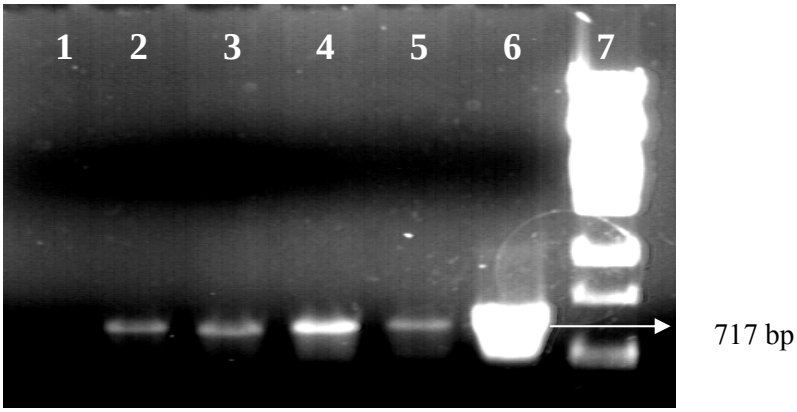

Figure 3.15. PCR analysis of T_1 sense putative transgenic plants using *nptII* primers. The samples used in PCR;

Lane 1. S2 **Lane 2.** S6 **Lane 3.** S7 **Lane 4.** S17

Lane 5. S19 **Lane 6.** pAO-S **Lane 7.** λ PstI marker

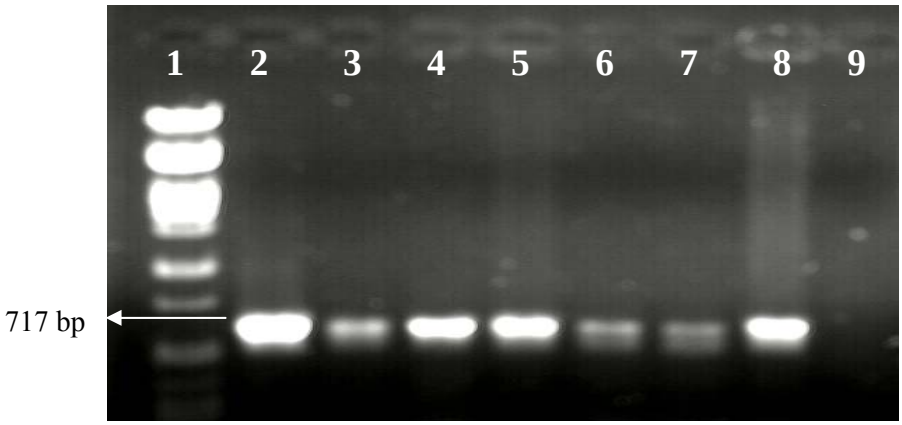

Figure 3.16. PCR analysis of T_1 antisense putative transgenic plants using *nptII* primers. The samples used in PCR:

Lane 1. λ PstI marker **Lane 2.** pAO-A **Lane 3.** A1 **Lane 4.** A2

Lane 5. A4 **Lane 6.** A8 **Lane 7.** A9 **Lane 8.** A17 **Lane 9.** Wild Type Control

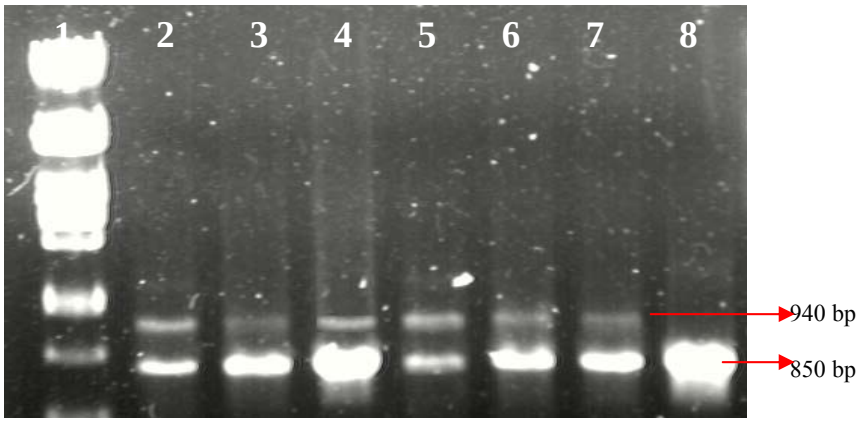

Figure 3.17. PCR analysis of T_1 antisense putative transgenic plants using *acc oxidase* primers. The samples used in PCR:

Lane 1. λ PstI marker **Lane 2.** A1 **Lane 3.** A2 **Lane 4.** A4

Lane 5. A8 **Lane 6.** A9 **Lane 7.** A17 **Lane 8.** pAO-A

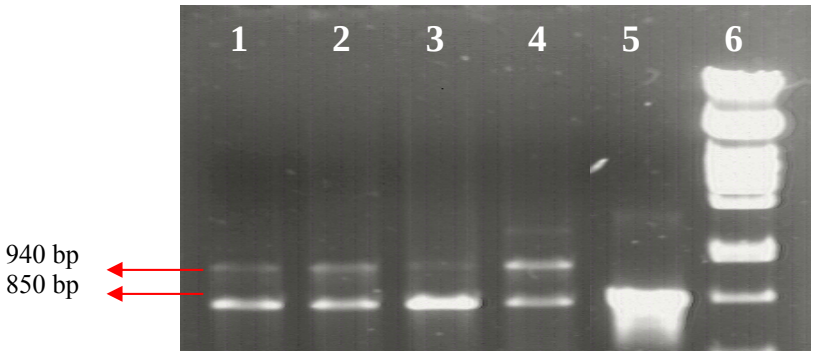

Figure 3.18. PCR analysis of T_1 sense putative transgenic plants using *acc oxidase* primers. The samples used in PCR:

Lane 1. S6 **Lane 2.** S7 **Lane 3.** S17

Lane 4. S19 **Lane 5.** pAO-S **Lane 6. .** λ PstI marker

In all lines with the exception of S2, *nptII* fragment was amplified (Figure 3.15 and Figure 3.16). Similarly Figure 3.17 and Figure 3.18 indicated the presence of 850 bp torenia *acc oxidase* fragments in T_1 generation. There was also another amplified fragment (~940 bp), which represents the tobacco *acc oxidase* gene amplification. *acc oxidase* specific primers used in this study, amplified both torenia and tobacco *acc oxidase* genes. *acc oxidase* gene sequences of torenia and tobacco are present in Appendix C and Appendix D, respectively. Those results are the indicative of inheritance of torenia *nptII* and *acc oxidase* gene in T_1 progeny.

3.3.2. Expression Analysis of *acc oxidase* in T_1 Transgenic Plants

Northern Blot Analysis was carried out to determine the expression of *acc oxidase* in T_1 transgenic plants. For this analysis total RNAs were isolated from transgenic lines (A1, A17, S7, S19) and control plants. After spectrophotometric measurement for concentration standardization 3 µg of total RNA was electrophoresed and photographed (Figure 3.19). 20 µg of total RNA from A1 and A17 and 12 µg of total RNA from S7 and S19 lines were electrophoresed in 1%

agarose gel and then RNAs were transferred to a nylon membrane. After pre-hybridization, hybridization and stringency washes, detection reagent was applied to membrane and signals were detected as described in section 2.2.7.4. Expressions of *acc oxidase* gene in antisense and sense transgenics are shown in Figure 3.20 and Figure 3.21.

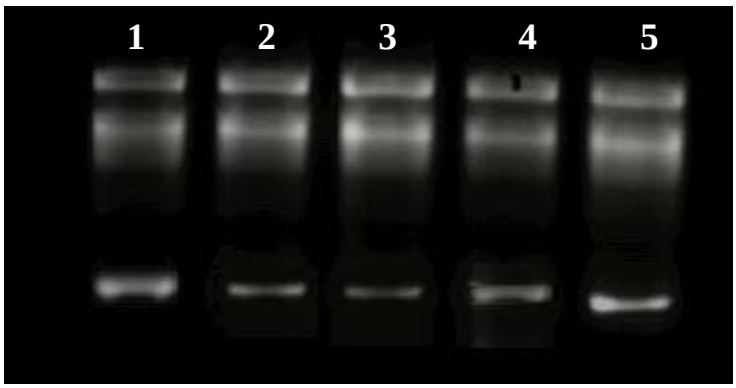

Figure 3.19. Total RNA isolated from transgenic and wild type plants.
Lane 1. A1 **Lane 2.** A17 **Lane 3.** S7 **Lane 4.** S19 **Lane 5.** Wild Type

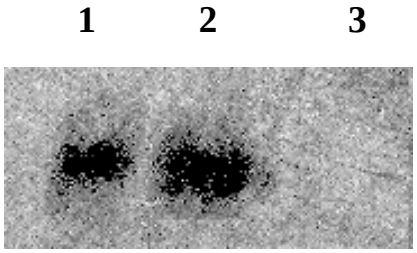

Figure 3.20. Northern blot analysis of T_1 antisense transgenic tobacco plants.
Lane 1. A1 **Lane 2.** A17 **Lane 3.** Wild Type Control

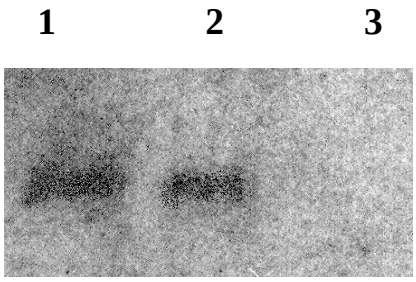

Figure 3.21. Northern blot analysis of sense T_1 transgenic tobacco plants.
Lane 1. S7 **Lane 2.** S19 **Lane 3.** Wild Type Contol

The results of Northern Blot Hybridization revealed that, a new transcript was present in transgenic lines, A1, A17, S7 and S19, used in the experiment but not in wild type plant. In addition to tissue culture studies and PCR analysis, which showed the presence of new genes (*nptII* and *acc oxidase*) in transgenic plants genome, Northern Blot Hybridization revealed that acc *oxidase gene* not only present but also expressed in both sense and antisense T_1 transgenic plants. Our results are consistent with the results of Aida and his co-workers results (Aida *et al.,* 1998) that transferred torenia sense and antisense transgenes to torenia and obtained expression of both transformants.

3.3.3. Mendelian Inheritance Pattern

The segregation pattern of transferred gene in T_1 transgenic tobacco plants were determined by germinating them in selective media containing 75 mg/L kanamycin. For this purpose nearly 100 seeds were used for each line and the numbers of survivors in selective media were counted and the results are given in Table 3.1. The expected ratio for Mendelian inheritance is 3:1 and the data was analyzed by Chi-Square Analysis with p ($\chi^2_1 \leq 3.841$) = 0.95. The appearances of antisense and sense transgenic seed in selective media are shown in Figure 3.22, and Figure 3.23, respectively.

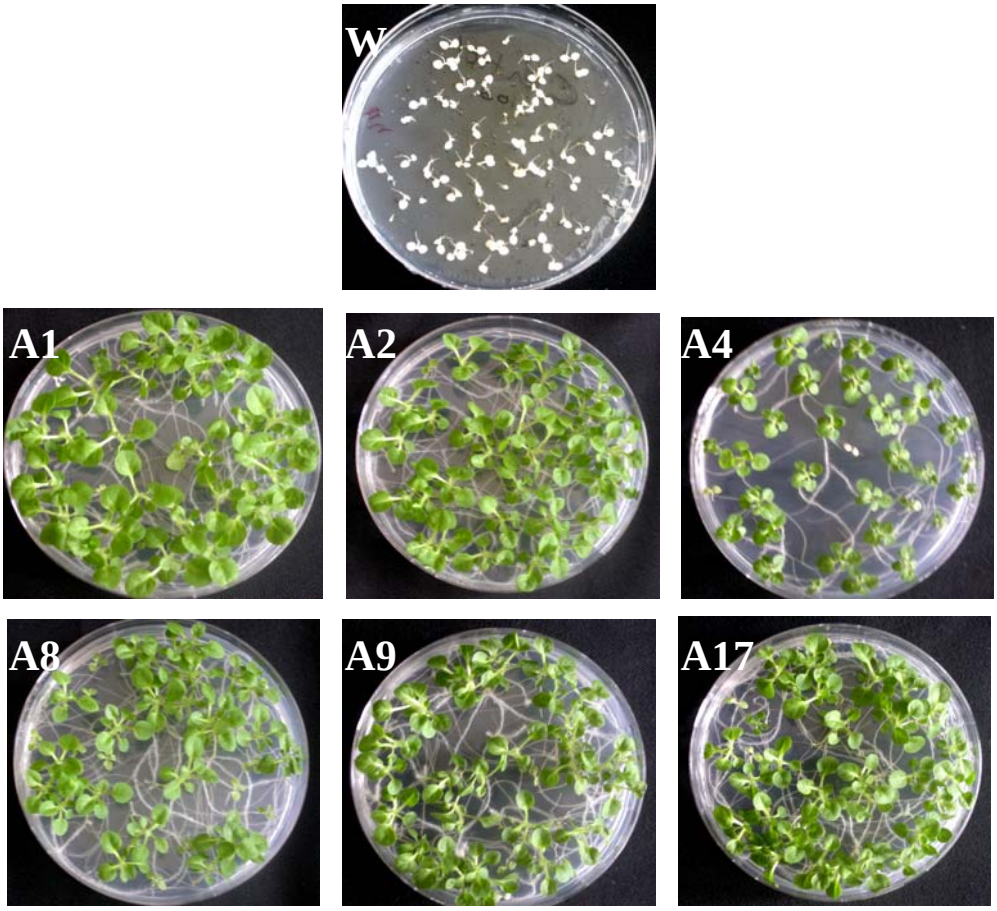

Figure 3.22. Photographs of T_1 antisense transgenic plants selected on 75 mg/L kanamycin containing medium. Photographs were taken after 6 weeks.

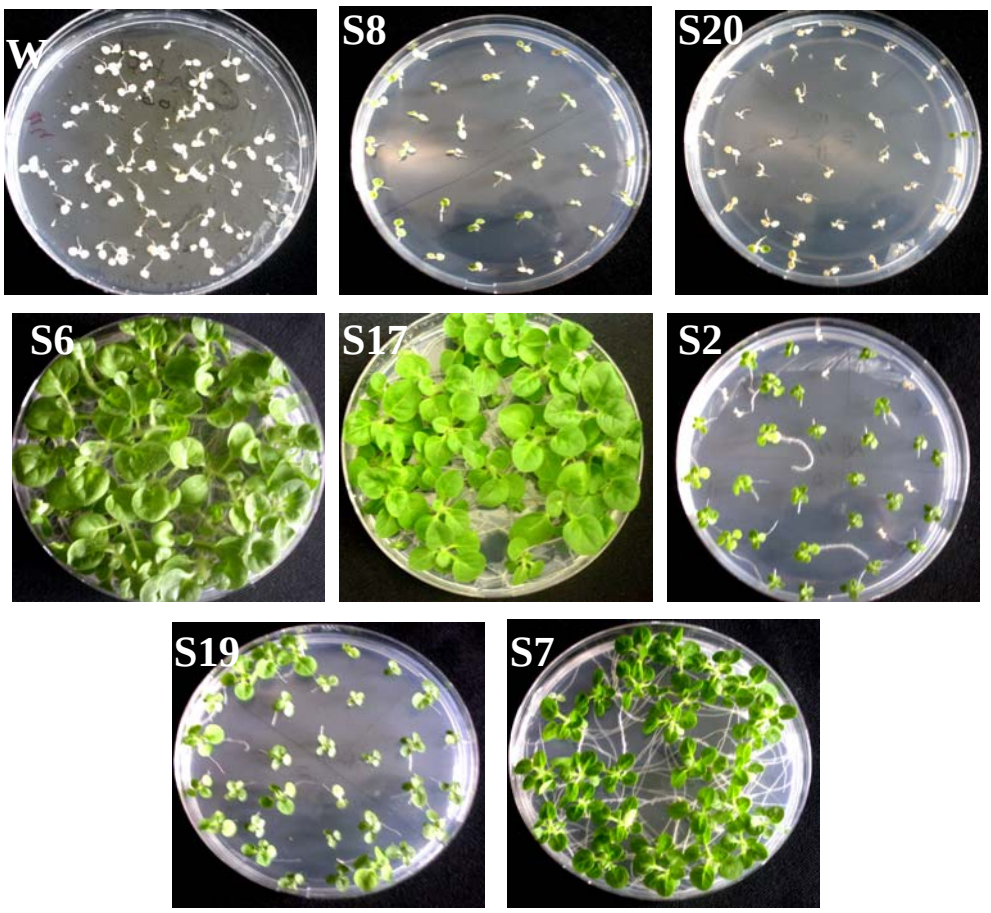

Figure 3.23. Photographs of T_1 sense transgenic plants selected on 75 mg/L kanamycin containing medium. Photographs were taken after 6 weeks.

From Figure 3.22 and 3.23 it can be seen that while all of the antisense lines (A1, A2, A4, A8, A9 and A17) and 5 sense lines (S6, S17, S7, S19 and S2) were germinated on selective medium, two sense lines (S8 and S20) and wild type control seeds did not germinate and grow on selective medium. Two lines (S2 and S19) grow slowly but were able to survived and grow on selective medium so for these lines T_1 seeds were obtained and used in molecular studies.

Table 3.1 shows the numbers of germinated T_1 seeds in selective medium, χ^2 results and the decisions can be concluded from the results. According to Mendelian Inheritance Analysis in 6 lines the gene was inherited according to Mendelian 3:1 ratio while 7 lines do not obey the ratio. Among the lines that do not obey the ratio, S8 and S20 seem to be lines that do not contain *nptII* gene and they can be categorized as escapes.

In our study we used the dominant characteristic of kanamycin resistance to determine the inheritance of *acc oxidase* gene in T_1 progeny in a manner analogous to Mendelian inheritance of a single dominant nuclear gene. For antisense and sense transgenics, A1, A8, A9, S7, S17 and S19 seemed to be carrying a single copy of *acc oxidase* gene in their genome. The high ratios for other lines, A2, A4, A17 and S6 can be due to the possibility of insertion and integration of more than one copy of the gene. While PCR analysis of T_0 progeny gave positive results, S2 seeds of T_1 progeny germinated and grow very slowly in selective medium as compared with other transgenic lines. It is escape nature was further verified with PCR analysis of T_1 progeny.

Table 3.1. Mendelian Inheritance Analysis of T_1 progeny of tobacco seeds. $p(\chi^2_{0.95,1}) \leq 3.841$

	Total Seed Number	Number of Germinated Seeds	χ^2	Decision
A1	118	81	1,9068	Fail to reject
A2	106	103	27,786	Reject
A4	107	100	18,7134	Reject
A8	103	85	3,11	Fail to reject
A9	72	53	0,074	Fail to reject
A17	76	67	7,0175	Reject
S2	109	97	11,38	Reject
S6	105	92	8,917	Reject
S7	102	81	1,059	Fail to reject
S8	103	0	231,75	Reject
S17	103	77	0,00324	Fail to reject
S19	113	87	0,239	Fail to reject
S20	109	1	319,05	Reject

3.3.4. Ethylene Measurements of T_1 Transgenic Tobacco Flowers

In the first part of the assay, wild type control flowers were used to determine the incubation period after cutting the flower to measure the ethylene amount. Ethylene production from flowers at different incubation periods are shown in Figure 3.24.

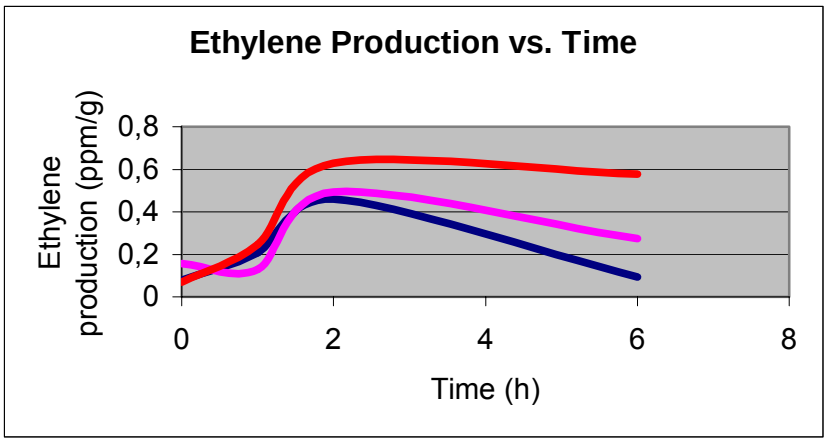

Figure 3.24. Ethylene production from flowers after 24 hours of wounding. The experiment was conducted with several control flowers and a representative data is given.

From Figure 3.24 it can be seen that 2 hours is the time at which the flowers produce maximum ethylene. Our results are similar to the results with torenia (Aida *et al.,* 1998) and parsley (Ella *et al.,* 2003) and in further studies ethylene measurements were carried out after 2 hours of incubation at growth chamber. A1 and S7 lines were chosen for ethylene measurement studies as all of the tissue culture and molecular studies confirmed their transgenic nature. For A1 line two flowers and for S7 line five flowers were used and for each flower

ethylene amount were measured for 2 or 3 times. Figure 3.25 and Figure 3.26 show the percent change of ethylene in T_1 transgenic A1 and S7 lines, respectively.

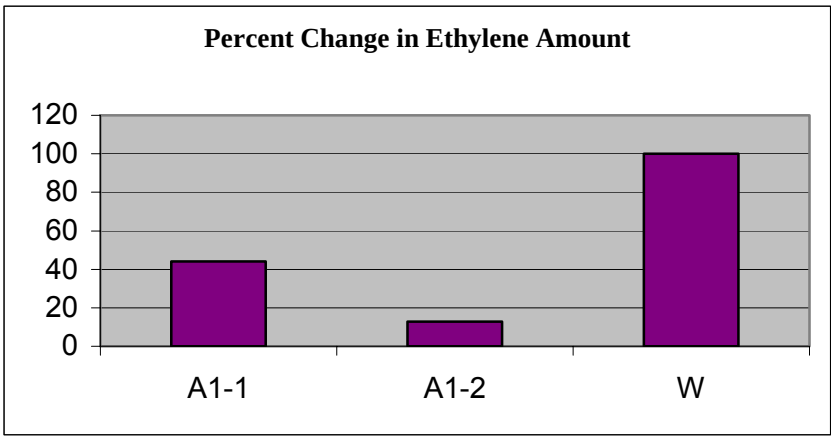

Figure 3.25. Percent change of ethylene amount in A1 line and wild type control. A1-1 and A1-2 are two different flowers from A1 transgenic line Ethylene production (1.8 ppm/g) in non-transformed plants was taken as 100%.

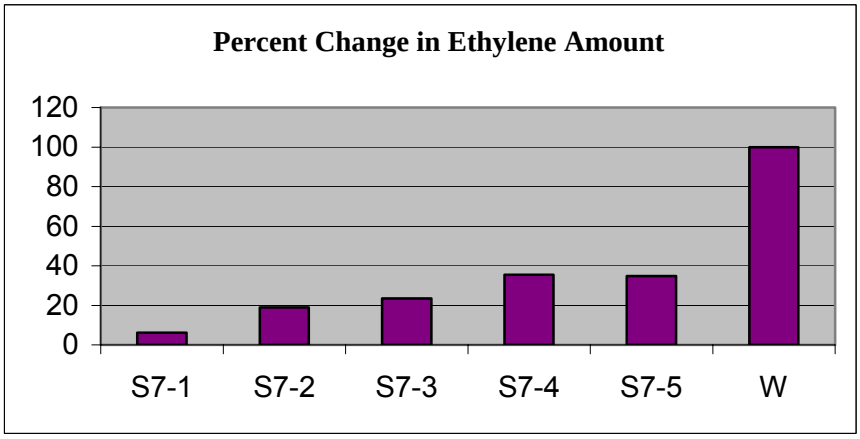

Figure 3.26. Percent change of ethylene amount in S7 line and wild type control. S7-1, S7-2, S7-3, S7-4 and S7-5 are five different flowers from S7 transgenic line. Ethylene production (1.8 ppm/g) in non-transformed plants was taken as 100%.

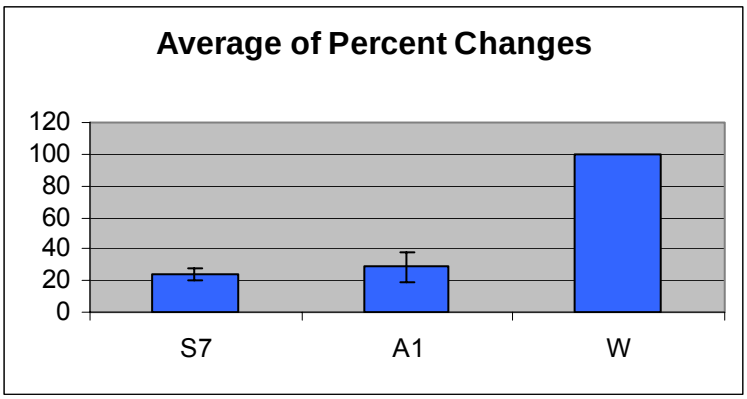

Figure 3.27. Average of percent changes of ethylene amount in S7 and A1 lines.

With respect to ethylene production, flowers from wild type plant exhibited higher amount than transgenic lines A1 and S7. It can be seen from Figures 3.25 and 3.26 that inhibition level of ethylene production from flowers varies from flower to flower. Reduction in ethylene production from A1 flowers changes from 55% to 87% and from S7 flowers changes from 64% to 93%. In literature very similar results were obtained for melon by Ayub and his coworkers from 44% to 67% (Ayub *et al.,* 1996) and for torenia by Aida and his coworkers from 32% to 54% (Aida *et al.,* 1998). From Figure 3.27 it can be seen that there was not an important change between antisense and sense transformation with respect to ethylene production. These results suggest that the existence of torenia *acc oxidase* transgene reduces the mRNA level of endogenous *acc oxidase* and eventually resulted in reduced ethylene production.

3.3.5. Overall Evaluation of Transgenic Lines according to the Tested Parameters

6 independent antisense and 7 independent sense transgenic T_0 lines were obtained at the end of *Agrobacterium*-mediated transformation of tobacco with torenia *acc oxidase* gene. T_1 progeny of S8, S20 and S2 were shown to be escapes

and all other lines were seemed to carrying the torenia *acc oxidase* gene. Several assays were conducted to test T_0 and T_1 transgenic lines and overall results are shown in Table 3.2.

PCR with *nptII* and *acc oxidase* primers and Northern Blot Analysis suggest that A1 and S7 are truly transgenic lines in which torenia *acc oxidase* gene is expressed, leading to reduction of *acc oxidase* mRNA level and ethylene production. Mendelian inheritance analysis showed that the gene was inherited according to Mendelian 3:1 ratio, suggesting a single transgene copy number in those lines.

Table 3.2. Overall evaluation of transgenic lines according to the tested parameters. **NA**: Not applicable.

	T_0 PCR *nptII*	T_1 Seed Germination Analysis	T_1 PCR *nptII*	T_1 PCR *acc oxidase*	3:1 Ratio	Northern Blot Analysis	Percent Ethylene Reduction
A1	+	+	+	+	+	+	+
S7	+	+	+	+	+	+	+
A17	+	+	+	+	-	+	NA
S19	+	+	+	+	+	+	NA
A2	+	+	+	+	-	NA	NA
A4	+	+	+	+	-	NA	NA
S6	+	+	+	+	-	NA	NA
A8	+	+	+	+	+	NA	NA
A9	+	+	+	+	+	NA	NA
S17	+	+	+	+	+	NA	NA
S2	+	+	-	-	-	NA	NA
S20	+	-	NA	NA	-	NA	NA
S8	+	-	NA	NA	-	NA	NA

From expression analysis torenia *acc oxidase* expression was demonstrated in A17 and S19 while only in S19 the gene was inherited according to Mendelian 3:1 ratio. To make a more accurate conclusion about these A17 and S19 lines ethylene analysis should be performed. And for other lines, A2, A4, S6, A8, A9 and S17 besides ethylene analysis, expression analysis should be carried out.

CHAPTER IV

CONCLUSION

As a result of tobacco transformations 6 independent lines transformed with *acc oxidase* in antisense orientation, and 7 lines in sense orientations were obtained. T_1 generation were obtained from all transgenic lines.

In order to evaluate the transgenic nature of T_0 and T_1 plants several tests were conducted. For T_0 putative transgenic lines leaf disc assay, chlorophenol red assay and PCR analysis were performed. In leaf disc assay, nearly all of the putative transgenic leaf discs were able to form calli and give shoot in selective media containing 75 mg/L kanamycin. In chlorophenol red assay, all putative transgenic lines changed the color of the medium from red to yellow, indicating the transgenic nature of the explants. However, control plants could not survive on both assays. These results were supported with PCR analysis, which revealed the presence of gene of interest within the plant genome.

The T_1 seeds were placed on selective medium in order to test Mendelian inheritance pattern. In 6 lines the gene was inherited according to the Mendelian 3:1 ratio, while 7 lines do not obey the rule. Three lines, S2, S8 and S20 were considered as escape lines.

PCR analysis and Northern Blot Hybridization were carried out for T_1 transgenic plants. PCR amplification with both *nptII* and *acc oxidase* gene specific primers revealed positive results for all transgenic lines.

Northern Blot Hybridization was carried out for A1, A17, S7, S19 and control plants. The results of Northern Blot Hybridization also revealed that, there was a new transcript present in transgenic plants but not in control plant.

In T_1 transgenic A1 and S7 lines ethylene production by flowers were reduced by 55% to 87% in A1 and by 64% to 93% in S7 as compared with the wild type flowers. This result showed that both sense and antisense expression of torenia *acc oxidase* gene in tobacco resulted in reduced ethylene production, which may lead to extension of flower longevity. Moreover, there was not a significant change between antisense and sense transformation in terms of ethylene production.

As far as current literature is concerned, our data supported the idea that heterologous expression of torenia *acc oxidase* gene in antisense and sense orientations inactivates the gene of interest in tobacco.

The studies to be conducted in the future work would include:

i. Northern Blot Hybridization of other transgenic lines,
ii. Confirmation of gene integration into the plant genome via Southern Blot Hybridization,
iii. ACC Oxidase enzyme assay
iv. Further bioassays of T_1 and T_2 plants.
v. Engineering of commercially important ornamental plants (e.g. carnation) with *acc oxidase* antisense and sense genes to extend flower life.

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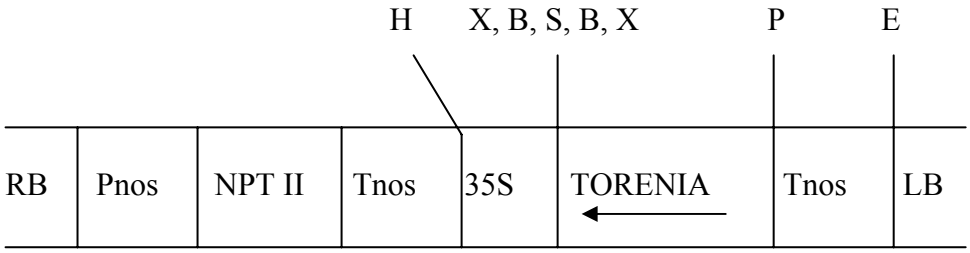
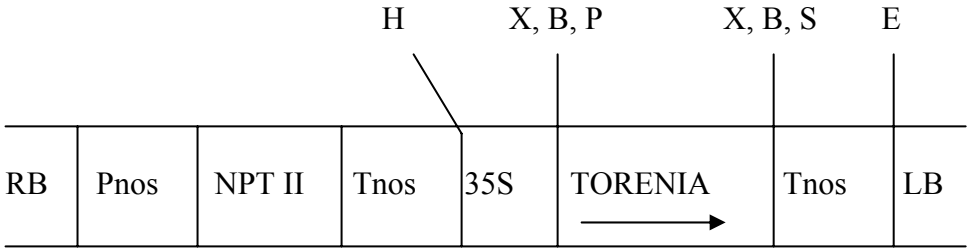
APPENDIX A

STRUCTURE OF BINARY VECTORS pAO-A AND pAO-S



PAO-A, carrying a fragment of torenia *acc oxidase* gene in antisense orientation



PAO-S, carrying a fragment of torenia *acc oxidase* gene in sense orientation

APPENDIX B

PRIMERS

Primers used during PCR amplification:

***nptII* primers:**

5’ GAGGCTATTCCCGGCTATGACT 3’
3’ ATCGGGAGCGGCGATACCCTA 5’

***acc oxidase* primers**

5’ GATGCTTGTGAGAACTGGGG 3’
3’ GCTTCAAATCTTGGCTCCTT 5’

APPENDIX C

TORENIA FOURNIERI mRNA for ACC OXIDASE, PARTIAL CDS

```
1   ttctttgagctattgaatcacgggatttctcccgagtttatggacactgtggagaggcta
61  accaaagatcattacaagaaatgcatggaactaaggttcaaagagatggttgctagcaaa
121 ggactcgaagctgttcaatcccaagcagacgacatagattgggaaagcacttttacgtg
181 aaacatctccctgaatctaacatctcagagatccctgatcttgatgatgactacaggaag
241 gtaatgaaggagtttgcagctcaaatagagaaactagccgaggagttactagatttactg
301 tgtgagaaccttggacttgaaaagggctatctcaagaaagtattttacggctcgaaaggc
361 ccacccttggcactaaggtgagcaactatccaccgtgtcccaagccagatttcatcaag
421 gggcttcgtgctcacactgatgctggtgggatcatacttttgttccaagacgataaagta
481 agcggcttgcagcttctcaaagatggcgaatgggtcgatgttccaccgatgaagcactcc
541 attgtcatcaaccttggtgatcagctcgaggtgattacgaatgggaagtacaagagcgtg
601 atgcacagggtcattgcacagacagatggtaacagaatgtcgattgcatcgttttacaat
661 cctgggactgatgctgtgatctactcagcgcctgctttggttgagaaagaagaggagaag
721 aaagatctgtacccaaagtttgtgtttgatgactacatgaagctctatgctgggctcaag
781 tttcaggcc
//
```

APPENDIX D

NICOTIANA TABACUM mRNA for ACC OXIDASE, COMPLETE CDS

```
1    cggattttattttattgtattacattttaacgcacttaaaaacacacatactttaccaa
61   gaaagatatggagaacttcccaattatcaacttggaaaagctcaatggttctgagagagc
121  tgacaccatggaaatgattaaggatgcttgtgagaactggggcttctttgagttggtgaa
181  ccatgggattccacatgaagtaatggatacagtggagaaaatgacaaagggacattacaa
241  gaagtgcatggaacagagatttaaagaattggtggccagcaaaggtcttgaagctgtgca
301  agctgaggttactgatttggattgggaaagcactttcttttacgccatcttcctgtttc
361  taacatttgtgaagtacctgatctcgatgatcaatacagggaagtaatgagagattttgc
421  tcaaagattagaaaaattggcagaggagttactggacttgctatgtgaaaatcttggcct
481  tgaaaaaggctacctgaaaaaaatcttttatgggacacaaggtcccaattttggagctaa
541  ggttagcaactatccaccatgtccccaaccagatttgataaagggtctgcgcgcccacac
601  agatgctggtggcataatccttctcttccaagatgacaaagtaagcggccttcaactcct
661  caaagacggccaatggatcgatgttcctcccatgcgccactctattgtggttaaccttgg
721  cgaccaacttgaggtgatcaccaatgggaaatacaagagtgtgatgcacagagtgattac
781  acaaacagacgggactcggatgtcattagcttcattttataatccaggaagtgatgcagt
841  aatatttccagcaccaactttagttgagaaagaggcagaggaaagtaaagcaatttatcc
901  aaagtttgtgtttgatgataatatgaagttatatgctggactcaagtttcaagccaaaga
961  gccaaggtttgaagccatgatcaaggccatggaaactgtgaaaagtgatccagttgcaac
1021 tgcttaaattccaattcgagaggaaggatggggtttgaaaagaaaaggattcattttaga
1081 agtttttacaaattaaacctagctactatatatacattatttgctctttgtattgtgtgt
1141 ggtggaatcaagctattcccaaatattgtgatctgctgcatatgtagtaaagattgtatc
1201 tcaaataaacttccttctcattgaatttccaaaccg
//
```

APPENDIX E

BACTERIAL CULTURE MEDIA

Luria-Bertani Broth (1 L)

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Bacterial agar	15 g

The pH of the medium is adjusted to 7.4 and autoclaved at 121^0C for 20 minutes. LB medium is readily available from Sigma.

Yeast Extract Broth Medium (1 L)

Nutrient Broth	13.5 g
Yeast Extract	1 g
Sucrose	5 g
$MgSO_4.7H_2O$	493 mg (final concentration 2 mM)
Bacterial agar	15 g

The pH of the medium is adjusted to 7.2 and autoclaved at 121^0C for 20 minutes.

SOB Medium (1 L)

Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
0.250 M KCl	10 mL

The pH of the medium is adjusted to 7.0. After autoclaving the medium at 121^0C for 20 minutes 5 ml of 2 M $MgCl_2$ is added.

TB Buffer (100 mL)

HEPES	0.24 g
$CaCl_2.2H_2O$	0.221 g
KCl	1.864 g
$MnCl_24H_2O$	1.09 g

The pH of the medium is adjusted to 6.7. The medium is filter sterilized and stored at 4^0C.

APPENDIX F

TBE, TAE AND TE SOLUTIONS

10X TBE (Tris Borate) Buffer (1L)

Tris-base	108 g
Boric acid	55 g
0.5M EDTA (pH: 8.0)	40 mL

All the components are mixed and the volume is completed to 1 L with dH_2O.

50X TAE (Tris-acetate) Buffer (1 L)

Tris-base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH: 8.0)	100 mL

All the components are mixed and the volume is adjusted to 1 L with dH_2O.

1X TE (Tris-EDTA) Buffer (1 L)

Tris.HCl	10 mM
EDTA	1 mM

All the components are mixed and the volume is adjusted to 1 L with dH_2O.

APPENDIX G

PLASMID ISOLATION SOLUTIONS

Sol I:

50 mM Glucose

25 mM Tris.Cl (pH: 8.0)

10 mM EDTA (pH: 8.0)

Sol II:

0.2 N NaOH (freshly diluted from 10N stock)

1% SDS

Sol III

5 M Potassium Acetate	60 mL
Glacial Acetic Acid	11.5 mL
dH_2O	28.5 mL

STE

NaCl	0.1 M
Tris.Cl (pH: 8.0)	10 mM
EDTA (pH: 8.0)	1 mM

APPENDIX H

COMPOSITIONS OF PLANT TISSUE CULTURE MEDIA

From Sigma Plant Cell Cultures	**M5519**	**M9274**
COMPONENT	**mg/L**	**mg/L**
Ammonium Nitrate	1650	1650
Boric acid	6.2	6.2
Calcium Chloride Anhydrous	332.2	332.2
Cobalt Chloride.6H_2O	0.025	0.025
Cupric Sulfate.5 H_2O	0.025	0.025
Na_2EDTA	37.26	37.26
Ferrous Sulfate	27.8	27.8
Magnesium Sulfate	180.7	180.7
Manganese Sulfate.H_2O	16.9	16.9
Molybdic Acid (Sodium Salt).2 H_2O	0.25	0.25
Potassium Iodide	0.83	0.83
Potassium Nitrate	1900	1900
Potassium Phosohate Monobasic	170	170
Zinc Sulfate.7 H_2O	8.6	8.6
ORGANICS		
Agar		8000
Glycine	2	2
Myo-inositol	100	100
Nicotinic Acid	0.5	0.5
Pyridoxine.HCl	0.5	0.5
Sucrose		30000
Grams of powder to prepare 1 liter	4.4	42.4

APPENDIX I

CTAB DNA ISOLATION SOLUTIONS

2X CTAB Extraction Solution (100 mL)

CTAB (Hexadecyl-trimethyl amonium bromide)	2 g
1 M Tris.Cl (pH: 8.0)	10 mL
0.5 M EDTA (pH: 8.0)	4 mL
5 M NaCl	28 mL

All are dissolved in dH_2O and volume is completed to 100 mL.

5X CTAB Extraction Solution (100 mL)

CTAB (Hexadecyl-trimethyl amonium bromide)	5 g
1 M Tris.Cl (pH: 8.0)	10 mL
0.5 M EDTA (pH: 8.0)	4 mL
5 M NaCl	28 mL

All are dissolved in dH_2O and volume is completed to 100 mL.

APPENDIX J

RNA ISOLATION SOLUTIONS

TRI Reagent (110 mL)

Guanidine thiocyanate	23.7 g
100 mM Na-citrate pH: 7.0	12.5 mL
35% Sarcosyl	715 µL
β-mercaptoethanol	340 µL
Water saturated phenol	50 mL
2 M Potassium acetate pH: 4.8	10 mL

Guanidine thiocyanate and 100 mM Na-citrate pH: 7.0 are dissolved in DEPC-treated distilled water and the volume was completed to 45 mL. Then 35% sarcosyl and β-mercaptoethanol was added and the volume was completed to 50 mL. The solution was autoclaved. 50 mL water saturated phenol was added onto the solution in hood and 10 mL sterile 2M potassium acetate pH: 4.8 was added.

Water Saturated Phenol (50 mL)

50 mL phenol

150 mL DEPC-treated dH_2O

50 mL phenol and 50 mL water are mixed and shaken for a few minutes. When two phases are separated from each other take the upper phase. Repeat this three times.

Tris Equilibrated Phenol pH: 8.0

50 mL phenol

100 mL 1 M Tris.Cl pH: 8.0

100 mL 0.1 M Tris.Cl pH: 8.0

Equal amount of 1M Tris.Cl and phenol was added. The bottle was shaken until a milky appearance was obtained and bottle was put onto a flat surface for phase separation. Upper phase was taken and some sample was taken from the lower phase and the pH of the phenol was measured by pH test papers. After 1 M Tris.Cl 0.1 M Tris.Cl was used until pH will be equal to 8.0.

APPENDIX K

NORTHERN BLOT ANALYSIS SOLUTIONS

10X MOPS (500 mL)

2.05 g Sodium acetate

1.46 g EDTA

20.92 g MOPS (3-N-[Morpholino] propane sulfonic acid)

All components were mixed and the volume was completed to 500 mL. PH was adjusted to 7 and the solution was filter sterilized.

Sample Buffer (for 500 µL, prepare freshly)

250 µL 100% formamide

85.75 µL 37% formaldehyde

50 µL 10X MOPS

2.5 µL ethidium bromide

All components were mixed the volume completed to 500 µL.

Formaldehyde Gel (for 150 mL)

1.5 g agarose

15 mL 10X MOPS

25.7 mL 37% formaldehyde

Agarose and MOPS were dissolved in DEPC-treated distilled water with microwave oven. 2 µL of ethidium bromide were added and the gel was cooled for

some time. Then formaldehyde was added under hood and the gel was poured under hood. The gel was run with 1X MOPS as buffer.

Transfer Buffer (20X SSC)

Tri-sodium citrate	88.23 g
NaCl	175.32 g

All are dissolved in 800 mL dH_2O and pH is adjusted to 7-8 then the volume is completed to 1 L with distilled water.

Pre-hybridization and Hybridization Buffer

Hybridization buffer	
NaCl	0.25 mL/cm^2 hybridization buffer
Blocking regent	4%

NaCl was added into hybridization buffer and the solution was stirred at room temperature. Then Blocking reagent was added and the solution was mixed for 1-2 hours at room temperature until complete dissolving.

Primary Wash Buffer (1L)

		Final concentration
Urea	120 g	2 M
SDS	1 g	0.1%
0.5 M sodium phosphate (pH: 7.0)	100 mL	50 mM
Sodium chloride	8.7 g	150 mM
1 M magnesium chloride	1 mL	1 mM
Blocking reagent	2 g	0.2%

All the components were added and the volume was completed to 1 L with distilled water. Primary wash buffer can be kept at 2-8 ^{0}C up to one week.

Secondary Wash Buffer (for 500 mL)

Tris-base	60.5 g
Sodium chloride	56 g

All components were dissolved in water and the volume is completed to 500 mL.

Secondary Wash Buffer Working Solution

Secondary wash buffer was diluted 1:20 and 2 mL/L of 1M magnesium chloride was added (2 mM magnesium in solution).

Secondary wash buffer can not be stored, it should be used immediately