GENETIC MANIPULATION OF TOBACCO AGAINST OSMOTIC STRESS VIA PROLINE OVERPRODUCTION

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Prof. Dr. Tayfur Öztürk
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Suzan Kıncal
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

Dr. Laszlo Szabados
Co-Supervisor

Prof. Dr. Hüseyin Avni Öktem
Supervisor

Examining Committee Members

Prof. Dr. Meral Yücel

Prof. Dr. Haluk Hamamcı

Doç. Dr. Sebahattin Özcan

Prof. Dr. Gülay Özcengiz

Prof. Dr. Hüseyin Avni Öktem

ABSTRACT

GENETIC MANIPULATION OF TOBACCO AGAINST OSMOTIC STRESS VIA PROLINE OVERPRODUCTION

Eyidoğan (İnci), Füsun

Ph.D., Department of Biotechnology

Supervisor: Prof. Dr. Hüseyin Avni Öktem

Co-Supervisor: Dr. Laszlo Szabados

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Most plants grow in environmental conditions that are to a considerable degree, unfavourable for plant growth. One of the most commonly induced responses in all organisms undergoing water deficit is the production and/or accumulation of so called compatible osmolytes. In plants proline accumulation has been well correlated with tolerance to salinity and drought. Therefore, genetic manipulation of plants towards proline overproduction might enhance their tolerance against salt and drought stress.

The aim of the present study was to develop osmotolerant transgenic tobacco plants via metabolic engineering for proline overproduction.

For this purpose, coding sequences of *Arabidopsis thaliana* Δ^1-pyrolline-5-carboxylate synthetase (AtP5CS) the key enzyme of proline biosynthetic pathway in plants, and/or a proline feedback insensitive mutant of the same gene that was obtained by random mutagenesis experiments was transferred to tobacco via *Agrobacterium tumefaciens* mediated gene transfer system.

T_0, T_1, and T_2 progeny of transgenic tobacco plants carrying the wild type AtP5CS gene were analysed for stable integration of transgenes, segregation patterns and osmotolerance under salt and drought stresses at seedling level and under greenhouse conditions. Compared to the controls most transgenic lines exhibited a higher proline accumulation, germination rate, lowered MDA content, better root development and capsule formation under salt and drought stress conditions.

Furthermore, a remarkably higher proline accumulation (up to 8 fold) with respect to control was observed in transgenic tobacco plants that were transformed with the mutated AtP5CS gene.

Taken all together, our results demonstrate an enhanced osmotolerance in transgenic plants engineered for proline overproduction.

Key Words: Proline, Δ^1-pyrolline-5-carboxylate synthetase, P5CS, Osmotic Stress, Osmotolerance, Transgenic tobacco.

ÖZ

TÜTÜNÜN PROLİN MİKTARININ ARTTIRILMASI YÖNTEMİYLE OZMOTİK STRESE KARŞI GENETİK MANİPULASYONU

Eyidoğan (İnci), Füsun
Doktora, Biyoteknoloji Bölümü
Tez Yöneticisi: Prof. Dr. Hüseyin Avni Öktem
Ortak Tez Yöneticisi: Dr. Laszlo Szabados

Haziran 2001, 184 sayfa

Bir çok bitki gelişmeleri için uygun olmayan koşullar altında büyür. Su yetersizliği altında bütün organizmalarda ortak olan cevaplardan biri osmolitlerin üretimi ve/veya akümülasyonudur. Bitkilerin tuzlanma ve kuraklığa toleransı ile prolin akümülasyonu arasında bağlantı olduğu gösterilmiştir. Bu nedenle, bitkilerin tuzlanma ve kuraklığa dirençli hale getirilmeleri prolinin fazla üretimini olası kılan genetik modifikasyonlar ile sağlanabilmektedir

Bu çalışmanın amacı metabolizma mühendisliği yöntemi ile prolini daha fazla üretebilen ve ozmotik strese dirençli transgenik bitkilerin geliştirilmesidir.

Bu amaçla, bitkilerde prolin biyosentezinde anahtar enzim olan Δ^1-Pyrollin-5 Karboksilat Sentetaz (P5CS)'ın sentezinden sorumlu *A. thaliana* geni ve aynı genin rastgele mutagenez yolu ile elde edilen prolin geri dönüşüm inhibisyonuna dirençli mutant formu, *Agrobacterium* yöntemi ile tütün bitkilerine transfer edilmiştir.

AtP5CS genini taşıyan T_0, T_1 ve T_2 transgenik bitkiler tuz ve kuraklık stresi altında hem fide seviyesinde hem de sera koşullarında, aktarılan genlerin bitki genomuna integrasyonu, açılımları ve bitkilerin osmotoleransları açısından analiz edilmişlerdir. Tuz ve kuraklık stresi altında kontrol bitkileri ile karşılaştırıldığında transgenik bitkilerin çoğunun daha fazla prolin akümülasyonu, çimlenme oranı, düşük MDA derişimi, daha iyi kök gelişimi ve kapsül oluşumu gösterdiği saptanmıştır.

Buna ek olarak mutant AtP5CS geni ile transfer edilen bitkiler normal büyüme koşullarında kontrole göre 8 kata kadar daha yüksek prolin akümülasyonu göstermektedirler.

Elde edilen bulgular bir bütün olarak ele alındığında transgenik bitkilerde prolinin fazla üretilmesinin osmotoleransı arttırdığı gözlenmektedir.

Anahtar Kelimeler: Prolin, Δ^1-Pyrollin-5-Karboksilat Sentetaz, P5CS, Ozmotik Stres, Ozmotolerans, Ozmoprotektan,

To my family for all of their support dedicated to me

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ABREVIATIONS

P5CS	Δ^1-Pyrolline-5-carboxylate Synthetase
P5CR	Pyrolline-5- Carboxylate Reductase
PDH	Proline Dehydrogenase
Pro	Proline
At	*Arabidopsis thaliana*
MDA	Malondialdehyde
NaCl	Sodium Chloride
ABA	Abscisic Acid
GK	Glutamyl kinase
GPR	γ-Glutamyl phosphate Reductase
CaMV35S.....	Cauliflower mosaic virus 35 S promoter
DHPro	3,4-Dehydroxyproline
MS	Murashige and Skoog
YEB	Yeast Extract Broth
LB	Luria-Broth
PCR	Polymerase Chain Reaction
BA	Benzyl aminopurine
NAA	Naphtalene acetic acid
Ti	Tumour inducing
T-DNA	Transferred DNA
SEM	Standart error of Mean

CHAPTER I

INTRODUCTION

During growth and development, plants are subjected to a variety of environmental stresses. For plants, stress can be defined as any factor that inhibits plant growth. Stress due to drought, waterlogging, salinity, extremes of temperature and metals are common throughout the world. These environmental conditions are unfavorable for plant growth.

For each environmental factor there are maximum and minimum levels and beyond these levels, plants cannot survive. Since plant productivity is greatly affected by environmental stresses, learning about the biochemical and molecular mechanisms by which plants tolerate environmental stresses is necessary for genetic engineering approaches to improve crop performance under stress.

1.1. Environmental Stress Factors Effecting Plant Productivity

All stress factors may induce potentially injurious strains in the plant, either reversibly by inhibiting metabolism and growth, or irreversibly by injuring or killing the cells. Although it is possible to eliminate all of the stresses to which a plant may be exposed, it is possible to modify them to the strains that they are capable of producing. When a stress acts on a plant, it may produce an injury in different ways. First, it may induce a direct stress injury and can be recognised by speed of its development. In such cases, the plant may be killed by very brief exposures to the stress (seconds and minutes). Secondly, the stress may produce a reversible strain and, therefore, not injurious itself. If maintained

for a long enough time, this reversible strain may give rise to an indirect irreversible strain that results in injury or death of the plant. Indirect injury may be recognised by the long exposure (hours or days) to the stress before injury is produced. Thirdly a stress may injure a plant, not by the strain it produces, but by giving rise to a second stress. A high temperature, for instance, may not be injurious of itself, but may produce a water deficit that may injure the plant. This may be called secondary stress injury.

Environmental stresses are of two main types, biotic and abiotic (physicochemical) ones. Biotic stresses include infection or competition by other organisms. Physicochemical (abiotic) stresses include light, temperature (heat, cold), water, radiation, chemical (salts, ions, heavy metals, herbicides) and mechanical factors. Among these, osmotic stress is a common denominator of a variety of stress conditions including salt, drought and cold.

1.2. Osmotic Stress

Why do plants not thrive in environments of lower water potential, i.e. under conditions of salinity, drought and cold? To understand the answer of this question, researchers investigated the osmotic stress phenomena. The osmotic strength of the environment is one of the physical parameters that determine the ability of organisms to proliferate in a given habitat.

Osmotic stress is used to refer to a situation where insufficient water availability limits plant growth and development. It can result from drought or from excessive salt in water, chilling and freezing which may also lead to osmotic stress due to reduced water absorbtion and cellular dehydration induced by ice formation. Basic cellular responses to osmotic stress, however, appeared to be conserved among all plants. These responses are summarised in Figure 1.1. One of the cellular responses, such as osmotic adjustment by accumulating products (Table 1.1) such as synthesising compatible osmolytes, is common even to all cellular organisms (Csonka and Hanson, 1991).

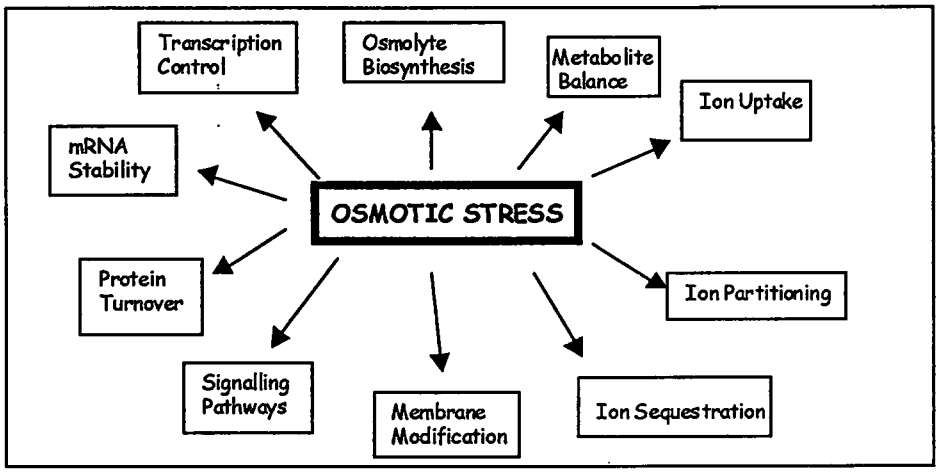


Figure 1.1 Different responses of plants to water deficit

Table 1.1 Accumulated products after osmotic stress

Product group	Specific Compound	Suggested functions
Ions	Potassium	Osmotic adjustment Macronutrient requistion Sodium exclusion/export
Proteins	LEA/dehydrins	Osmoprotection
	Osmotin	Pathogenesis-related proteins
	SOD/Catalase	Radical detoxification
Amino acids	Proline	Osmotic adjustment
	Ectoine	Osmoprotection
Sugars	Sucrose	Osmotic adjustment
	Fructans	Osmoprotection, carbon storage
Polyols	Acyclic(e.g. mannitol)	Carbon storage, osmotic adjustment
	Cyclic (e.g. pinitol)	Osmoprotection, osmotic adjustment Radical Scavenning
Polyamines	Spermine,spermidine	Ion Balance, chromatin protection
Quartenay Amines	Glycine betaine	Osmoprotection
	Alanine betaine	Osmoprotection
	Dimethyl-sulfonio propionate	Osmoprotection
Pigments and Carotenoids	Carotenoids	Protection against photoinhibition
	Anthocyanins,betalaines	

Not all accumulating products are found in all species,
but biochemical pathways are specific for orders or families of plants

1.2.1. Salt Stress

Salinity is the one of the serious factors causing osmotic stress. Salinity is a severe problem that effects 2 million km^2 of land used for agricultural production and around 30 to 50% of irrigated land, in the most productive areas of the world, such as Mediterrenian basin, California and Southern Asia. This is due to the cumulative effects over decades or centuries of adding water with some dissolved salts to the soils of arid regions (Gaxiola *et al.* 1992).

Salinity is a more complex phenomenon than a simple increase in sodium and chloride concentrations. The major contributing cations are Na^+, Ca^{+2}, Mg^{+2},K^+ and anions are Cl^-, SO_4^{-2}, HCO_3^-, CO_3^{-2}, NO_3^{-2}. There also exist trace ions including B,Sr, Li, Rb, Mo, and Al.

The deleterious effect of salt on plant cells has two components; osmotic stress and ion toxicity. The osmotic component is not specific for NaCl and results from the dehydration and loss of turgor induced by external solutes. Osmotic stress also results from desiccation and therefore it is a component of drought stress.

Crop plants are very sensitive to NaCl; the 0.15 M concentration found in animal fluids is very toxic to many crops. There is no absolute incompatibility, however, between plants and salt as demonstrated by the existence of halophytic plants, which can tolerate 0.5 M NaCl present in sea water (Flowers and Yeo, 1988). On the other hand, there is genetic potential for plants to tolerate salts but this potential is not present in crop plants.

1.2.1.1. Physiological and Biochemical Aspects of Salt Stress

Salt stress like many other abiotic stresses, inhibits plant growth. Slower growth is an adaptive feature for plant survival under stress because it allows plants to rely on multiple sources (e.g. building blocks and energy) to battle with stress. In nature, the extent of salt tolerance often appears to be inversely related to growth rate. One cause of growth rate reduction under stress is inadequate photosynthesis owing to stomatal closure and consequently limited CO_2 uptake. These responses result in conservation of water and avoidance of ion toxicity. Dehydration often leads to irreparably destructive events in proteins and cellular membranes. Salinity induces changes in the shoot/root ratio with consequences of increasing root tissue to absorb more water. But the overall growth is reduced to limit water loss in first place.

Plant survival under salt stress results partly from the maintenance of full photosynthetic capacity by the leaves, thus allowing a rapid recovery after rehydration (Chaves,1991). Reduced photosynthesis in stressed plants results from a combination of factors, including increased stomatal and mesophyll resistance and a decrease in total leaf area. As indicated by Yencey *et al.* (1982), there is a striking case of convergent evolution in solving the osmotic problem by all organisms. This occurs via synthesis of special organic solutes (osmolytes) that are well tolerated by intacellular enzymes and accumulate at high cytoplasmic concentrations to restore cell volume and turgor.

Plant cells exposed to salt undergo osmotic adjustment via a double mechanism: accumulation of NaCl at the vacuole and accumulation of organic solutes at the cytoplasm (Flowers and Yeo 1988, McCue and Hanson, 1990).

The plasma membrane H^+-ATPase is the primary pump of plant cells that drives all secondary transport systems (Serrano, 1990) The *in vivo* activity of this

ATPase, as assessed by proton efflux measurements, seems to increase during osmotic adaptation in carrot (Reuveni et al., 1987) and tobacco (Watad *et al.* 1986) cultures. Another aspect of the adaptation of plant cells to salinity is the alteration of the structure of the cell wall (Zhong and Lauchli, 1988). A decrease in the cellulose-extension framework explains the weakening of this structure in salt-adapted cells.

1.2.1.2. Molecular Aspects of Salt Stress

A variety of genes have been reported to respond to osmotic stress. These genes function both to protect cells from water deficit by production of important metabolic proteins and to regulate genes for signal transduction in stress response (Figure 1.2). These gene products can be classified into two groups. First group includes functional proteins for stress tolerance, whereas second group includes regulatory proteins to provide stress response.

The first group of genes and their products responsible from stress tolerance can be summerized as;

a) Water channel proteins (Aquaporins) mostly involve in movement of water through membranes.

b) Enzymes required for biosynthesis of various osmoprotectants are synthesized from group I genes. These osmoprotectants include, sugars, proline, glycine-betaine and sorbitol.

c) Proteins that may protect macromolecules and membranes include, LEA, osmotin, dehydrins, antifreeze proteins, chaperon and mRNA binding proteins

d) Thiol protease, Clp protease and ubiquitin are the examples for proteases synthesized for protein turnover.

e) Detoxification enzymes like superoxide dismutase (SOD), glutathione-S-transferase, soluble epoxide hydroxylase, catalase and ascorbate peroxidase.

f) Transport proteins such as Na^+/H^+ transporter.

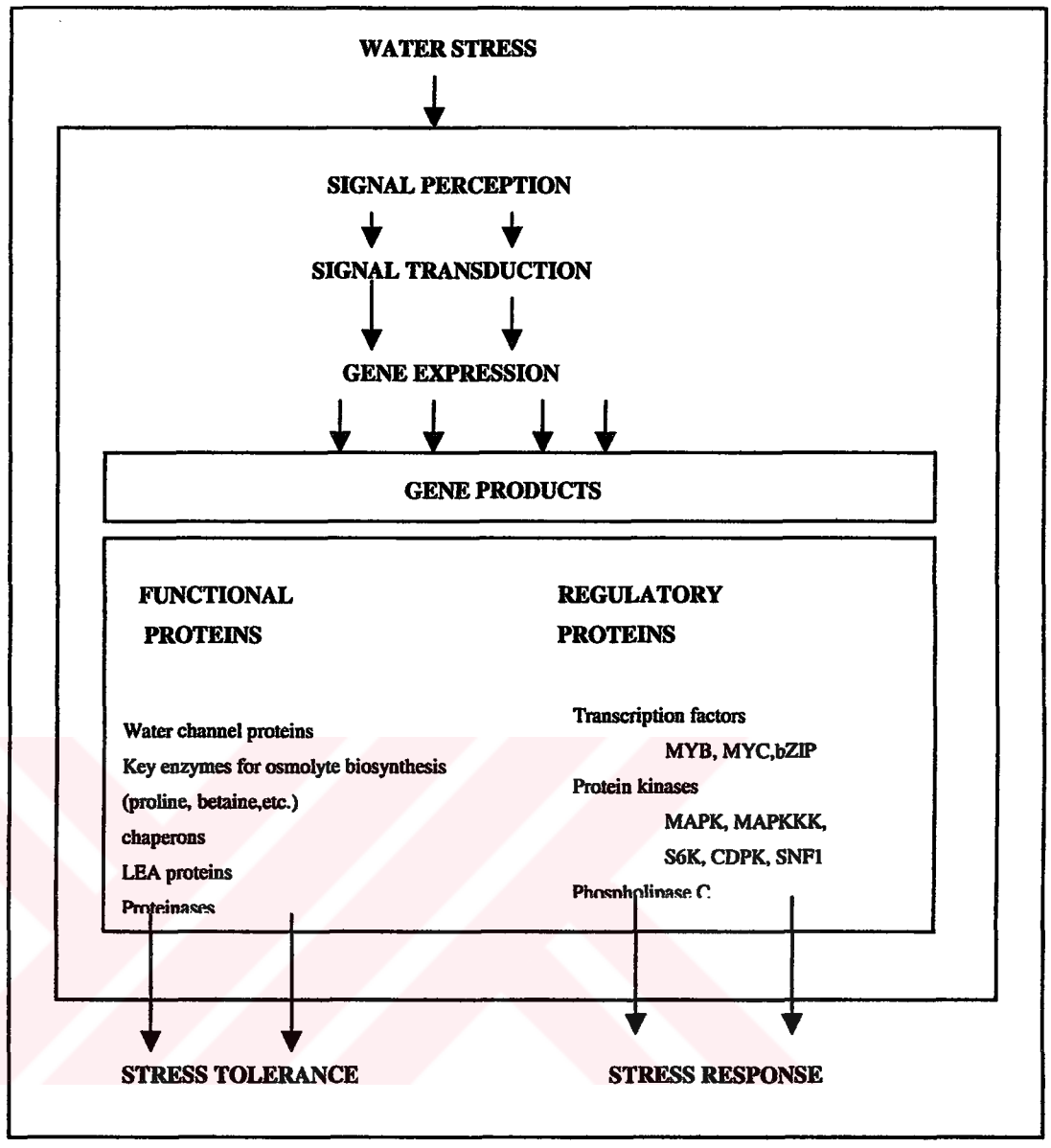


Figure 1.2 Function of water-stress inducible gene products in stress tolerance and stress response.

The second group of genes function in stress response and for regulation of signal transduction and gene expression. These include;

a) Protein kinase, MAPK, MAPKK, MAPKKK, CDPK

b) Transcripton factors, DRE1A, DREB1B etc.

c) PhospholipaseC important in PIP turnover.

d) Phosphates, i.e. calcineurin.

Salt stress induce a large number of different genes. These genes are responsible for salt tolerance of the plants. Therefore these normal components of plant adaptation could help to improve salt tolerance via manipulation of their expression.

The proteins induced by salt stress and their suggested function can be seen in Table 1.2.

Table 1.2 Proteins induced by salt stress and their proposed functions

Name	Function
Osmotin	Antifungal
LEA and RAB proteins	Desiccation protection
HS proteins	Heat shock protection
ASI	Amylase subtilisin inhibitor
WGA	Lectin
MA16	RNA regulation
Oleosins	Oil body stabilisation
TSW12	Lipid transfer protein
PEP Carboxylase	CAM metabolism
salT	Na+ accumulation
7a clone	Ion channel
Ca+2- ATPase	Ca+2 homeostatis
Aldose reductase	Sorbitol synthesis
Methyl transferase	Pinitol synthesis
Betaine- aldehyde synthesis	Betaine synthesis
Pyrolline-5-Carboxylase synthetase	Proline Biosynthesis

The enzymes involved in proline, sorbitol and betaine synthesis seem logical adaptations to salt stress. The LEA and RAB proteins may be general protectors of native protein structures during stress caused by water loss. One limitation of the search for salt-induced genes is that only abundant proteins and mRNAs can be isolated. Adaptations of tobacco cells to salt induce the accumulation several proteins, particularly one with apparent molecular weight of 26kD. This protein called osmotin and known to accumulate in the vacuolar inclusion bodies of cells that are adapted to NaCl.

Signal transduction during the induction of genes by salt and osmotic stress seems to involve the plant hormone, Abscisic Acid (ABA). While some of the water-stress-inducible genes dependent on the ABA, the others do not. Therefore, besides ABA-dependent pathways, ABA-independent pathways are also involved in water stress response. As shown in Figure 1.3, it is now hypothesised that at least four independent signal pathways function in the activation of stress-inducible genes under dehydration conditions (Shinozaki and Yamaguchi-Shinozaki 1996): two are ABA dependent (pathways I and II) and two are ABA independent (pathways III and IV).

In pathway I, biosynthesis of protein factors is necessary for the expression of water-stress-inducible genes. Such as, induction of rd22 is mediated by ABA and requires protein synthesis for ABA dependent expression. Rd22 promoter contains MYC and MYB (conserved motifs of DNA binding proteins), but not ABA Responsive Elements (ABREs). ABA-inducible bZIP proteins are also involved in pathway I.

In pathway II, genes do not require protein synthesis for their expression. They contain ABREs (PyACGTGGC) in their promoter regions. An ABRE functions as a *cis* acting DNA element involved in ABA regulated gene expression. ABREs were first identified in wheat *Em* and rice *rab* genes., and the ABRE-DNA binding protein EmBP-1 was shown to encode a bZIP protein.There are other cis- acting elements other than ABRE that function in ABA responsive gene expression.

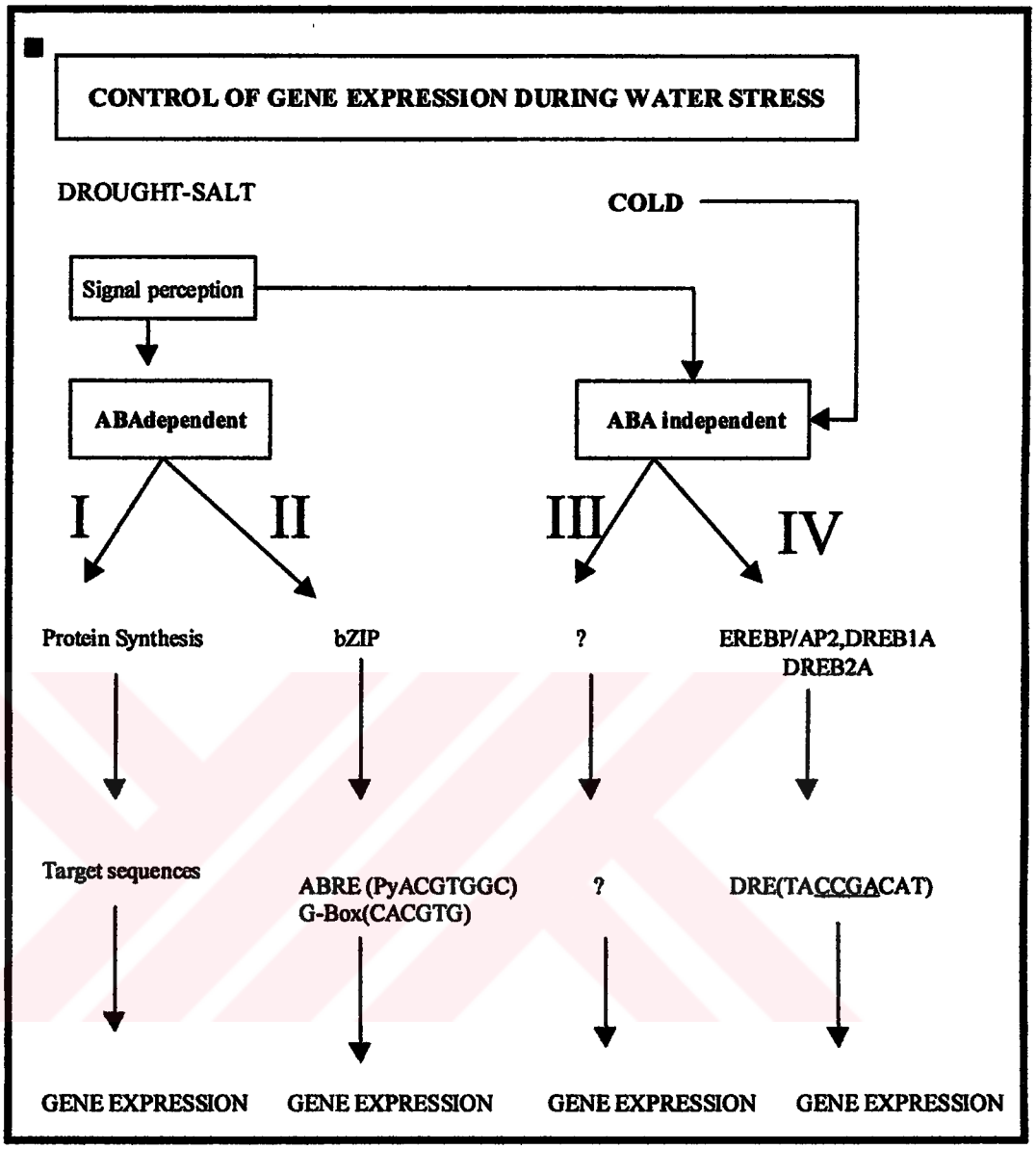


Figure 1.3 Signal Trasduction pathways between the perception of a water stress signal and gene expression

There are several drought inducible genes that do not respond to either cold or ABA treatment. These genes include rd19 and rd21. This is accepted as ABA-independent pathway III.

Several genes are induced by osmotic stress in ABA mutants. This suggested that these genes do not require ABA for expression (pathway IV), and respond to exogenous ABA (Thomashow,1994, Bray 1997). These genes are rd29 and cor47. A 9 bp conserved sequence, TACCGACAT, termed DRE, is essential for the regulation

of the induction of rd29 under osmotic stress conditions but does not function as ABRE. DRE-related motifs, including C-repeat, which contains a CCGAC core motif are involved in drought and cold responsive but ABA-independent expression.

ABA seems to induce many of the genes responsive to osmotic and salt stress (Hetherington and Quatrano,1991). The observation that ABA accelerates adaptation of cultured tobacco cells to salt (LaRosa *et al.* 1985) suggests a very important role of ABA in salt tolerance at cellular level. There is more specific effect of ABA on salt stress than on generalized osmotic stress. The mechanism of ABA action have not yet been clarified. The promoter of genes responsive to this hormone contain a core consensus sequence (CACGTG) essential for induction and recognized by a leucine-zipper protein (Mundy *et al.* 1990). ABA treatments and salinity stress have been shown to increase cytoplasmic free Ca^{+2}, which could activate Ca^{+2} dependent protein kinases (CDPK).

1.2.2. Drought Stress

There are regions in the world, where average rainfall is 5 mm or less. Of course this is not the case for all areas in the world, but drought problem exists all around the world. Water availibility changes from year to year. The problem of water availibility will increase every year because of global warming. The water limited productivity of plants, depend on the total amount of available water and the water use efficiency of the plant. Water stress has several effects on growth.

1.2.2.1. Physiological and Biochemical Aspects of Drought stress

If plants can not escape drought, they must resist it by either avoiding dehydration or tolerating dehydration. Drought mainly causes specific limitation on leaf expansion. The earliest responses to stress appear to be mediated by biophysical

events rather than by changes in chemical reactions due to dehydration (Bohnert *et al.* 1992). When water content of the plant decreases, the cells shrink and the cell walls relax which results in turgor, resulting in decreased leaf area as an early response to water deficit along with limitation of size of the individual leaves, and also decreased number of the leaves. Mild water deficit also effect the development of the root system. Water stress usually affects both stomatal conductance and photosynthetic activity in the leaf. Water stress decreases photosynthesis and the consumption of assimilates in the expanding leaves; as a consequence, water stress indirectly decreases the amount of photosynthate exported from the leaves. Since the accumulation of ions during osmotic adjustment appears to occur mainly within the vacuoles, some other compatible solutes must accumulate in the cytoplasm to maintain water potential equlibrium in the cell. Osmotic adjustment develops slowly in response to tissue dehydration. It is not clear whether osmotic adjustment is an independent and direct response to water deficit or a result of some other factor such as decreased growth rate (Table 1.3).

Table 1.3 Response of plants to drought (Ludlow, 1992)

Response	Characteristic or mechanism
Drought Escape	Short life cycle, developmental plasticity
Drought Resistance	
-Dehydration Avoidance	Enhanced water uptake (e.g., deep roots) and reduced water loss (e.g. closed stomata, leaf movement, leaf area reduction)
- Dehydration Tolerance	Dessication tolerance osmotic adjustment

1.2.2.2. Molecular aspects of drought stress

Water stress causes many changes in metabolism and development that can improve fitness for a water-deficit environment. The numerous responses to water deficit are controlled by a number of genes with various different functions. When water lost from cell, growth inhibition and alterations of developmental pathways results in changes in gene expression. Many of the water deficit induced genes encode gene products predicted to protect cellular function (Fig 1.4). As in the salt stress, LEA genes are expressed during drought stress. The predicted functions of LEA proteins are sequestration of ions, protection of other proteins and membranes and renaturation of unfolded proteins.

ABA concentration is altered when there are changes in the environment that result in cellular dehydration. Studies using ABA–deficient mutants have been used to demonstrate that elevated levels of ABA are required for the expression of specific–drought-induced genes. Since some drought induced genes are not expressed in ABA-deficient mutants, accepted as ABA-requiring genes. ABA applications have also been used to show that there are a number of water deficit induced genes that do not respond to ABA application (Yamaguchi-Shinozaki *et al.* 1992) These genes may be induced directly by drought stress, or they may be controlled by other signalling mechanisms that may operate during water deficit. There are several DNA elements acting on genes, when there is a signal coming from the stress. An ABRE 5'-C/TACGTGGCG-3', controls transcription in response to ABA. Althought it was thought that this DNA sequence is found in many genes in response to ABA application, later it was shown that ABRE is not found in all of the ABA requiring genes (Plant *et al.* 1991, Michel *et al.* 1993). Therefore, it is believed that additional elements are required to control gene expression during water deficit.

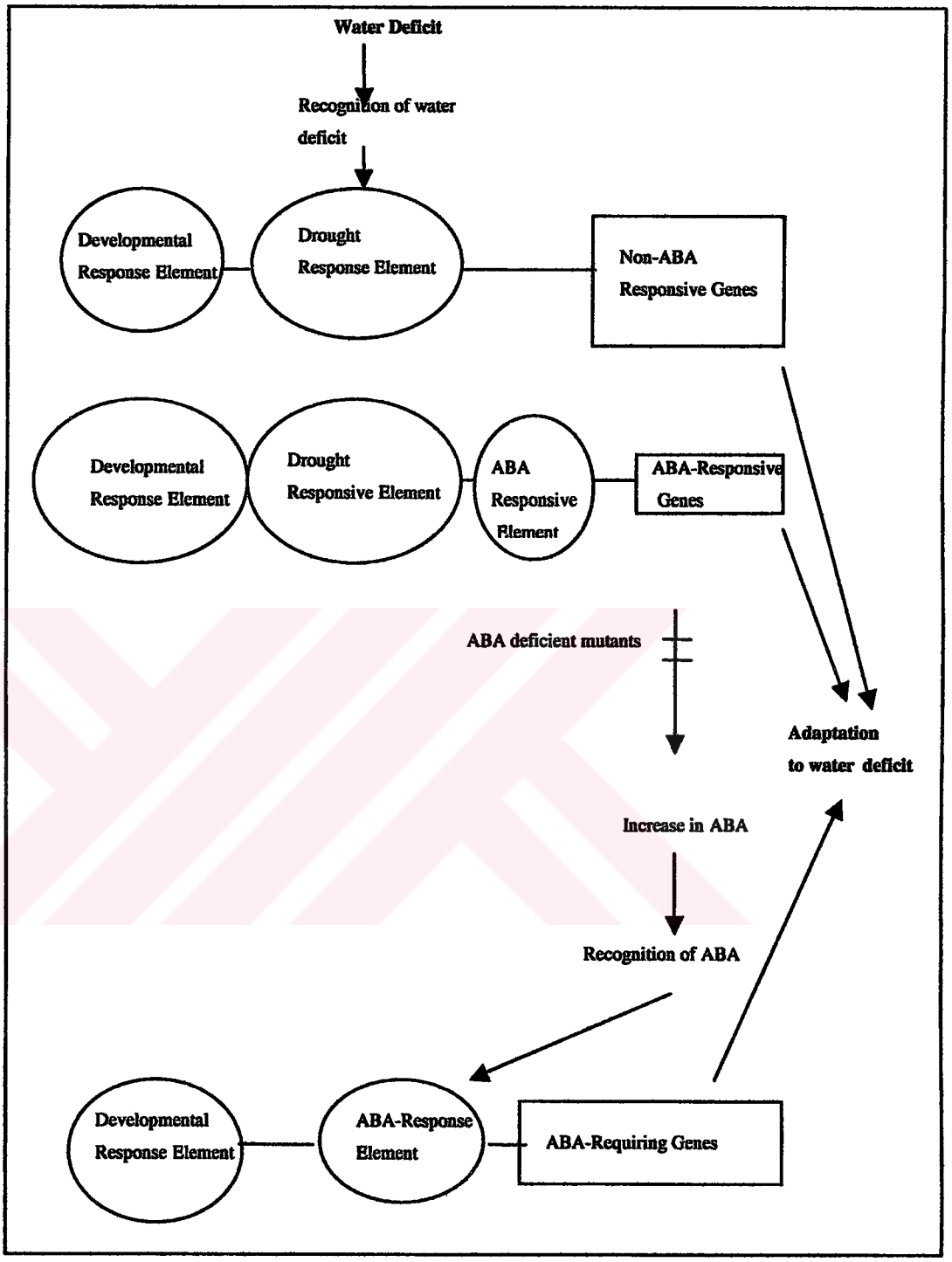


Figure 1.4 A pathway that results in the induction of genes that are ABA-requiring, ABA-responsive and ABA-non responsive.

1.2.3. Cold Stress

Every plant has an optimum temperature to grow with upper and lower limits. Cold stress can be divided into two; chilling and freezing stress. Chilling injury occurs in sensitive species at temperatures that are too low for normal growth but not enough for ice crystals to be formed in warm climates and are very sensitive to low nonfreezing temperatures. These plants include tobacco, cotton, soybean and maize. There are different responses of plants to duration of the low-temperature exposure, age of plant and species. When the tissue is cooled under optimum temperatures, ice formation occurs within the cell walls. If the tissue gets warmed this formation is not lethal. But, when this exposure is prolonged, the crystals in the walls continue to grow and cause lethal damages. Freezing temperature represents a major environmental constraint limiting growth and development. Most tropical and subtropical plant species lack the ability to adapt to freezing temperature and are typically injured by temperatures below 10 °C. In contrast, temperate plants have evolved mechanisms by which they can increase the ability to withstand the subsequent freezing temperatures in response to a period of low but nonfreezing temperatures. This process is called Cold Acclimation (CA) (Levitt, 1980). Tolerance to freezing temperatures is important in agriculture, where the ability to withstand late spring or early autumn frosts may determine success and yield of a crop.

1.2.3.1. Physiological and Biochemical Aspects of Cold Stress

The process of cold acclimation involves numerous physiological and biochemical changes. The changes under cold stress includes reduction of growth and tissue water content, a transient increase in ABA, changes in membrane lipid composition, increased level of antioxidants and accumulation of compatible osmolytes. Reduced leaf expansion, wilting and chlorosis are first signs od chilling stress. In extreme cases, browning and necrosis occur. Impaired protoplasmic

streaming, reduced respiration, photosynthesis and protein synthesis occurs in chilling sensitive tissues. All of these responses depend on the loss of membrane properties during chilling. Since low temperature causes irreversible changes in structure and functioning of membranes, ratio of saturated fatty acids to unsaturated fatty acids is very important(Xin and Browse, 2000).

Biochemical studies revealed a positive correlation between cold tolerance and a high proportion of cis-unsaturated fatty acids in the phosphatidyl-glycerol molecules of chloroplast membranes (Murata, 1983). Chilling resistant species tend to have lower proportions of saturated fatty acids, therefore lower transition temperatures enable them to withstand low temperatures. Accumulation of soluble sugars during cold acclimation is well documented in many plants and time course of sugar accumulation correlates with development of freezing tolerance during cold acclimation.

There are several roles of soluble sugars to protect plants from freezing injury, including functioning as cryoprotectans for specific enzymes, as molecules promoting membrane stability and as osmolytes to prevent excessive deyhdration during stress. The accumulation of betaines and proline is also important for tolerance of plants to cold stress.

1.2.3.2. Molecular Aspects of Cold Stress

Genetic evidence suggests that acclimation–induced cold tolerance is a quantitative character controlled by a number of additive genes (Nelson 1994). Some of these genes encode proteins with known enzymatic functions, such as alcohol dehydrogenase, phenyalanine ammonia lyase, chalcone synthase, fatty acid desaturase, catalase, a translational initiation factor and pyrolline-5-carboxylase. Some show similarity to a group of proteins involved in dehydration such as dehydrin- or LEA-like proteins, antifreeze proteins and molecular chaperones.

When the several genes were investigated in broad spectrum of plants, it was found that they contain conserved structural elements suggesting a conservation for functional reasons. A group of low-temperature-induced genes are homologous to LEA genes which are preferentially expressed during embryo maturation and encode mainly hydrophilic proteins (Skriver and Mundy-1990). This group of genes referred as COR (Cold regulated) genes (Thomashow 1999), but the same genes or homologous are referred as LT1 (low-temperature induced), CAS (cold acclimation specific), Kin (cold induced specific) and RD (responsive to desiccation) genes (Yamagushi-Shinozaki *et al.* 1992), as well.

1.3. Osmoprotectants

Most organisms increase the cellular concentration of osmotically active compounds, termed compatible solutes (osmoprotectants), during desication by either drought or accompanying external lowering of osmotic pressure, for example, increases soil salinity (Le Rudulier *et al.* , 1984, McCue and Hanson 1990, Delauney and Verma 1993). Some inorganic compounds and organic solutes can be included among compounds that operate in osmoregulation of higher plants. Accumulation of organic solutes causes internal regulation of the osmotic potential in a plant, resulting in an increase of water absortion by the roots. These compounds are compatible with proteins and membrane lipids. They are also used as important source of carbon and nitrogen source in higher plants. Organic osmoregulators can be synthesized or absorbed from the environment.

1.3.1. Structure of Osmoprotectants

Compatible solutes are hydrophilic in nature indicating that they can replace water at the surface of proteins, protein complexes, or membranes. These compounds tend to be uncharged at neutral pH. Several amino acids (proline, alanine, β-alanine and taurine), quartenary ammonium compounds (glycine betaine, prolinebetaine, β-alanine betaine, glycophosphorylcholine and choline-O-sulphate), the tertiary sulphonium compound β-dimethylsulphonipropionate and choline-O-sulfate, certain

carbohydrates (trehalose, glycerol, mannitol, sorbitol and pinitol) and polyamines (spermine) are synthesised in cells as osmoprotectants. Recently genes have been characterised leading to ectoine (1,4,5,6,- tetrahydro-2-methyl-4-pyrimidinecarboxylic acid), a compatible solute found in a number of halobacteria which shows exceptional protection of protein function *in vitro* assays (Galinski 1993, Louis and Galinski 1997). Structures of some osmolytes are shown in Figure 1.5.

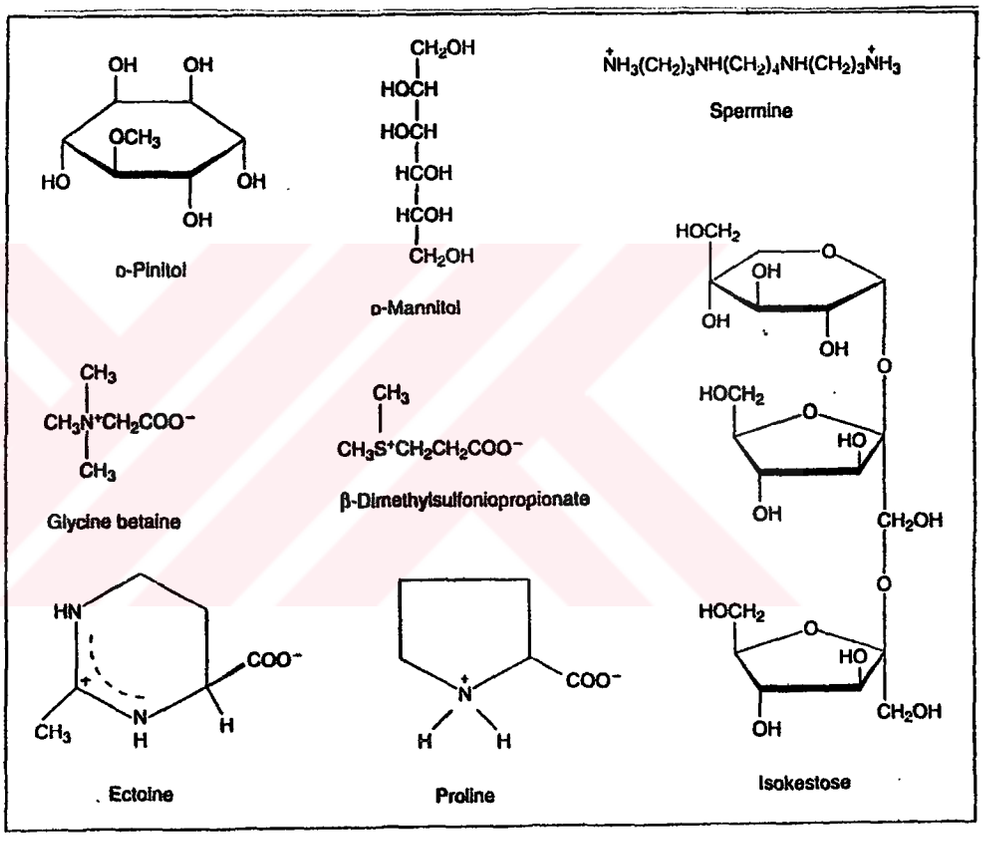


Figure 1.5 Structure of various osmoprotectants in plants

1.3.2. Function of Osmoprotectants

Perturbing solutes (such as inorganic ions) readily enter the hydration sphere of proteins, favoring unfolding. In contrast compatible solutes tend to be excluded from the hydration sphere of proteins and stabilize folded protein structures (Low 1985). Since they don't interfere with protein structure and function, they alleviate inhibitory effects of high ion concentrations on enzyme activity. Multitude of compatible solutes accumulate in response to osmotic stress.

The main function of a compatible solute may be the stabilisation of proteins, protein complexes or membranes under environmental stress. In *in vivo* experiments, compatible solutes at high concentrations have been found to reduce the inhibitory effects of ions on enzyme activity. The addition of compatible solutes increase the thermal stability of enzymes (Paleg *et al.* 1981), and prevented dissociation of the oxygen-evolving complex of photosystem II. Compatible solutes may also function as oxygen radical scavengers. Little is known about the relationship between compatible solute synthesis, water transport and ion uptake. Loss of turgor following water deficit caused either by lowering of water uptake through roots or continued evapotranspiration through stomata is likely a signal for compatible solute synthesis, possibly through a pathway that is similar to the yeast high osmolarity glycerol osmotic signalling pathway (Shinozaki and Yamaguchi-Shinozaki 1997).

Two therotical models have been proposed to explain protective or stabilising effects of compatible solutes on protein structure and function.The first is termed the ' preferential exclusion model' according to which compatible solutes are largely excluded from the hydration shell of proteins. The second model , the 'preferential interaction model', in contrast, emphasizes interactions between compatible solutes and proteins (Bohnert *et al.* 1999).

1.3.3. Types of the Osmoprotectants

From the osmoprotectants, amino acids which are products of protein hydrolysis, such as proline, glutamic acid and alanine, can be used in *de novo* protein synthesis or as osmoregulators. Betaines, including prolinebetaine, 4-hydroxyproline and glycine betaine are quartenary ammonium compounds, accumulated during salt stress in higher plants. These compounds are derived from the sequential path of proline metabolism. 3- dimethylsulfoniopropionate (DMSP) is a sulphur analoge of betaine and like betaine compatible with enzyme functioning *in vitro*. Osmotic stress induces genes that encode enzymes involved in betaine synthesis, such as aldose reductase and betainealdehyde dehydrogenase. High levels of diamines and polyamines are synthesized under stress, such as putrescine, spermine and spermidine. Among these compounds, especially spermine neutralize free radicals, regulates pH and protects membranes from ionic interactions.

Protein synthesis is one of the cellular processes particularly susceptible to osmotic stress. The presence of soluble proteins in the cytoplasm help osmoregulation. Protease inhibitors are synthesized after extended dehydration or severe salt stress.

Osmotic stress induces some specific genes to be expressed. These genes are LEA, RAB and SOS genes. LEA and RAB proteins show high hydrophilicity due to large quantity of glycine. These proteins have the ability to retain water, exclude ions and chaperone molecules. Besides these proteins, the synthesis of other kinds of proteins are stimulated by osmotic stress; 7 clone, forms an ionic channel, MA16 proetin regulates RNA, oleosine stabilizes the oil bodies, TSW12 protein affects lipid transfer, lypooxygenases, PEP Carboxylase acts in CAM (Crassulacean acid metabolism), Ca-ATPase acts on calcium homeostatis, osmotin has antifungal properties, amylase-subtisilin inhibitor, protease inhibitors, wheat germ agglutinin which is a lectin, OSR40 protein has an important effect on root adaptation to stressful environment, Alfin1 is a putative zinc finger regulatory protein, DREB2A

protein induced by dehydration and salt stress. Soluble sugars can also be accumulated in the leaves under osmotic stress.

Sucrose and trehalose are the examples. Trehalose, a non reducing disaccharide of glucose accumulates in a large number of organisms in response to different stress conditions (Elbein 1974). Polyols, glycerol, sorbitol and mannitol are alcohols that acts like osmoprotectant in response to salt stress soluble sugars are more concentrated in plants and degraded by production of glycerol and glycerol is capable of increasing the strength of hydrophobic regions . It was reported that mannitol, sorbitol, glycerol, proline, ononitol and pinitol were active free radical scavengers, although at different concentrations *in vitro*, while glycine betaine was not able to scavenge radicals (Bohnert 1999). Proline is widely distributed among organisms and accumulation have been observed not only in plants but also in eubacteria, marine invertebrates, protozoa and algae. Proline is an organic solute and synthesised under salt and water stress conditions.

1.4. Proline As an Osmoprotectant

The amino acid proline is a dominant organic molecule that accumulates in many organisms. From bacteria to maize, there is a strong correlation between increased cellular proline levels and the capacity to survive under water deficit, high temperature, freezing, heavy metals and high environmental salinity (Delauney and Verma 1993, Yancey *et al.* 1982, Nanjo *et al.* 1999). Proline accumulation is a common metabolic responses of higher plants to water deficits, and salinity stress, and has been the subject of numerous reviews (Stewart, 1981, Rhodes, 1986; Delauney and Verma, 1993, Samaras *et al.* 1995, Taylor, 1996).

This highly water soluble amino acid is accumulated by leaves of many halophytic higher plant species grown in saline environments (Stewart and Lee 1974, Treichel 1975, Briens and Larher 1982), in leaf tissues and shoot apical meristems of plants experiencing water stress (Boggess *et al.* 1976, Jones *et al.* 1980), in

desiccating pollen (Lansac *et al.* 1996), in root apical regions growing at low water potentials (Voetberg and Sharp, 1991), and in suspension cultured plant cells adapted to water stress (Handa *et al.* 1986, Rhodes *et al.* 1986), or NaCl stress (Treichel 1986, Binzel *et al.* 1987, Rhodes and Handa 1989, Thomas *et al.* 1992).

Proline protects membranes and proteins against the adverse effects of high concentrations of inorganic ions and temperature extremes (Pollard and Wyn Jones 1979, Paleg *et al.* 1984, Gibson *et al.* 1984, Santarius 1992). Proline may also function as a protein-compatible hydrotrope, and as a hydroxyl radical scavenger (Smirnoff and Cumbes 1989).

Exogenously supplied proline is osmoprotective for bacteria, facilitating growth in highly saline environments (Csonka 1989, Strom *et al.* 1983, Csonka and Hanson 1991). Accumulation of proline in the cytoplasm is accompanied by a reduction in the concentrations of less compatible solutes and an increase in cytosolic water volume (Cayley *et al.* 1991; 1992).

Selection for hydroxyproline-resistant mutants of barley and winter wheat has succeeded in identifying lines that accumulate greater quantities of proline than wild-type (Kueh and Bright 1981; Dorffling *et al.* 1993). However, it appears that the concentrations of proline accumulated by these mutants may be an order of magnitude smaller than required to produce a significant physiological effect on osmotic stress tolerance (Lone *et al.* 1987). In winter wheat the hydroxyproline-resistant lines are significantly more frost tolerant than wild-type (Dorffling *et al.* 1993). Salt tolerant and polyethylene glycol resistant mutants of *Nicotiana plumbaginifolia* have been derived from protoplast culture and appear to have enhanced proline accumulation in comparison to wild-type (Sumaryati *et al.* 1992).

Proline synthesis is implicated as a mechanism of alleviating cytoplasmic acidosis, and may maintain NADP/NADPH ratios at values compatible with metabolism (Hare and Cress, 1997). Rapid catabolism of proline upon relief of stress may provide reducing equivalents that support mitochondrial oxidative phosphorylation and the generation of ATP for recovery from stress and repair of stress-induced damage (Hare and Cress 1997).

1.4.1. Localisation of Proline in Plant Cells

The proline accumulated in response to water stress or salinity stress in plants is primarily localised in the cytosol (Ketchum *et al.* 1991, Pahlich *et al.* 1993). Induction of proline accumulation is summarised in Table 1.4 .

In cell cultures of tobacco adapted to 428 mM NaCl, proline represents over 80% of the free amino acid pool. Assuming uniform distribution of the proline in total intracellular water volume this amino acid is present at levels in excess of 129 mM (Binzel et al, 1987). If confined to the cytoplasm, however, the concentration of proline could exceed 200 mM in these cells and therefore contribute substantially to cytoplasmic osmotic adjustment (Binzel *et al.* 1987). Similarly, the cytosolic proline concentration of salt stressed *Distichlis spicata* cells (treated with 200 mM NaCl) are estimated to be more than 230 mM (Ketchum *et al.* 1991).

In the apical millimeter of maize roots, proline represents a major solute, reaching concentrations of 120 mM in roots growing at water potential of -1.6 MPa (Voetberg and Sharp, 1991). The accumulated proline accounts for a significant fraction (~50%) of the osmotic adjustment in this region. Proline accumulation in maize root apical meristems in response to water deficits involves increased proline deposition to the growing region, and appears to require abscisic acid (ABA) (Ober and Sharp, 1994)

Although maize roots are known to synthesise proline, at present it is unclear whether increased deposition of proline in the apical region is a consequence of increased transport to the apex via the phloem, or *de novo* synthesis of proline in the apex.

Table 1.4 Induction of proline accumulation during osmotic stress in plants

Species	(proline) fold increase	Stress condition
Algae		
Sticococcus bacillaris	140	1234mosmol kg-1
Dicots		
Mesembryanthermum nodiflorum (cell suspension culture)	7	400mM NaCl
Nicotiana tabacum L.	4.4	428 mM NaCl
Tobacco cell suspension culture		
Nicotiana sylveristeris	46	150mM NaCl
tobacco salt resistant strain		
Nicatiana tabacum L.	20	200 mM NaCL
leaves		
Spinacia oleracea L.	11	-2mPa
Solanum tuberosum L.	9	10% PEG
potato cell suspension culture		
Lycopersicon esculentum	319	25% PEG
tomato cell suspemsion culture		
Arabidopsis thaliana	8	120 mM NaCL
Medicago sativa L.		
roots	8	150mM NaCL
bacteriods	13	150mM NaCL
cytosol	11	150mM NaCL2-day drought
Vicia faba L.	9	2 -day drought
Glycine max L.	11	200mM NaCL
leaves		
nodules	3	2-day drought
Monocots		
Triticum aestivum L.	195.4	-3.6mPa
apex and leaves		
Hordeum vulgare	3	-1.5 mPa

1.4.2. Roles of Proline

Role of proline is not restricted as osmoprotectant in plants but it serves as nitrogen source that can be utilized during recovery. Degradation of proline in mitochondria is directly coupled to the respiratory electron transport system and ATP production. Proline may also function as hydroxyl radical scavenger (Smirnoff and Cumbes 1989) and may stabilise membranes by interacting with phospholipids (Rudolph *et al.* 1986), buffer in cellular redox potential (Hare *et al.* 1998), cryoprotective to cells (Santarius 1992) and even as a stress related signal. Several studies suggest that proline accumulation in response to osmotic stress is localised primarily in cytosol (Ketchum *et al.* 1991). Principally, accumulation of Pro can be achieved in three different ways (a) *de novo* synthesis in the affected cells (Rhodes *et al.* 1986, Voetberg and Sharp 1991) (b) decreased degradation, (c) specific transport systems that distribute Pro to the locations of need (Rentsch *et al.* 1996)

1.4.3. Proline Synthesis

It was shown that the increase in proline concentration in stressed plants is primarily due to stimulation of proline biosynthesis. Salt induced proline synthesis is not always rapid, beginning only when cell injury is evident (Hanson *et al.* 1977, Delauney and Verma 1993). There are differences in the proline synthesis in bacteria and plants.

The expression of the gene encoding P5CS is strongly induced during water deficit in plants (Yoshiba *et al.* 1995), and is thought that this enzyme catalyses the rate limiting step in proline biosynthesis (Delauney and Verma 1993). Proline biosynthesis is regulated by feedback inhibition of P5CS enzyme by proline. It was reported that P5CS is encoded by 2 differentially regulated genes in *A. thaliana*, such that P5CS1 is expressed in most plant organs, and P5CS2 is expressed in dividing cells (Strizhov *et al.* 1997).

1.4.3.1 Proline synthesis in bacteria

The pathway of proline biosynthesis was first correctly outlined in *E.coli* more than 40 years ago (Vogel and Davis 1952). In bacteria such as *E.coli, Salmonella typhimurium*, proline biosynthesis from glutamate is catalysed by three enzymes: γ-glutamyl kinase (GK), γ-glutamyl phosphate reductase (GPR) (also called glutamic semialdehyde dehydrogenase) and Δ^1-pyrolline-5-carboxylate reductase (P5CR), encoded by genes proB, proA and proC, respectively (Figure 1.6) (Adams and Frank 1980, Csonka and Baich 1983, Hayzer and Leisinger 1980, 1981, Smith *et al.* 1984).

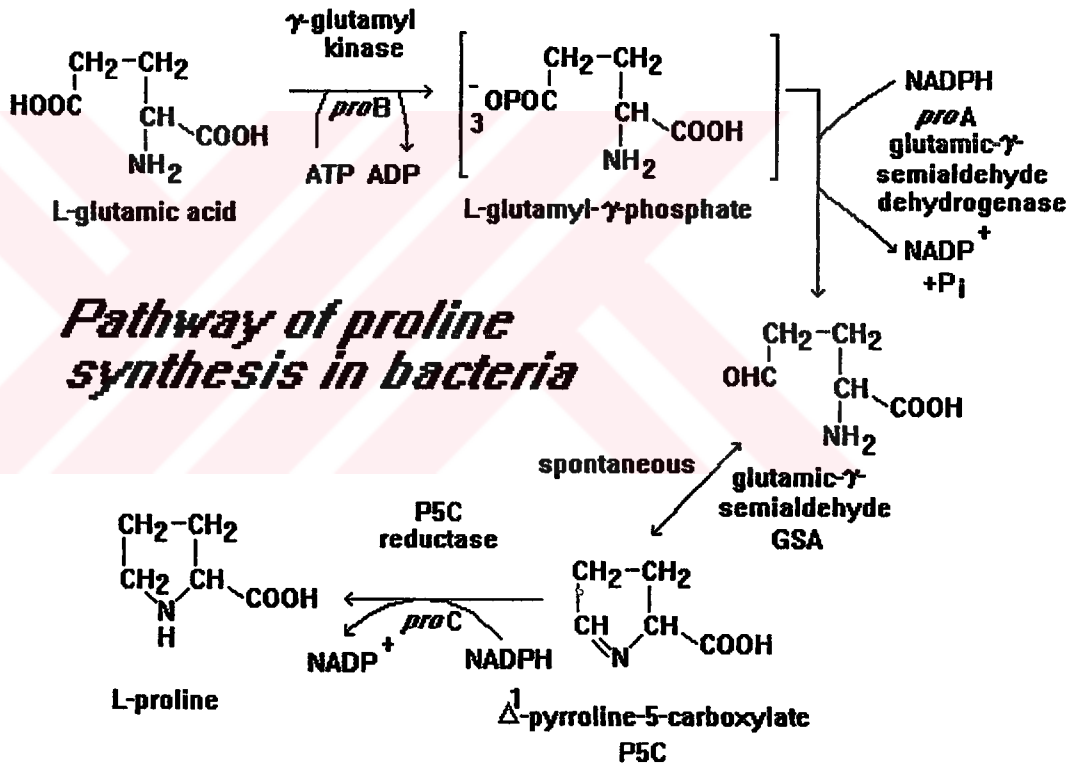


Figure 1.6 Metabolic pathway for proline synthesis in bacteria

The step from GSA to P5C is reversible and spontaneous. The reaction catalysed by GK is irreversible. The product of the reaction: γ-glutamyl phosphate is extremely labile. The GK of *E.coli* is inhibited by proline (Baich 1969, Rushlow *et al.* 1984, Smith 1985) and has a relatively low affinity to glutamate. Proline overproducing mutants have a GK with markedly altered feedback inhibition (Csonka 1981, Rushlow *et al.* 1984, Smith 1985). The yeast proline biosynthesis pathway appears to be similar to the bacterial pathway.

1.4.3.2. Proline Synthesis in Plants

A similar pathway for proline synthesis from glutamate has been assumed to operate in plants. The biosynthetic pathway to Pro from Glu is thought to involve conversion of Glu to Pro via the intermediates γ-glutamyl phosphate, GSA and P5C, as in the case in *E.coli*, because a cDNA clone for P5C synthetase (P5CS) was isolated from mothbean (*Vigna aconitifolia*) by complementation of a mutant of *E.coli*, a recombinant P5CS protein , expressed in E.coli, with both γ-GK and GSA dehydrogenase activities (Hu *et al.* 1992) (Fig 1.7). cDNAs for plant P5CS have been isolated from mothbean (Hu et al. 1992), *Arabidopsis thaliana* (Yoshiba *et al.* 1995, Savoure *et al.* 1995) and rice (Igarashi *et al.* 1997). Plants also synthetase proline from ornithine, a conversion which could proceed via two routes, both involving transamination of ornithine followed by cyclization and reduction. Isotope tracing experiments showed that the pathway via ornithine is not important for Pro synthesis during osmotic stress (Fujita *et al.* 1998).

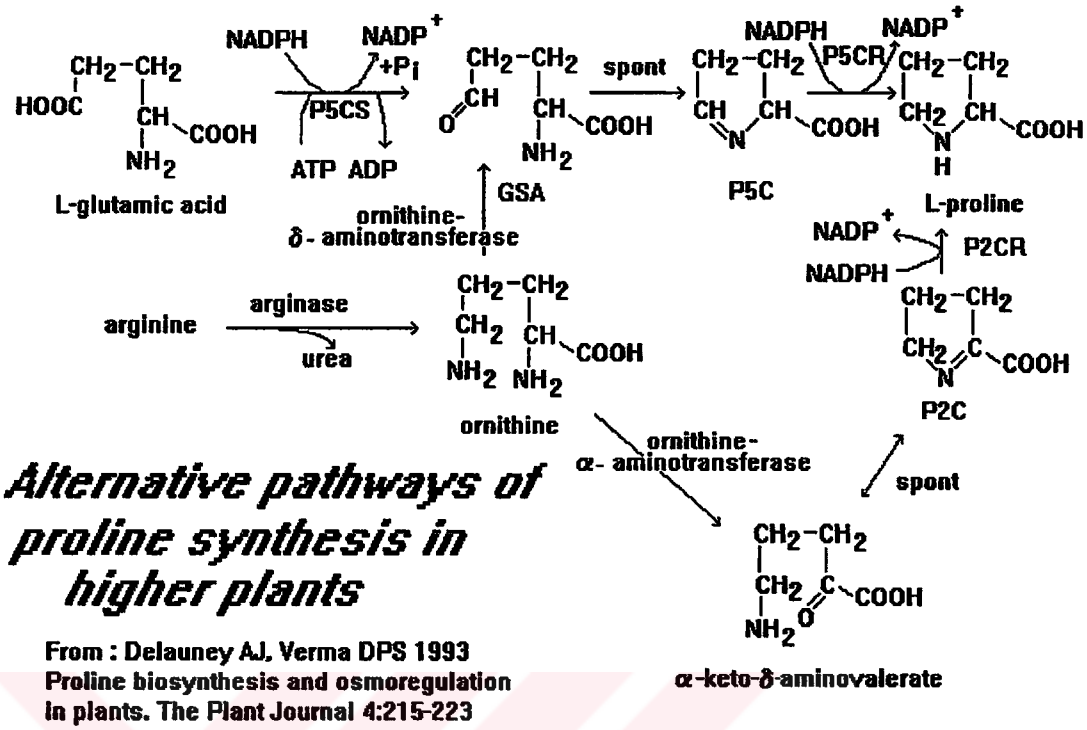


Figure 1.7 Metabolic pathway for proline in plant cells

Pyrroline-5-carboxylate reductase (P5CR) has been identified and characterised in several plant species (Treichel 1986, LaRosa *et al.* 1991). A chloroplast localisation of this enzyme has been reported in pea (Rayapati *et al.* 1989).

However, there have been no reports of GK or GPR activities in higher plants since the first report of the occurrence of *in vitro* synthesis of ^{14}C-GSA from ^{14}C-glutamate, Mg^{2+}, ATP and NADPH in cell free extracts of beet. The enzyme activity described is similar to that reported for the pyrroline-5-carboxylate synthetase (P5CS) activity of mammalian cells (Wakabayashi and Jones 1983), where a bifunctional enzyme catalyses the first two steps of proline biosynthesis.

Substantial progress has been made in recent years in the cloning of cDNAs encoding the proline biosynthetic enzymes from higher plants by complementation of proline-requiring mutants of *Escherichia coli* (Verma *et al.* 1992).

1.4.4. Regulation of Proline Biosynthesis

A cDNA encoding P5CR was cloned by direct complementation of an *E. coli proC* proline auxotroph with a soybean nodule cDNA expression library (Delauney and Verma, 1990), facilitating the isolation of P5CR homologs from *Pisum sativum* (Williamson and Slocum, 1992) and *Arabidopsis thaliana* (Verbruggen *et al.* 1993).

P5CR transcripts increase in abundance in response to osmotic stress, indicating that P5CR gene transcription is under control of osmotic stress (Delauney and Verma 1990, Williamson and Slocum 1992, Verbruggen *et al*, 1993). The P5CR gene of *Arabidopsis* is developmentally regulated. The P5CR promoter directs strong GUS expression in root tips, the shoot meristem, guard cells, pollen grains, ovules and developing seeds (Hua *et al.* 1997).

The P5CR cDNA from soybean, when over-expressed in tobacco, did not result in a significantly increased proline level in the transgenic plants, despite a 100-fold greater P5CR activity than wild-type (Szoke *et al.* 1992). Thus, P5CR may not be the rate-limiting step in proline accumulation (LaRosa *et al.* 1991, Delauney and Verma 1993).

A cDNA encoding both GK and GPR was isolated from a mothbean cDNA expression library employing *E. coli proA*, *proB* and *proBA* proline auxotrophs, and screening for cDNAs, which permit growth in the absence of proline. This cDNA was found to encode a bifunctional enzyme pyrroline-5-carboxylate synthetase (P5CS) (Hu *et al.* 1992). The single major open-reading frame of this cDNA encodes a polypeptide of 73.2 kDa which has two distinct domains exhibiting 55.3% overall similarity to *E. coli* GK and 57.9% similarity to *E. coli* GPR.

Proline synthesis in plants is more complex since there are two pathways which may be regulated at the levels of both enzyme activity and gene expression. Early experiments indicated that there is end product inhibition in unstressed tobacco leaves (Boggess *et al.* 1976). When the glutamate pathway was tested in plants, it

was found that mothbean P5CS enzyme is inhibited in *E.coli*. It was allosterically inhibited and less sensitive to feedback inhibition than *E.coli* GK enzyme.The expression of the gene for P5CR appears not to be enhanced to any significant extent by dehydration, high salinity, or treatment with ABA (Yoshiba *et al.* 1995). Expression of the P5CR cDNA in transgenic tobacco resulted in a 200 fold increase in the P5CR activity, but the proline level in transgenic plants was not significantly altered (Szoke *et al.* 1992) This result indicated that P5CR is not the rate limiting enzyme in proline biosynthesis in plants.

The GK of the bifunctional P5CS of *Vigna* is inhibited by proline (50% inhibition with 6 mM proline) (Hu *et al.* 1992). This enzyme appears to be much less sensitive to feedback inhibition than the wild-type GK of *E. coli* (Hu *et al.* 1992).

Northern analysis indicate that the P5CS gene is induced (particularly in leaves) by treatment of *Vigna* plants with 200 mM NaCl (Hu *et al.* 1992). Desiccation, salinity stress and ABA dramatically induce P5CS mRNA transcript abundance in *Arabidopsis* (Yoshiba *et al.* 1995, Stritzov *et al.* 1997). Savoure *et al.* (1997) suggest that the expression of the proline biosynthetic genes are dependent upon at least two signal transduction cascades; one triggered by exogenously applied ABA in the absence of stress, and the other triggered by cold and osmotic stress independently of exogenously applied ABA.

Hare and Cress (1997) argue that the different subcellular localisation of proline biosynthesis (cytoplasm) and oxidation (mitochondrion), the NADPH cofactor preference of the biosynthetic enzymes, and NADH cofactor preference for the proline oxidation pathway, would enable proline biosynthesis to enhance activity of the cytoplasmic oxidative pentose phosphate pathway, and provide a mechanism of interconversion of the phosphorylated and non-phosphorylated pools of pyridine nucleotide cofactors. They suggest that the osmoprotective effects of proline accumulation may be of secondary importance to the associated metabolic implications of proline synthesis and degradation. Further investigations of transgenic plants engineered for altered proline synthesis and/or catabolism clearly

need to consider not only the consequences on absolute proline level and the biophysical effects exerted by elevated cytoplasmic proline concentrations, but also the fluxes to and from the proline pool (Hare and Cress, 1997).

In growing tissue, the proline deposition rate will be equal to the combined rates of proline synthesis, proline release from protein, and proline import, minus the combined rates of proline catabolism, proline export, proline utilisation in protein synthesis, and the rate of pool dilution caused by water uptake during growth (Rhodes and Handa, 1989; Voetberg and Sharp, 1991). The latter will in turn be determined by fundamental plant water relations of the growing region, including water potential, solute potential, yield threshold, turgor, and potential difference between the xylem and growing cells as well as the levels of growth inhibitory metabolites such as ABA, whose synthesis or compartmentation may respond rapidly to changes in turgor and/or metabolism (e.g. intracellular pH) (Rhodes, 1987). The feedback loops in this system are clearly complex, with ABA and osmotic signals altering expression of genes encoding proline biosynthesis enzymes, proline and osmotic signals altering expression of PDH, proline feedback inhibiting its own synthesis, and proline *per se* (if accumulated to sufficiently high levels) contributing to solute potential, and hence turgor and growth maintenance. The full promise of transgenic plants engineered for proline metabolism in understanding of proline's role(s) in stress resistance will not be realised likely until attention is given to the growing regions as well as mature tissues (Kavi-Kishor et al, 1995).

The consideration of growing and non-growing tissues and the fluxes between them will be essential in testing the intriguing scheme recently proposed by Hare and Cress (1997) in which proline and P5C might act as an intercellular signalling system. In this scheme, proline produced from P5C in an "effector" tissue is transported via the phloem to a "target" tissue characterised by a high energy requirement, where proline degradation generates reducing equivalents needed to drive TCA cycle activity. The P5C or glutamate generated may then be translocated back to the effector tissue where conversion to proline regenerates $NADP^+$ needed to prime the oxidative pentose phosphate pathway.

P5C or glutamic-5-semialdehyde, or a molecule derived from them, is implicated as a regulator of other osmotic stress responsive genes, including dehydrins and *sal*T (a NaCl, drought, ABA and proline-inducible gene that it a sensitive indicator of abiotic stress) in rice (Iyer and Caplan, 1998). The proline metabolites appear to be more potent inducers of *sal*T than proline (Iyer and Caplan, 1998).

Plant vacuole plays an important role in the accumulation and transportation of Pro during stress. The expression of P5CS gene is independent of ABA upon exposure to cold and osmotic stress, even though expression of this gene can be triggered by treatment with exogenous ABA (Savoure *et al.* 1997). The observations suggest that the expression of the gene for P5CS can be induced both in ABA-independent and ABA dependent pathway, under dehydration conditions. Two different *cis* acting elements appear to function in ABA dependent and –independent gene expression under water stress, The ABRE (ABA responsive element) functions as a *cis* acting element is involved in ABA responsive transcription. By contrast, the DRE (dehydration responsive element) has been involved in ABA independent gene expression under dehydration, high salinity and low temperature conditions.

The transcriptional regulation of the level of Pro under dehydration and rehydration is shown schematically in Figure 1.8. P5CS gene is induced by dehydration and repressed by rehydration. P5CR gene expression is slightly upregulated by dehydration. These results show that induction of P5CS has a main role in Pro synthesis.

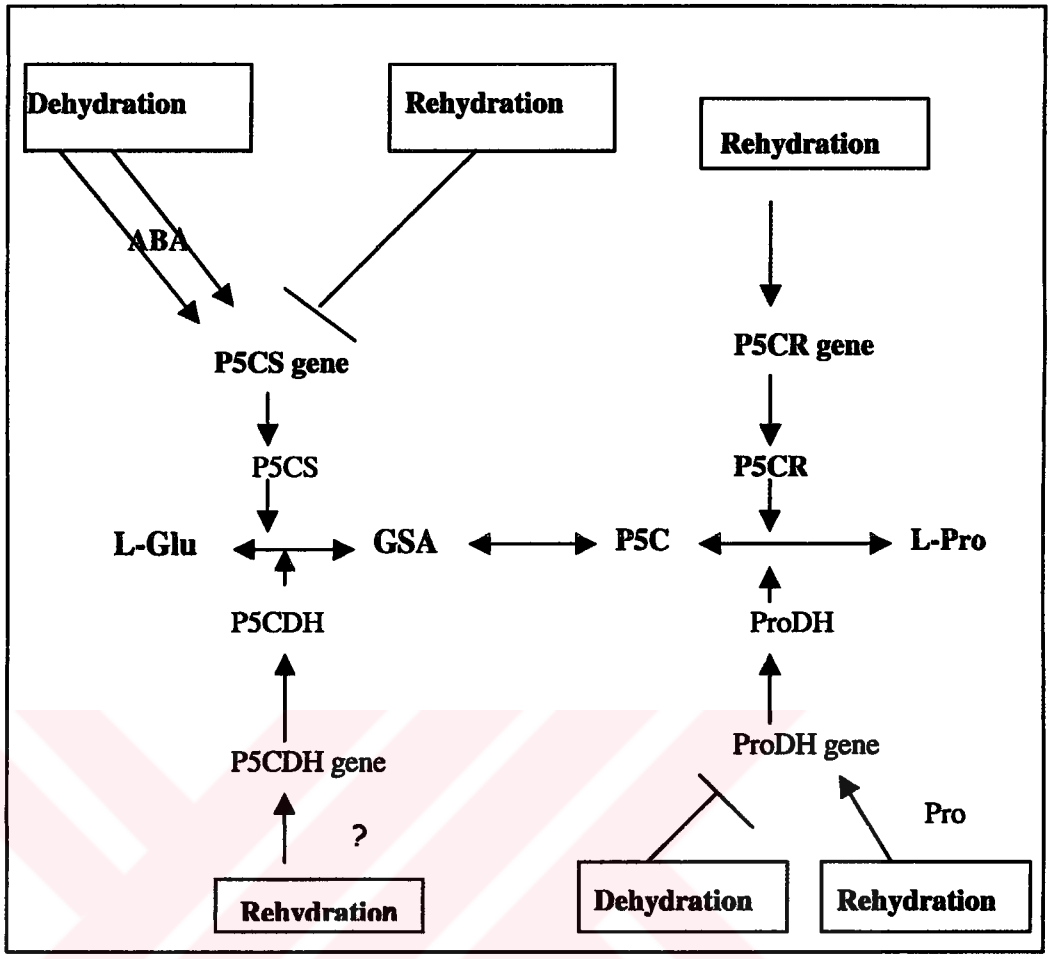


Figure 1.8 Effect of dehydration and rehydration on proline metabolism

1.4.5. Proline Degradation

L-proline is metabolised to Glu by two enzymes, Proline dehydrogenase (ProDH) and Δ^1-pyrolline-5-carboxylate dehydrogenase (P5CDH), which catalyse reactions that are the reverse of those catalysed by P5CR and P5CS, respectively, in the biosynthesis of Pro (Fig 1.6). Proline dehydrogenase is localised on the matrix side of the inner membrane of mitochondria. ProDH seems to donate electrons directly to the respiratory electron transport system (Elthon and Steward 1981, 1982). cDNAs encoding ProDH were isolated from *Arabidopsis, Brassica napus* (Kiyosue *et al.* 1996, Verbruggen *et al.* 1996). Kiyosue *et al.* isolated a cDNA clone (erd5) for ProDH from a cDNA library of one-hour–dehydrated plants by differental screening. Verbruggen *et al.* and Peng *et al.* found a conserved sequence of ProDH in the *Arabidopsis* EST database by using the aminoacid sequences of ProDH from

Saccharomyces cerevisiae and from D*rosophilia melanogaster*. The ProDH protein of *Arabidopsis* is 34.5% and 23.6% homologous to those of *Drosophilia and S. cerevisiae*, respectively. The expression of ProDH was strongly induced by rehydration after dehydration for 10 hr, but not by cold or heat stress (Kiyosue *et al.* 1996). The expression of the gene for ProDH was induced by exogenous L-Pro and D-Pro. On the other hand expression was repressed by osmotic stress.

Dehydration of plants cause osmotic stress and then elevated levels of proline in plant cells. When plants rehydrated, the expression of this gene became inducible by Pro because of the removal of osmotic stress and absence of repression. The activity of ProDH is regulated at both enzyme activity and gene expression levels. Research is still being performed for the analysis of the promoter of gene for ProDH and identification of cis-acting elements that are involved in the Pro-inducible expression and repression of this gene by osmotic stress.

1.4.6. Mechanisms of Stress-Induced Proline Accumulation

In vivo labeling studies with ^{14}C-labeled precursors (Boggess *et al.* 1976), ^{13}C-glutamate (Heyser *et al.* 1989a,b) or ^{15}N (Rhodes *et al.* 1986; Rhodes and Handa, 1989) suggest that glutamate is a major precursor of osmotic stress-induced proline accumulation in plants.

Osmotic stress results in an increase of proline biosynthesis rate (Boggess *et al.* 1976, Stewart 1981, Rhodes *et al.* 1986; Rhodes and Handa, 1989). Proline accumulation may in part involve induction (Peng *et al.* 1996) and/or activation of enzymes of proline biosynthesis, possibly coupled with a relaxation of proline feedback inhibition control of the pathway (Boggess *et al.* 1976, Stewart 1981, Delauney and Verma 1993), decreased proline oxidation to glutamate (Stewart *et al.* 1977, Stewart and Boggess 1978, Elthon and Stewart 1982) mediated at least in part by down-regulation of proline dehydrogenase (Kiyosue *et al.* 1996, Peng *et al.* 1996), decreased utilization of proline in protein synthesis (Boggess and Stewart 1980, Stewart 1981) and enhanced protein turnover.

Water deficits induce dramatic increase in the proline concentration of phloem sap in alfalfa (Girousse *et al.* 1996), suggesting that increased deposition of proline at the root apex in water stressed plants (Voetberg and Sharp, 1991) could in part occur via phloem transport of proline (Girousse *et al.* 1996). A proline transporter gene, *ProT2*, is strongly induced by water and salt stress in *Arabidopsis thaliana* (Rentsch *et al.* 1996). Homologous proline transporter genes have been identified in tomato; *LeProT1* is strongly expressed in mature and germinating pollen, and may encode a general transporter for compatible solutes (Schwacke *et al.* 1999). LeProT1 transports proline and GABA with low affinity and glycinebetaine with high affinity (Schwacke *et al.* 1999).

Stress-induced proline accumulation is inhibited by both cycloheximide and cordycepin in *Arabidopsis* (Verbruggen *et al.* 1993). In contrast, because cycloheximide is inhibitory to 200 mM NaCl-induced proline accumulation in *Distichlis spicata* cells, whereas actinomycin D is not inhibitory, Ketchum *et al.* suggested translation but not transcription is necessary for production of proline in stressed cells.

1.5. Strategies to Develop Osmotic Stress Resistant Transgenic Crop Plants

Understanding the mechanisms for the stress tolerance is very important for the development of the resistant plants. Transgenic plants with improved tolerance to osmotic stress have been produced using various genes (Table 1.3). For the production of osmotic stress resistant transgenic plants both functional and regulatory genes were transformed to plant cells. Functional proteins are involved in water stress tolerance and adaptation, on the other hand regulatory proteins function in gene expression and signal transduction in stress response.

There are several strategies to develop water stress tolerance and stress response in plants. These can be categorised as,

1.5.1.Oxidative Stress Related Genes

First strategy is the protection againist toxic by products (free radicals) via expressing detoxification enzymes. Transgenic plants have been developed expressing oxidatice-stress-induced genes. Tomato and tobacco plants were developed which were overexpressing Cu/Zn –SOD but failed to induce any protection againist superoxide toxicity. (Tepperman and Dunsmuir 1990). However, Gupta showed that the overexpression Cu/Zn SOD in tobacco provided resitance with respect to photosynthesis under drought and chilling stress. Overexpression of the MnSOD decreased the cellular damage againist osmotic stress (Bowler *et al.* 1991). When the coding region of the yeast mitocondrial Mn-SOD gene was fused with chloroplast targetting signal of glutamine synthetase gene and transformed to rice protoplasts, the increased level of ascorbate peroxidase and high levels of chloroplast SOD in the transformant together with increased salt resistance was observed in rice (Tanaka *et al.* 1999).

The overexpression of chloroplast glutamine synthetase enhanced the tolerance to salt (0.15 M NaCl) in transgenic rice plants. Thus it was shown that enhancement of photorespiration conferred resistance to salt (Hishida *et al.* 2000). Nt107 cDNA encoding glutathione-S-transferase constitutively expressed in tobacco showed better growth than control plants under salt and cold stress (Roxas *et al.* 1997).

Overexpression of cell wall associated peroxidase (TPX2) which is an enzyme involved in modifying cell wall architechture, improved germination under osmotic stress in tobacco plants (Amaya *et al.* 1999).

In another study, peroxisomal membrane bound ascorbate peroxidase encoding gene from *A.thaliana* (APX3) introduced in tobacco plants. The expression of APX3 in tobacco can protect plants from oxidative stress resulted from aminotriazole (AT) treatment (acting on peroxisomes), but not from paraquat treatment (acting on chloroplast) (Wang *et al.* 1999).

Rapid accumulation of toxic products from reactions of reactive oxygen species with lipids and proteins significantly contribute to the damage of crop plants under abiotic stresses. Sorbitol is sugar alcohol of glucose. The conversion of glucose to its sugar alcohol is catalysed by aldose reductase Aldose reductase is also active on 4-hydroxynon-2-enal, a known cytotoxic lipid peroxide degradation product. A gene encoding for aldose reductase was isolated and transformed to tobacco, transformed plants showed better tolerance with respect to control under heavy metal and paraquat stress (Oberschall. 2000). GutD gene, encoding a key enzyme (glucitol-6-phosphate dehydrogenase) of sugar alcohol metabolic pathway in *E.coli*, was transformed to maize. The synthesis and accumulation of sorbitol were detected in transgenic maize plants and this lead to an increased tolerance to 200 mM NaCl stress (Yan *et al.* 1999).

1.5.2. Osmolyte Synthesizing Genes

The second strategy was the osmoprotectant engineering via transferring osmolyte producing enzymes. Since the accumulation of the osmolytes is one of the main responses during osmotic stress, engineeringly increased osmolyte content is a promising strategy to protect plants againist stress. Transgenic plants have been produced containing proline, mannitol, glycine betaine, trehalose, etc. These experiments showed that there was reduction on the effects of stress in transgenic plants.

Mannitol:

Tarczynski *et al.* (1993) produced transgenic plant containing *E.coli mtlD* gene encoding mannitol-1-phosphate dehydrogenase accumulated mannitol. These plants showed accumulation of mannitol and tolerance to salt stress. Same gene was transferred to *Arabidopsis* and similar response was observed (Thomas *et al.* 1995). In the presence of 250 mM salt, mannitol containing plants have a growth advantage when compared with controls in terms of less fresh weight loss, more leaf and root production and better height gain. Hovewer, Karakas *et al.* (1997) reported that there were no growth changes between transgenic and control plants under drought stress.

Recently, *mtlD* gene was introduced to the cytosol of rice plants by an imbibition technique to overproduce mannitol. The growth and germination of imbibed seeds were increased transiently (Lee *et al.* 2000).

Glycine betaine:

Glycine betaine accumulates in chloroplasts and cytoplasm. There was a genetic evidence that glycine betaine improves salinity tolerance of barley and maize (Grumet and Hanson 1986, Rhodes *et al.* 1989). The synthesis of glycine betaine occurs from choline. Lilius *et al.* (1996) introduced *E.coli* betA gene to tobacco. This gene is encoding for choline dehydrogenase. The bacterial choline oxidase gene from *Arthobacter globiformis* was isolated and transferred to *Arabidopsis*. Transgenic *Arabidopsis* plants showed better response to salt and cold stress (Hayashi *et al.* 1997,Alia *et al.* 1998).

Establishment of genetically engineered rice plants with the introduction of choline oxidase gave higher glycine betaine contents than control plants. In two types of transgenic plants in which *codA* targeted to chloroplasts and cytosol, showed different levels of glycine betaine and photosynthetic inhibition under stress conditions.

It was also indicated that the subcellular compartmentalisation of the biosynthesis of glycine betaine was a critical element in the efficent enhancement of tolerance to salt and cold stress in genetically engineered rice (Sakamoto *et al.* 2000). Holstrom *et al.* (2000) indicated that, induction of glycine betaine production into tobacco was associated with increased stress tolerance probably due to improved protection in photosynthetic apparatus of transgenic tobacco plants.

Trehalose:

Trehalose is a nonreducing disaccharide of glucose and is found in bacteria, fungi, insects and some plant species. Trehalose is rare sugar in higher vascular plants. Yeast trehalose–6-phosphate synthetase gene (TPS1) was transferred to tobacco, and transformed plants showed improved drought tolerance and phenotypic alterations (Romero *et al.* 1997). Although the trehalose protective effect remains unclear at the molecular level, correlative evidence suggets that trehalose stabilises proteins and membrane structures (Iwashashi *et al.* 1995).

Pinitol and ononitol:

The cyclic sugar alcohol pinitol and ononitol are stored in a variety of species when exposed to salt conditions (Bartels and Nelson 1994). Myo inositol is methylated to ononitol and then epimerization to pinitol. An inositol methyl transferase (IMT) gene was introduced to tobacco under constitutive promoter (Vernon et al. 1993, Sheveleva et al 1997), which resulted in accumulation of methylated inositol conferring tolerance to salt and drought stresses.

Fructans:

Fructans are polyfructose molecules that are produced by many plants and bacteria. A gene encoding a bacterial fructan synthase isolated from *Bacillus subtilis* transformed to tobacco, and transformed plant performed better under PEG mediated osmotic stress than control plants (Pilon-Smits *et al.* 1995). Same gene was used to produce transformed sugarbeet plants. Transgenic plants showed better growth under drought stress than untransformed beets (Pilon-Smits *et al.* 1999).

Polyamines:

Polyamines are small, ubiquitous, nitrogenous cellular compounds that accumulate under stress conditions. Cultivars having salt tolerance contained higher levels of polyamines (Bajaj *et al.* 1999).

Transgenic carrot overproducing ornithine decarboxylase, which converts ornithine to diamine putrescine, withstanded osmotic stress for a short period. Capell *et al.* (1998) overexpressed the oat arginine decarboxylase gene in rice. Plants had improved trought tolerance in terms of chlorophyll loss. However, it is not clear yet how polyamines provide protection againist osmotic stress.

Heat-shock protein DNAK/Hsp 70 from halotolerant cyanobacterium *Aphanothece halophytica* overexpressed in cytosol of tobacco cells. The growth rate of the transgenic plants was better than nontransformed ones under 0.6 M NaCl stress. Similar results were observed for stomatal response, photosynthetic activity and sodium content of the transformed plants (Sugino *et al.* 1999).

Table 1.5 Stress responses of transgenic plants expressing various genes involved in stress tolerance

Gene	Gene product and function	TR plant	Performance of TR
Genes encoding enzymed that synthesize osmoprotectants			
bet A (glycine betaine synthesis)	choline dehydrogenase	tobacco	Increased tolerance to salt
codA	choline oxidase	Arabidopsis,rice	Seedlings were more tolerant to salt
IMT1	myo,inositol-O-methyltra,nsferase	tobacco	Improved performance under drought stress shown by better photosynthetic rate
mtlD	mannitol-1-phosphate dehydrogenase	tobacco	6-week-old plants showed better growth in terms of percent change of weight and fresh weight under high salinity
		Arabidopsis	Enhanced seed gemination under salt
		tobacco	increase in dry weight under salt stress
otsA	trehalose-6-phosphate-synthetase	tobacco	Increase in dry weight and more efficient photosynthesis under drought stress
otsB	trehalose-6-phosphate phosphatase		
P5CS	Pyrolline-5-carboxylae synthetase	tobacco	Enhanced biomass and flower development
		rice	Transgenic seedlings exposed to 0.1 M NaCl for 5 days and 8-week-old plants subjected to water stress had increased biomass than control
sacB	fructosyl transferase	tobacco	3-week-old plants showed better growth under PEG treatment
TPS1	trehalose-6-phosphate-synthetase	Tobacco	Increased drought tolerance with better survival of leaves after withholding by water
Adc	arginine decarboxylase	rice	Minimised chlorophyll loss under drought stress
Odc	Ornithine decarboxylase	carrot	Transgenic cell lines could withstand high salt ove a (cell line) short period
LEA or LEA related genes			
HVA1	group 3 LEA protein	rice	Better growth under 100mM NaCl and 200mM Mannitol
COR15a	cold-induced gene	Arabidopsis	Increased freezing tolerance of chloroplasts and protoplasts
Regulatory genes			
CBF1	Transcription factor	Arabidopsis	Increased cold tolerance as determined by the electrolyte leakage test and whole plant survival rate
DREB1A	Transcription factor	Arabidopsis	Increased salt, drought and cold tolerance
Oxidative stress-related genes			
Nt107	glutathione-S-transferase	tobacco	Transgenic seedlings grew faster than controls when Subjected cold and salt stress
SOD	Cu/Zn superoxide dismutase	tobacco,tomato	no protection against superoxide toxicity
	Cu/Zn superoxide dismutase	tobacco	retained 90% photosynthesis under chilling and high-light stress
	Fe superoxide dismutase	tobacco	Protected plant against ozone damage
	Fe superoxide dismutase	tobacco	No good response to salt stress
	Mn superoxide dismutase	tobacco	Reduced cellular damage under oxidative stress
	Mn superoxide dismutase	alfalfa	Increased tolerance to freezing , water deficit and better winter survival
Msfer	ferritin	tobacco	Increased tolerance to oxidative damage induced by iron excess or paraquat treatment

1.5.3. LEA or LEA Related Genes

Another strategy is the enhanced stress related gene expression via transfer of transcriptional factors. Lea or lea-related genes are expressed under stress conditions. When the barley Lea protein HVA1 is expressed constitutively in rice, second generation showed significantly increased tolerance to salt and water-deficit conditions. The precise mode of action of HVA1 gene under drought conditions is not clear. Lea-related gene Cor15a was constitutively expressed in *Arabidopsis* and enhanced the freezing tolerance that indicates the constitutive expression of Cor15a affects the cryostability of the plasma membrane. The overproduction of glyoxalaseI showed significant tolerance to high salt (Veena, Reddy *et al.* 1999). Since the products of the regulatory genes regulate gene expression and signal transduction, overproduction of these genes can activate other stress related genes. CBF1 gene, whose product is a transcriptional activator that binds to a cis-acting repeat (CRT)/(DRE) was introduced to *Arabidopsis* plants. The overexpression of these genes induced Cold responsive gene expression and increased freezing tolerance (Jaglo-Ottosen *et al.* 1998). In another study, cDNA encoding DERB1A was transformed to *Arabidopsis*, overexpression of this gene activated the expression of many stress-tolerance genes such as rd29A (similar to LEA protein), Kin1, Cor6.6, Cor15a, rd17 and P5CS. In all cases transgenic plants are more tolerant to osmotic stress.

Other approach is the isolation and transfer of genes directly implicated in salt tolerance mechanisms. Several halotolerance genes (HAL) have been isolated from *S. cerevisea* (Serrano and Gaxiola 1994). The overexpression of HAL genes was demonstrated to improve growth of yeast under salt stress (Gaxiola *et al.* 1992). Transfer of the HAL1 to melon (Bordas *et al.* 1997) and HAL2 to tomato (Arrilaga *et al.*, 1998) resulted a significant increase of *in vitro* salt tolerance to the transgenic plants. Recently HAL2 gene was transferred to citrus plants and succesful transformants were obtained (Cervera *et al.* 2000).

Last strategy is the enhanced ion compartmentalization via Na+/H+ antiport overexpression. Overexpression of a vacuolar Na+/H+ antiport from *A.thaliana* in *Arabidopsis* plants promoted sustained growth and development in 200 mM NaCl treatment (Apse *et al.* 1999). Most recently, a rice gene encoding calcium dependent protein kinase (CDPK) OsCDPK7 overexpressed in rice and conferred osmotic stress tolerance (Saijo 2000).

1.6. Development of Stress Tolerant Transgenic Plants Through Proline Expression

Christian (1955) first reported that exogenous proline could alleviate the inhibition of growth of *S.oranienburg* that was due to osmotic stress. It was reported subsequently that a wide variety of osmotically stressed bacteria accumulate proline (Measures 1975). Other observations also indicated that proline is involved in tolerance to osmotic stress as a compatible osmolyte. The expression of genes for P5CS and P5CR has been analysed under dehydration conditions in mothbean and *Arabidopsis*. La Rosa *et al.* (1991) conculded that the P5CR reaction was not rate limiting and was not involved in NaCl dependent regulation of proline biosynthesis. It was also shown that 50-fold enhancement of soybean P5CR activity in transgenic tobacco plants did not result any significant increase in proline formation (Szoke *et al.* 1992). Since biosynthesis during stress has been demonstrated to be controlled by P5CS (Hu *et al.* 1992, Verbruggen *et al.* 1993 and Yoshiba *et al.* 1995), attempts to enhance osmotolerance by increasing proline accumulation centred on this enzyme.

The first study was the overproduction of mothbean P5CS in tobacco plants (Kavi Kishor *et al.* 1995). The overexpression of gene encoding mothbean P5CS in transgenic tobacco plants resulted in accumulation of proline up to 10 to 18 fold increase over control plants under dehydration stress. Transgenic plants showed enhanced biomass and flower development under salt stress conditions.

Later, the same gene was introduced in rice under the control of an ABA/stress-inducible promoter (Zhu *et al.* 1997). The transgenic rice plants

accumulated up to 2.5- fold more proline than control plants under stress, with higher biomass, root and shoot fresh weight observed under stress conditions.

Although transgenic tobacco and rice plants over-expressing P5CS have been obtained, and these appear to have increased resistance to both water deficits and salinity stress, insufficient data is available to conclude with certainty that proline accumulation in these transgenic plants contributes to their enhanced stress resistance via osmotic adjustment, or by some other mechanism(s) (Sharp *et al.* 1996). As discussed by Hare and Cress (1997), the controversial data reported for the water relations of these transgenic plants emphasize the need to investigate non-osmotic explanations for the phenotypes observed.

Since its specific role through plant is still unclear, to understand the essential role of proline, antisense transgenic *Arabidopsis* plants with P5CS cDNA was produced (Nanjo *et al.* 1999). Several transgenics accumulated proline at a significantly lower level than wild type plants, providing direct evidence for a key role of P5CS in proline production. The antisense transgenics showed morphological alterations in leaves and defect in elongation of inflorescences. The transgenics were also hypersensitive to osmotic stress.

However, elevated synthesis of the P5CS enzyme may, by itself be sufficient to significantly overproduce proline since P5CS is virtually completely inhibited (*in vitro*) by proline. Therefore a loss of feedback inhibition of P5CS may be necessary in addition to its increased production. The sensitivity of P5CS enzyme to end product inhibition by genetically. The mutation responsible for the existence of the proB74-encoded mutant GK to proline inhibition involves a change in aspartate to asparagine and much less sensitive to proline inhibition in *E.coli* (Csonka 1981, 1988, Dandekar and Uratsu 1988). Using these information and homologous sequences of P5CS gene site directed mutagenesis was performed with mothbean P5CS gene and found that when alanine converted to phenylalanine at 129th amino acid residue, there was a significant reduction in proline feedback inhibition (Zhang *et al.* 1995). When this mutated gene was transferred to tobacco plants under control

of 35S promoter, it was found that proline levels were higher in transgenic lines under stress conditions (Hong *et al.* 2000). Numerous proB mutations have been isolated from bacteria conferring different levels of proline overproduction (Csonka 1989), and it seems that the allosteric properties of the encoded mutant GK enzymes are modified in different ways. Ruslow *et al.* (1984) found another proline overproducing mutant with replacement of glutamate at 143 to alanine.

To see the function of proline degradation in the accumulation of proline antisense transgenic plants with *Arabidopsis* ProDH cDNA was produced. Several transgenics showed enhanced accumulation of proline. Antisense *Arabidopsis* transgenics were more tolerant to freezing and high salinity than wild-types (Nanjo *et al.* 1999). That was the first report of the efficient accumulation of proline by the supression of proline biosynthesis.

1.7. Aim of the Study

The ultimate goal of the present study was to develop salt/drought tolerant transgenic tobacco plants via metabolic engineering for proline overproduction.

For this purpose we focused on three different strategies:

i) First strategy aims, proline overproduction via transfer of AtP5CS gene under the control of CaMV35S promoter and Rubisco small subunit promoter.

ii) In the second strategy, we aimed the expression of a feedback insensitive P5CS enzyme in transgenic plants

iii) Third strategy aimed to inhibit proline degradation (thus increase in proline concentration) via antisense inhibition of proline dehydrogenase enzyme activity.

To achieve these goals, for the first strategy we aimed to transform tobacco plants with a P5CS gene originated from *Arabidopsis*.

For the second strategy, on the first place we tried to isolate feedback insensitive mutant form of the AtP5CS gene by random and PCR mutagenesis work followed by vector construction and transformation to tobacco plants.

For the last strategy, main goal was to isolate coding sequences of proline dehydrogenase gene from a tobacco cDNA library and construction of antisense vectors for further transformation studies.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strains

E.coli strain DH5α cells were used as competent cells. *E. coli* proline auxotroph CSH26 cells (Lac-proBA,thi,ara) and JM83 (λ^- ara (lac, proAB) were used for selection experiments. For mutation experiments, *E.coli* M2995 (mutD) strain was used. GV3101/MP90 and EHA105 *Agrobacterium tumefaciens* strains were used for plant transformation.

2.1.2. Plasmids

PC8-2b (Appendix A) is pBluescript SK^+ (Stratagene,USA), carrying a full-length cDNA encoding AtP5CS(Appendix F). PC8-2R is pBluescript SK^+, carrying a full-length cDNA encoding AtP5CS in reverse orientation. ROK2 (carrying CaMV 35S promoter-Nos terminator) and ROK8 (carrying Rubisco small subunit promoter-Nos terminator) (Appendix A) were used as binary vectors.

2.1.3. Culture Conditions

The complex medium used for bacteria was LB (Appendix B) supplemented with appropriate antibiotics: cefotaxime at 500mg/l, kanamycin at 50-75mg/L, ampicillin at 50-100 mg/l, on 1.5 % bacterial agar plates. YM and YEB cultures (Appendix B) were used for *Agrobacterium tumefaciens* strains. M63 minimal medium (Appendix B) was used for proline auxotroph *E.coli* CSH26 strain. The

bacterial plates were incubated at 37°C overnight and for further storage kept at 4°C for several weeks. A liquid aliquot of each bacterial clone carrying a specific plasmid was also kept at -80°C in 20% glycerol for longer storage. Different combinations and antibiotics were added to media according to purpose. Cultures were grown with aeration at 37°C or 28 °C.

2.1.4. Plant Material and Plant Tissue Culture Media:

Seeds of *Nicotiana tabacum* cultivar Samsun were used in the experiments.

All the media that were used during experiments were MS based media (Murashige and Skoog, 1962). Basically two different media were used, M9274 and M0404 (compositions are given in Appendix C). After addition of suitable hormones, pH was adjusted to 5.7-5.8 with NaOH and autoclaving was done at 121°C for 20min. Filter sterilized (0.2µm) antibiotics were added to the media. The composition and the purpose of the media is given in Table 2.1.

Table 2.1 The compositions and purpose of plant tissue culture media

Media	Composition	Purpose of Use
Liquid MS	M0404+3% sucrose	Diluting *Agrobacterium* culture before transformation
MSA	M9274+ 1mg/L Benzylaminopurine(BA)+ 0.1mg/L Naphtalenaceticacid(NAA)	Callus and shoot formation for control plants, Cocultivation for *Agrobacterium* treated plants
MSB	MSA+75mg/L Kanamycin or 5mg/L tetracycline+ 500mg/L Cefotaxime	Seletion of transformed plants and their regeneration
MSC	MSA (for control shoots) or MSB without hormones (for transgenic shoots)	Induction of root formation on regenerated shoots

2.1.5. Chemicals

The chemicals used in the preparation of solutions were all commercially available from Merck, Sigma, Oxoid and Fluka. The chemicals used in molecular biology studies were from MBI Fermentas.

2.2. Methods

2.2.1. Bacterial Transformation Studies

All vectors were first mobilized in *E.coli* cells. Then, pC8-2B plasmid was first mobilized to M 2995 *E.coli* strain. Colonies were selected on NaCl or DHPro containing M63 medium. To provide *Agrobacterium* mediated gene transfer to tobacco, they were then transferred to *Agrobacterium* EHA105 or GV3101 strains by electroporation.

2.2.1.1.Bacterial Growth

E.coli strains were grown at 37 °C in LB medium (Appendix B). EHA105 and GV3101 were grown for 1-2 days at 28 °C in YEB medium (Appendix B). 1.5% agar was added when solid media was needed. Filter (0.2μm) sterilized antibiotics were also included into the media for selection.

2.2.1.2. Competent Cell Preparation:

Competent cells were prepared according to Inoue *et al.* (1990). *E.coli* DH5α cells, taken from glycerol stock, were streaked on an LB plate and were cultured overnight at 37°C. About ten to twelve large colonies were isolated with a loop, and transferred to 250 ml of SOB medium (Appendix B) (2-liter flask was used for

efficient aeration). The culture was grown at 18 °C with vigorous shaking (200-250 rpm) until OD_{600} reached 0.6. After being kept in ice bath for 10 min, the cells were precipitated by centrifugation at 2500 g for 10 min at 4°C. The pellet was suspended in 80 ml of ice cold TB (Appendix B), and incubated in ice bath for 10 min. Then the second centrifugation was done in the same manner. The pellet was suspended in 20ml of TB and DMSO was added with gentle swirling to a final concentration of 7%. After incubation in ice bath for 10 min, the suspension was distributed into Eppendorf tubes in 1.5 ml parts and immediately chilled by immersing in liquid Nitrogen. The frozen competent cells can be stored at –80°C for a few months without a detectible loss in their competencies.

2.2.1.3. Bacterial Transformation

Frozen competent cells were thawed in ice and distributed into sterile eppendorf tubes (0.1 ml) for each transformation. 1-5µl (at least 10 ng) of plasmids were added and the cells were incubated in ice bath for 60 min. After that they were heat shocked without agitation at 42°C for 90 seconds to enhance the entrance of plasmids into the cells, followed by cold shock in ice bath for 5 min. Following the shock cells were suspended in 900 µl sterile LB medium and incubated by shaking at 37°C for about 45 minutes. At the end of the incubation the cells were centrifuged at 3000 rpm for 3 min. 900 µl of supernatat was removed and cells were resuspended in the remaining 100 µl solution . Finally they were spread on LB plates containing suitable antibiotics and incubated overnight. After the colonies were visible, plates were stored at 4°C.

2.2.1.4. Plasmid Isolation

Although several colonies were grown on selective medium after transformation not all of them contained the plasmid together with the desired foreign gene. Plasmid isolation on both mini and maxi scales was done to verify the true transformants.

2.2.1.4.1.Mini-Scale Plasmid Isolation:

5 ml of culture was grown in LB overnight at 37 °C. 1.5 ml of bacterial culture was taken in an eppendorf tube. Then centrifuged at 12000 rpm for 1 min at 4°C. Supernatant was removed and bacterial pellet taken. Pellet was resuspended in 100µl of Sol I (Appendix D) with vigorous vortexingfollowed by addition of 200 µl freshly prepared Sol II (Appendix D). Contents were mixed by inverting the tube. After incubation in ice, 150 µl of Solution III (Appendix D) was added and vortexed for 2 seconds. Tubes were stored in ice for 5 minutes. Equal volume of Chloroform:Isoamyl alcohol was added and mixed by vortexing. After centrifugation at 12000 rpm for 5 minutes, upper phase was taken into a new eppendorf tube. Double stranded DNA was precipitated with 2 volumes of 96% ethanol. Tubes were centrifuged for 5 min at 12000 rpm. Supernatant was removed and 1 ml of 70% ethanol was added. Tubes were centrifuged for 2 min at 12000 rpm. Supernant was removed and pellet was dried well. Then 50 µl of TE and RNAse (20mg/ml) were added. DNA was stored at -20°C until usage. Purity and integrity of the DNA was confirmed by spectrophotometry and agarose gel electrophoresis, respectively.

2.2.1.4.2. Midi Scale Plasmid Isolation

After inoculation of 50 µl of fresh culture to 250 ml LB+ antibiotic containing media, culture was grown at 37 °C overnight with shaking. This bacterial culture was centrifuged at 4000 rpm for 15 min at 4°C. Pellet was resuspended in 10 ml of Sol I by adding 1 ml of lyzozyme (10 mg/ml in 10mM Tris-Cl pH: 8) was added. After 15 minutes incubation at room temperature, 20 ml of Sol II was added. Tube was inverted gently and incubated at room temperature for 5 minutes. 15 ml of Sol III was added (ice-cold), tube was kept in ice for 10 minutes. After centrifugation at 4000 rpm for 15 minutes, supernatant was filtered through 4 layers of cheesecloth into a centrifuge bottle. 0.6X isopropanol was added. The solution was mixed well and kept at the freezer (-20°C) for 20 minutes. After centrifugation at 5000 rpm for 15 min at room temp, supernatant was discarded and pellet was rinsed with 70% ethanol. Pellet was dried well and 2 ml of TE buffer was added to dissolve the pellet.

For the purification of DNA, 2 ml of 5M LiCl was added to 2 ml of DNA solution. Mixture was incubated in ice for 5 min. Centrifugation was performed at 1000 rpm for 10 min. at room temperature. Supernatant was taken to another eppendorf tube. 1X volume of isopropanol was added to supernatant. Solution was incubated at -20°C for 10 min. After centrifugation, pellet was washed with 70% ethanol. Pellet was dried well dissolved in 1 ml of TE and RNAse. Solution was incubated at 37°C for 30 min. 1 ml Chloroform:Isoamyl alcohol was added to the solution. Mixture was vortexed well, then stored at room temperature for 5 min. Centrifugation was performed at 12000 rpm for 5 min. Upper phase was taken to a new eppendorf. 250 µl 10M Ammonium Acetate was added and 2 volumes of ethanol and 1 volume of isopropanol were mixed. Mixture was stored at -20°C overnight. After 10 min centrifugation, pellet was washed with 1 ml of 70% ethanol. Centrifugation was performed at 12000 rpm for 5 min. Resulting pellet was dried and dissolved in 1 ml of TE buffer.

2.2.4.1.3. Miniprep Isolation from *Agrobacterium*

Agrobacterium strain was grown in YEB medium containing appropriate antibiotics. Overnight grown bacteria was transferred into sterile 1.5 ml eppendorf tubes and centrifuged at 12000 rpm for 2 min to get the bacterial cells. The supernatant was poured off and 1 ml of Solution 0 (0.5M NaCl and 0.1% Sarcosyl in TE buffer) was added onto pellet and resuspended. Then the sample was centrifuged at 12000 rpm for 1 min and the supernatant was poured off. 100µl of Sol I (Appendix D) was added and the pellet was resuspended well. Freshly prepared 200 µl of solution II (AppendixD) was added and the sample was mixed slowly. After an incubation at 50-60°C for 5 min, 150 µl of Sol III (Appendix C) was added onto sample and incubated for 10 min on ice. Then equal volume of chloroform:isoamylalcohol (24:1 (v/v)) was added onto sample for extraction and mixed by vortexing for few seconds. The sample was centrifuged for 5 min at 12000 rpm and the clear upper phase was transferred in a new sterile eppendorf tube with a wide-bore pipette. For precipitation of double stranded DNAs, 2 volume of cold

absolute ethanol (%96) was added on to the sample and incubated at -20°C for at least 30 min. After a centrifugation at 12000 rpm for 5 minutes, supernatant was removed and 1 ml of 70% ethanol was added to wash the DNA. Then the sample was centrifuged at 12000 rpm for 2 minutes and the supernatant was removed well. The pellet was air dried and 50 µl of TE buffer containing RNAse A at a final concentration of 20 µg/ml was added and the pellet was resuspended. After an incubation period at 37°C for 30 min, the isolated plasmid was used to transform competent *E.coli* cells or for PCR analysis.

2.2.2. Optimization of Growth Conditions for Mutated Plasmid Carrying Bacteria

After the transformation of the AtP5CS containing plasmid to two *E.coli* auxotroph strains, cells were spreaded to LB and different concentrations of NaCl and Dehydroxyproline containing M63 media (Table 2).

Table 2.2 The test of selection conditions

CSH26	**GM83**
M63	M63
LB	LB
0,25mM DHP+M63	0,25mM DHP+M63
0,5mM DHP+M63	0,5mM DHP+M63
1mM DHP+M63	1mM DHP+M63
1,5mM DHP+M63	1,5mM DHP+M63
2mM DHP+M63	2mM DHP+M63
0,4M NaCl+M63	0,4M NaCl+M63
0,5M NaCl+M63	0,5M NaCl+M63
0,6M NaCl+M63	0,6M NaCl+M63
0,7M NaCl+M63	0,7M NaCl+M63
0,8M NaCl+M63	0,8M NaCl+M63
1M NaCl+M63	1M NaCl+M63

2.2.3. Random Mutagenesis

To obtain random mutagenesis with AtP5CS cDNA, two different methods were used; These include mutator bacteria and PCR method.

2.2.3.1. Mutator Bacteria:

pC8-2b plasmid was transferred to *E.coli* mutator strain M2995 (mutD). After transformation, miniprep plasmid isolation was performed from growing colonies. These isolated plasmids were transferred to *E.coli* auxotroph strain and tested under different concentrations of the Dehydroxyproline containing M63 media.

2.2.3.2. PCR Mutagenesis

Using the plasmid pC8-2, PCR was performed. The error rate (mutation frequency of the Taq polymerase (10^{-4})) can be increased by using higher concentrations of the Mg^{+2} ion and nucleotide concentrations.

Optimized conditions for the PCR are;	Total volume(100 µl)
Template DNA : pC8-2b plasmid (1ng)	1µl
Primer1 : (T3) (100µg/µl)	1µl
Primer2: (T7) (100µg/µl)	1µl
Buffer (10X)	10µl
dNTP (4mM)	5µl
$MgCl_2$(25mM)	5µl
Taq polymerase(5U/µl)	0.5µl
dH_2O	77µl

PCR conditions are given below.

Table 2.3 PCR cycling conditions for mutagenesis

95°C	5min	
95°C	1 min	30 cycle
55°C	40 sec	
72°C	40 sec	
72°C	5 min	

The amplified fragment was isolated from the agarose gel using MBI Fermentas, DNA Exraction Kit, and digested with KpnI and Xba I enzymes and ligated to pBSK. The ligation product was transferred to *E.coli* auxotroph strain and tested for the resistance of the colonies to DHPro.

Colonies which were growing on the DHPro containing media, were taken and plasmid isolation was performed on these colonies. These isolated plasmids were transferred to CSH26 strain again to see whether resistance comes from plasmid or bacteria and their growth was tested on the 1 mM DHPro containing M63 media.

2.2.4. Mutation Selection Studies

2.2.4.1. Growth Curves

Growth of the selected colonies was checked in M63 and 0.8 M NaCl containing M63 media. Growth was performed at 28°C for the expression of the AtP5CS gene since this plant gene was active at this temperature. Growth was monitored for 5 days sampling each day with density of the bacteria measured at OD_{600}.

2.2.4.2. Mannitol Selection

Growth of the selected colonies were evaluated on M63 media with different concentrations of mannitol (0.5M, 0.6M, 0.8M, 1M). Each colony was grown in 3 ml of LB containing Ampicillin (50 mg/l). Then their density was measured at 600

nm. Same concentration of the bacteria was applied on the mannitol containing solid media according to the density. After 2 days of incubation at 28°C, their growth zone was measured using Image Analyser. Success of mutations were evaluated according to the growth zone of colonies in mannitol containing media..

2.2.5. Sequence Analysis

Sequence Analysis of the promising mutants was done at laboratories of Dr. Laszlo Szabados, Biological Research Center, Szeged, Hungary using ABI PRISM 377 DNA sequencer (Applied Biosystem).

2.2.6. Construction of Plant Transformation Vectors:

Plasmid pC8-2R containing AtP5CS gene in reverse orientation was digested with KpnI and SacI restriction enzymes. AtP5CS gene is cloned in sense orientation into derivatives (ROK2 and ROK8) of the binary vector pBIN19. PROK2 contains strong, nominally constitutive, 35S promoter from cauliflower mosaic virus and pROK8 contains light regulated promoter from tobacco rbcS gene encoding the small subunit of ribulose-1,5-biphosphate carboxylase.

For the random mutants, pC8-2b plasmid was digested with KpnI and Xba restriction enzymes and ligated to pROK2 and pROK8 plasmids.

2.2.7. Proline Dehydrogenase Partial Cloning

In order to find the partial sequence of proline dehydrogenase from tobacco, the primer was designed and using PCR method, 2 fragments were obtained. These fragments were sequenced and compared with other databases.

2.2.7.1. Amplification of λZap II Libraries

E.coli XL1-Blue cells were grown overnight from a single colony in 2xYT (Appendix B) liquid medium, containing $MgCl_2$ and 0.2% maltose. 100X dilutions

was made by transferring 5 µl aliquots into 500µl of SM (Appendix B) buffer. Then phage was stored at 4°C. Standard 2X YT plates were prepared. In a 10 ml of test tube, 100µl diluted phage was added to 250µl bacterium and mixed immediately. This mixture was incubated at room temperature for 20 min. 3 ml of 50°C 2x YT top agarose was added to the infected bacterium and after mixing carefully, mixture was layered over the plate immediately. Plate was incubated at 37°C. When plaques were visible, phage titers were established. For the amplification, 1.5-2 ml of bacterium was mixed with $2x10^5$ phage particles. Purification of λ DNA was performed according to Sambrook *et al.* 1989.

Lysed cultures was brought to room temperature and pancreatic DNAseI and RNAse were added (final conc 1µg/ml). Solution was incubated 30 min at room temperature. Solid NaCl was added to final concentration 1 M. Cultures was kept in ice for 1 hour. Centrifugation was performed at 11000 g for 10 min at 4°C. Supernatant was taken and solid PEG 6000 was added to final concentraion 10% w/v. This was dissolved by slow stirring at room temperature. Solution was incubated in ice at least one hour. Centrifugation was performed at 11000 g for 10 min at 4°C. Pellet was resuspended in SM. Equal amount of chloroform was added and centrifugation was performed at 3000 g for 15 min at 4°C. Aqueous phase was taken and centrifuged at 18000 rpm for 3 hours at 4°C. Pellet was dissolved in 1 ml of SM buffer. After overnight incubation at 4°C, 20mM EDTA, 50 µg/ml Proteinase K and 0.5% SDS were added. Mixture was incubated at 56°C for 1 hour. Digestion mixture was cooled to room temperature. Equal amount of phenol was added. Mixture was centrifuged at 3000 g for 5 min at room temperature. Aqueous phase was transferred to a new tube. Aqueous phase was extracted with 50:50 mixture of phenol chloroform. Then aqueous phase was extracted with equal volume of chloroform. 0.3 M Sodium acetate was added to the solution. Solution was mixed with 2 volumes of ethanol. This mixture was incubated at room temperature for 30 min. Centrifugation was performed at 12000 g for 5 min. Pellet was taken and mixed with 70% of ethanol. Then another centrifugation was performed at 12000g for 2 min at 4°C. Pellet was redissolved in TE (pH:7.6) and used for further experiments.

2.2.7.2. Primer Design and Synthesis

Degenerate oligonucleotide primer from conserved sequences of proline dehydrogenase of *A. thaliana* were used to identify *Nicotiana tabaccum* proline dehydrogenase cDNA.

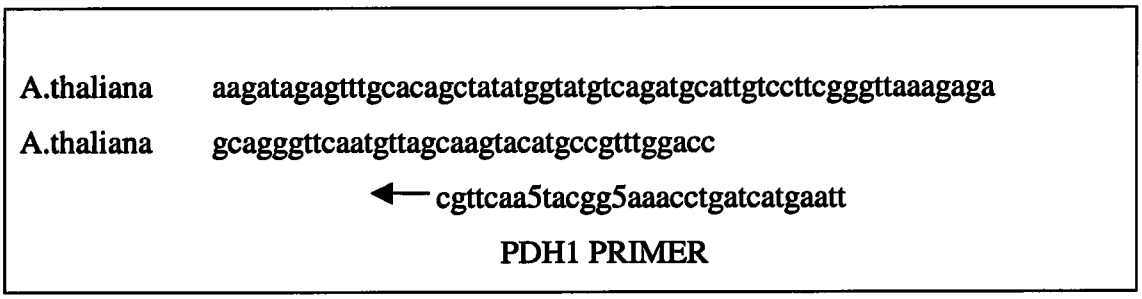


Figure 2.1 Specific primer designed from the homologous sequence of Arabidopsis thaliana.

2.2.7.3. PCR Conditions to Amplify Proline Dehydrogenase from λZAP Tobacco cDNA Library

Two sequential PCR were performed. In the first PCR; pdh1 primer and T3 universal primer were used to amplify a DNA fragment from λZAP tobacco cDNA library, In the second PCR, SK primer and the product of the first PCR were used as primers. Amplified fragments were isolated from agarose gel with isolation kit and ligated to pBSK for sequencing. Sequencing was performed as described in section 2.2.5.

Table 2.4 Two PCR cycling parameters for the amplification of proline dehydrogenase from λZAP tobacco cDNA library

1st PCR	2nd PCR
Template: λZAP tobacco cDNA	Template: Product of 1st PCR
Primer: Pdh1 primer and T3 primer	Primer: Pdh1 and SK primer
95°C 5min	95°C 5min
95°C 1 min	95°C 1 min
55°C 40 sec 30 cycle	55°C 40 sec 30 cycle
72°C 40 sec	72°C 40 sec
72°C 5 min	72°C 5 min

2.2.8. Transformation Studies

Electroporation technique was used to transfer the plasmids to *Agrobacterium* cells. The P5CS gene was transformed to tobacco cells by leaf disc transformation method.

2.2.8.1. Transformation of Vectors to *Agrobacterium* by Electroporation

Electroporation is a technique involving exposing cells to an electric field for a short duration, in order to faciliate the cross membrane transfer of material.

2.2.8.1.1. Preparation of Master Cultures:

Agrobacterium cells were streaked onto a YM plate and incubated at 30°C for 36-48 hour. A well-separated colony was used to inoculate 50 ml YM broth in a 500 ml flask and cells were grown with vigorous aeration at 30°C until OD_{550}=0.2. 5 ml culture were transferred into a chilled 15 ml falcon tube containing 5 ml YM storage medium, mixed well and chilled on ice for 10 min. 0.5 ml of mixed cells were transferred in to ice-chilled 1 ml nalgene cryotube, and quickly freezed in liquid nitrogen at –70 °C.

2.2.8.1.2. Preparation of Electro Competent Cells:

Agrobacterium cells were streaked from a master culture onto YM plate and incubated at 30°C for 36-48 hour. Well separated colony was used to inoculate 50 ml of YM broth in a 500 ml flask, and cells were grown with aeration at 30°C until OD_{550}= 0.2. 100 µl of the cultured cells were inoculated in to 1.5 L of YM broth and cells were incubated with vigorous aeration at 30°C until OD_{550}=1.0. Cells were

centrifuged at 2600g for 10 min. Pellets were washed two times by resuspending cells in 500 ml of ice-cold glycerol. Cells were centrifuged at 2600 g for 10 min. Resulting pellet was resuspended in ice-cold 10% glycerol to a final volume of 1.5 ml.

2.2.8.1.3. Electroporation

Frozen *Agrobacterium* cells were thawed on ice. 1 ml of YM broth was added to falcon tubes at room temperature. 40 µl of electro competent cells and 1 µl (1-2 ng) DNA were mixed and transferred to 0.1 cm cuvette cooled to 4°C. An electric field 16.7 kv/ cm was applied with 4ms time constant. Cells were transferred to the YM broth in a 15 ml falcon tube. Remaining cells were resuspended with 200 µl YM broth. Cells were incubated at 30°C for 3 hours with shaking at 250 rpm. 100 µl of sample was plated on to selective YM plate. From positive colonies plasmids were isolated, digested and tested on agarose gels.

2.2.8.2. Transformation of Tobacco

In this part of the experiments, the A*grobacterium* cells that were transformed previously, were used to obtain transgenic tobacco plants via *Agrobacterium* mediated gene transfer method. The steps of plant regeneration and transformation are as follows;

2.2.8.2.1. Leaf Disc Transformation:

During transformation studies, a modified (Öktem *et al.* 1994) "leaf disc transformation " protocol of Horsch *et al.* (1985) was used. *Agrobacterium* strains were inoculated in YEB medium (Appendix B) and were grown at 28-30°C overnight with shaking. After that, they were diluted with liquid MS medium (M0404+ 4% sucrose) to an A_{600} of 0.4-0.6. Freshly prepared leaf discs were incubated with this mixture for about 10 min. They were mixed gently several times. At the same time in a separate tube, control leaf discs (prepared from untreated plants) were treated with liquid MS without bacteria. Before transferring the explants to MSA medium, leaf discs were blot dried with sterile filter papers. After 2 days of cocultivation at 25°C with 16 hours photoperiod, the *Agrobacterium* treated discs then taken to MSB medium for selection and regeneration. Elimination of *Agrobacterium* cells after cocultivation was done by adding 500mg/L Cefotaxime in to the media. Plates were refreshed every 2 weeks. After formation of callus and shoots in kanamycin (50-75 mg/L) (for ROK plasmids) selective medium, shoots were cut point attachment to callus and transferred to baby jars with MSC medium to induce root formation and further development. Plantlets were transferred to soil and grown in green house. When flower buds emerged, they were enveloped with nylon bags to prevent cross pollination.

2.2.9. Analysis of Potential Transgenic Plants

To verify the integration and expression of the transferred DNA, transformed plants were analysed by different methods which include PCR analysis, germination and regeneration on kanamycin.

2.2.9.1. PCR Analysis

Screening of putative transgenic tobacco lines was done with PCR (Polymerase Chain Reaction). Genomic DNA was isolated from these lines under special care to avoid contamination with foreign DNA, using standard method. These studies were performed in order to verify the integration of transferred gene into the genome of transformed plants. Genomic DNAs of 9 independent putative transgenic plants were isolated and amplified with nptII and P5CS specific primers (Appendix E).

2.2.9.1.1 Genomic DNA Isolation

The genomic DNAs from fresh leaf tissues were isolated by using the Nucleospin Plant DNA Extraction Kit of Macherey Nagel, according to Manufacturer's recommendations.

2.2.9.1.2. PCR Conditions

For each PCR reaction 200-500 ng of DNA was mixed with a master mix solution containing 20 pmols of gene specific primer (Appendix E), 0.2mM dNTP mix, Taq DNA polymerase reaction buffer and 2 units of Taq DNA polymerase in a total volume of 30-50 µl. Reaction tubes were mixed gently but instantly and then temperature cycling parameters were activated just after addition of immersion oil as barrier againist evaporation. Cycling parameters are given in Table 2.5.

Table 2.5 PCR cycling parameters for NPTII and P5CS primers

95°C	5min	
95°C	1 min	
47°C	45 sec	30 cycle
72°C	30 sec	
72°C	5 min	

An aliquot of amplification products were separated on 1 % agarose gels in Tris-borate buffer under constant voltage of 80 Volts. Gels are visualised under UV transilluminators and documented by Image Analyser.

2.2.10. Analysis of T_1 Progeny

Biochemical, physiological and molecular analysis were performed with T_1 progeny plants. These include proline, MDA assay, wet/dry weight measurement, capsule number.

2.2.10.1 Proline Assay

Proline determination from the leaves of T_0 plants was performed according to Bates *et al.* 1973. Leaves (200 mg tissue) were weighed and homogenized 1 ml in 3% sulphosaliysilic acid. Then centrifuged at 15000 rpm (microfuge) for 15 min at 4°C. 200 µl acid ninhydrin (0.31 gr ninhydrin, 7.5 ml acetic acid, 5 ml 6M phosphoric acid, freshly prepared), 200 µl 96% acetic acid, 100 µl supernatant, and 100 µl 3% sulphosalysilic acid were mixed and incubated at 100°C for 1 hour. Then sample were mixed with 1 ml toluene for spectrophotometric determination at 520 nm.

2.2.10.2. MDA Assay

About 0.2 g of tissue was homogenised in 1 ml of 5% (w/v) trichloroacetic acid and the homogenate was centrifuged at 12000 g for 15 min at room tempertaure. The supernatant was mixed with an equal volume of 2-thiobarbituric acid (0.5% in 20% (w/v) trichloroacetic acid), and the mixture was boiled for 25 min at 100°C, followed by centrifugation for 5 min at 7500 g, to clarify the solution. Absorbance of the supernatant was measured at 532 nm and corrected for nonspecific turbidity by subtracting the OD_{600}. MDA contents were calculated using an extinction coefficient 155 M^{-1} cm^{-1}.

2.2.10.3. Wet/Dry Weight Measurements

After growing of the plantlets for 2 months under stress and normal conditions, their roots were excised and weighted and photograph was taken. Then roots were dried in oven at 60°C overnight and their weight was measured for dry weight measurements.

2.2.10.4. Seed Germination Test

The seeds of tobacco plants were surface sterilised before placing on growth medium. Seeds were sterilised in 30% hypochlorite solution for 20 minutes in an eppendorf tube with shaking. Then hypochlorite was removed by rinsing with sterile distilled water for 3 times. Surface sterilised seeds were placed on 0, 1% and 1.5% NaCl containing MS medium plates (Appendix C). Seeds cultured on MS medium without NaCl served as control. Only positive control plants were placed on nonselective growth medium. The baby jars were incubated at 25± 2°C with 16 hours light and 8 hours dark-light cycle.

2.2.10.4. 200mM NaCl Treatment

Seedlings were germinated in 75 mg/l kanamycin containing media. Then transferred to a liquid MS medium containing 200mM NaCl. The treatment continued for 5 days. After 5 days the leaves from plantlets were taken for proline analysis.

2.2.10.5. 0.4M NaCl Treatment

Seedling were transferred to the soil in pots in green house. After short adaptation of the plants to the green house, at 4 leaf stage, first treatment started with 0.4M NaCl. Salt solution was added to cups under pots every 2 days. Normal irrigation was done for the control plants. 0.4M NaCl treatment continued for 2 months. Leaf samples were taken for proline and MDA Assay, then plants are watered for 1 week to remove roots for wet and dry weight measuremens.

2.2.10.6. Drought Stress Applications

Drought treatment was applied only after adaptation of plants to green house. Control plants were watered every 2 days to field capacity. Water stress initiated (day 0) by withholding watering. Since no further water was added, plants were subjected to progressive drought stress during growth. In contrast to osmotic stress and rapid desiccation treatments generally used, this technique developed by Vartanian 1981) aimed to mimic the physiological conditions that plants experience in the field during natural drought periods. In addition, the progressive drought stress allowed us to reveal any adaptive potental of the plants. Treatment continued for 1 month. Samples were taken for proline and MDA Assay, then plants are watered for 1 week to remove roots for wet and dry weight.

2.2.10.7. Mendelian Inheritance Pattern

For analysis of the inheritance pattern seeds of individual plants were well mixed and surface sterilised. Then seeds were placed on selective growth medium containing 75 mg/L Kanamycin in petri plates. After two weeks of germination on the same plate they were counted and percent germination was calculated from the data. Chi square analysis was carried out to evaluate whether the inheritance pattern follows Mendelian inheritance.

2.2.10.7. PCR Analysis

PCR Analysis was performed as described in 2.2.9.1.2. using DNA of the T_1 plantlets.

2.2.11. Analysis of T_2 Progeny

Seed germination test, proline MDA assay and Mendelian inheritance pattern test were performed on T_2 progeny.

2.2.12. Color Induction on Chlorophenol Red Medium

Leaf discs of sterile plants grown in jars placed on hormones containing selective MS medium with 15 mg/l indicator chlorophenol redalong with 0.1 mg/l BAP and 0.01 mg/l NAA. Color change from red to yellow was observed in 3 weeks. They were recorded by taking photographs.

2.2.13 Statistical Analysis

One way ANOVA was used for the calculation of standard error of mean of the independent experiments using MINITAB software statistical programme.

All experimental procedure for transformation and analysis of transgenic plants containing AtP5CS gene is summarised in Figure 2.2.

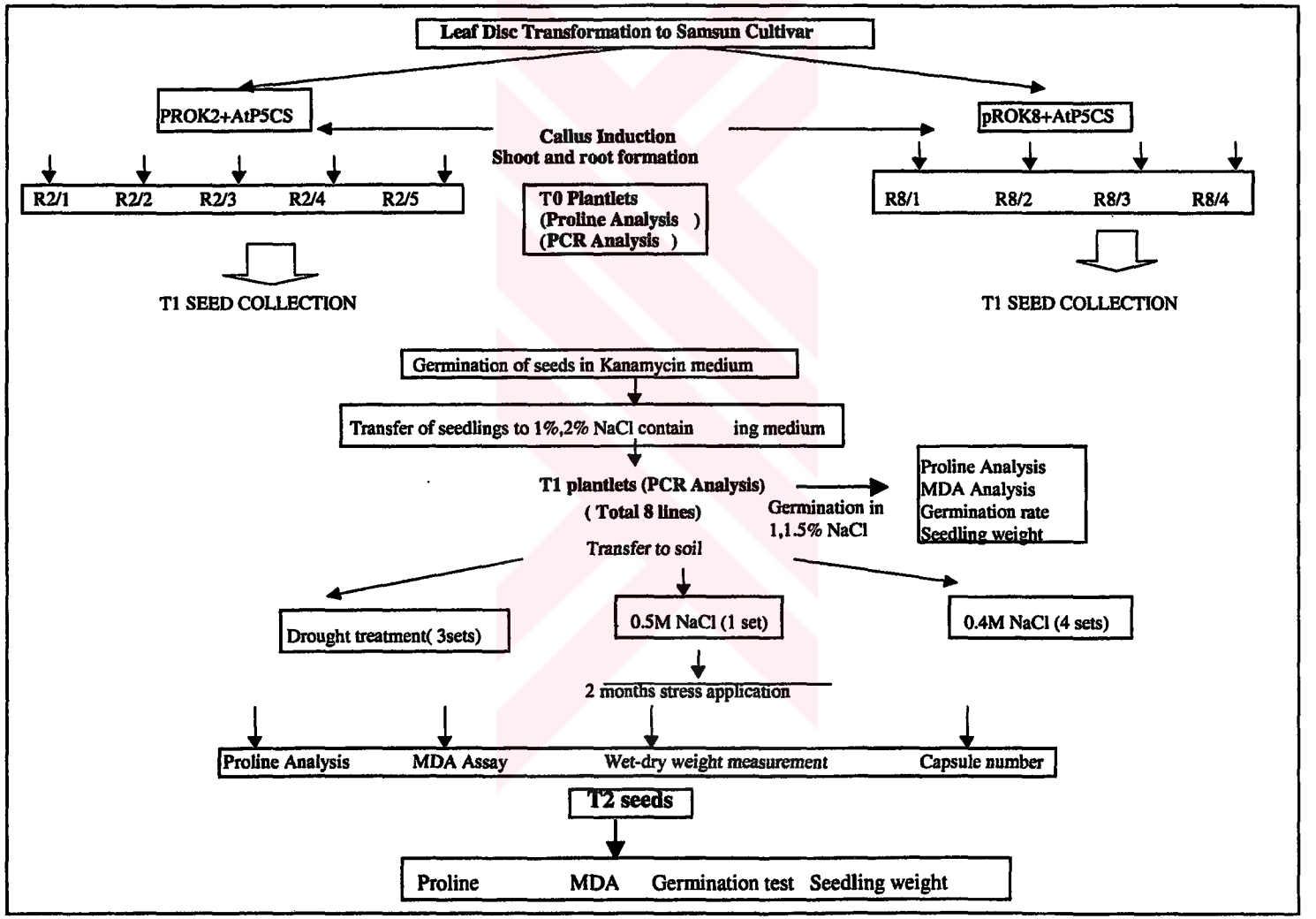


Figure 2.2 Outline of transformation studies with AtP5CS gene

CHAPTER III

RESULTS

In this study, we conducted experiments towards development of salt/drought tolerant transgenic tobacco plants via proline overproduction. For this purpose three different alternative strategies were employed. Two of the strategies aimed to increase proline content via enhancing proline biosynthetic pathway by transfer of wild type or mutated AtP5CS gene. Third includes partial cloning of tobacco proline dehydrogenase gene and construction of an antisense vector to block proline degradation in transgenic plants. Therefore, we have analysed the expression of the AtP5CS gene in transgenic tobacco plants carrying *Arabidopsis thaliana* originated gene in two different binary vectors. The attempts to manipulate this gene were also made to avoid feedback inhibition via random mutagenesis. The isolation of the partial proline dehydrogenase gene from tobacco was another subject of this study.

3.1 Optimisation of selection criteria for mutagenesis

In order to increase the accumulation of proline, mutation on the P5CS gene that would remove proline feedback inhibition was necessary. There are different strategies for the mutation experiments. In this study random mutagenesis was employed for this aim. In order to provide the best conditions for mutagenesis, the first step was to choose selective agents. First selective agent, proline toxic analogue, 3,4-Dehydroxyproline (DHPro) is an inhibitor of proline biosynthesis. Therefore when it is found in the media, it prevents accumulation of free proline and inhibits cell growth by incorporation of the amino acid analogue in cellular proteins. If the concentration of the proline is high in the cell due to the inhibition of feedback mechanism, dehydroxyproline can not be incorporated in proteins. Second selective

agent NaCl, creates osmotic stress in the cells. When dose of stress increase, the accumulation of proline occurs. Since the end product proline, blocks P5CS enzyme, the cells die. Two *E.coli* auxotroph strains (CSH26 and JM 83) were tested to find inhibitory dose for the growth of the bacteria in minimal media. Two selective media containing NaCl and Dehydroxyproline in different concentrations were tested. Both strains were grown well in LB and M63 media. When different concentrations of NaCl were added to the media, the growth stopped at 0.6 M NaCl containing media (Table 3.1). Similarly, it was found that 1 mM DHPro, was inhibitory concentration for growth (Table 3.2). Therefore 0.6 M NaCl and 1 mM DHPro were chosen as a selective dose for the further mutation experiments.

Table 3.1 Growth of AtP5CS transformed proline auxotroph bacteria on different concentrations of NaCl. [a] (+++) indicates >500 colonies, (++) indicates >100 colonies, (+) indicates < 100 colonies per plate.

Media	Bacterial Strain	
	CSH26	JM83
LB	+++[a]	+++
M63	+++	+++
M63+ 0.4M NaCl	++	++
M63+ 0.5M NaCl	+	+
M63+ 0.6M NaCl	-	-
M63+ 0.7M NaCl	-	-
M63+ 0.8M NaCl	-	-

Table 3.2 Growth of AtP5CS transformed proline auxotroph bacteria on different concentrations of DHPro. [a] (+++) indicates >500 colonies, (++) indicates >100 colonies, (+) indicates < 100 colonies per plate. Numbers in parenthesis indicates the colony count per plate.

Media	Bacterial Strain	
	CSH26	JM83
LB	+++[a]	+++
M63	+++	+++
M63+ 0.25 mM DHPro	++(120)	-
M63+ 0.5 mM DHPro	+(26)	-
M63+ 1mM DHPro	-	-
M63+ 1.5 mM DHPro	-	-

3.2 Random Mutagenesis of the AtP5CS Gene

In order to obtain a proline feedback insensitive mutant form of AtP5CS gene, two random mutagenesis approaches were used. From these, (with mutD mutagenesis) 69 mutants were obtained and from PCR studies 2 mutants having better growth than control were obtained.

3.2.1. Random Mutagenesis by Mutator Bacteria

The AtP5CS gene carrying pC8-2b plasmid was transferred to MutD mutant M2995 bacteria. MutD mutant *E. coli* strain shows an increased spontaneous mutation rate since ε-subunit of DNA polymerase III holoenzyme is specified by the mutD (dna Q) gene.

Mutants were selected as strains resistant to toxic proline analogue 3,4-dehydroxyproline. When the proline is produced at high levels, it can antagonise the toxic analogue.For selection of resistant cells, CSH26 were spread on minimal media supplemented with 1mM DHPro. 36 colonies resistant to toxic analogue were obtained (Table 3.3). As another selection, cells were spread on minimal media containing 0.6 M NaCl. From these plates 33 colonies were obtained (Table 3.3).

Therefore, a total of 69 mutants were obtained using two different selection criteria; 0.6 M NaCl and 1mM DHPro. These strains containing mutated derivatives of AtP5CS were further analysed for their enhanced osmotolerance on liquid media supplemented with 0.8M NaCl (Table 3.4). To verify the mutation, plasmids were isolated from each of the 69 colonies and transferred into empty CSH26 strain. This back transformation proved that all 69 mutants have mutation on AtP5CS.

Table 3.3 Growth of proline auxotrophic bacteria on 1mM DHPro and 0.6 M NaCl when transformed with mutated AtP5CS from mutator bacteria by random mutagenesis. Numbers indicate the number of colonies on selective media.

	1mM DHPro		**0.6 M NaCl**	
	Bacterial Strain		**Bacterial Strain**	
Plate Number	**CSH26**	**GM83**	**CSH26**	**GM83**
1	4	-	15	-
2	6	9	-	-
3	-	6	10	-
4	2	-	3	5
5	9	-	-	-

Table 3.4 Relative growth of bacteria carrying mutated gene. The growths were measured in minimal media (M63) containing 0.8 M NaCl at 28 °C. The growth rate of the each mutant was divided to that of wild type to calculate relative growth.

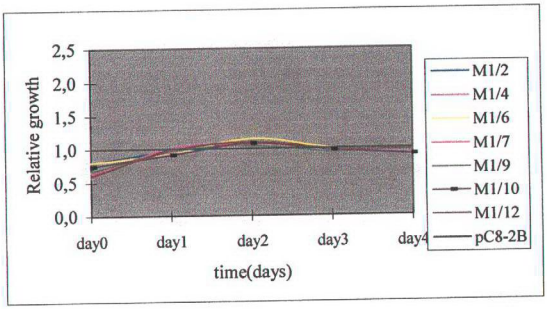
Mutant strain	Relative growth	Mutant strain	Relative growth
M1	0,87	M36	0,08
M2	1,1	M37	0,15
M3	0,73	M38	0,58
M4	1,44	M39	0,05
M5	1	M40	0,1
M6	1,18	M41	0,21
M7	1,3	M42	0,63
M8	1,11	M43	0,38
M9	1,22	M44	0,02
M10	0,98	M45	0,04
M11	1	M46	0,74
M12	1,28	M47	0,33
M13	1	M48	0,25
M14	1	M49	0,13
M15	0,93	M50	0,23
M16	1	M51	0,97
M17	1	M52	0,97
M18	1	M53	0,97
M19	1	M54	0,89
M20	1	M55	0,93
M21	0,4	M56	0,97
M22	1,4	M57	1
M23	0,1	M58	0,9
M24	0,8	M59	0,91
M25	1	M60	0,94
M26	1	M61	0,06
M27	0,4	M62	0,1
M28	0,7	M63	0,67
M29	0,6	M64	0,8
M30	0,4	M65	0,73
M31	0,02	M66	1
M31	0,01	M67	0,6
M33	0,01	M68	0,51
M34	0,2	M69	0,84
M35	0,35	Wild type	1

Among all the resistant colonies obtained with mutagenesis in selective media, of the 69, there were only seven that after 5 days of incubation were judged to give rise better growth than CSH26 carrying wild type plasmid. Therefore, these seven colonies were further analysed. The generation time of all strains was nearly same when grown in minimal medium (Figure 3.1a). However, when the medium was supplemented with 0.8 M NaCl or 1 M NaCl , the relative growth of mutants was better than wild type (Figure 3.1. b,c). The growth rate was measured for 4 days and at 28°C, because the *A. thaliana* originated enzyme is active at 28°C. The derivatives of CSH26 were tested for enhanced osmotolerance on solid media containing 1M mannitol in minimal media. Also same colonies were tested in liquid minimal media containing 1mM DHPro. Table 3.5 lists the growth rate of promising mutants and wild type (2b) in minimal media supplemented with 1mM DHPro and 1M mannitol. The growth rates of the mutant #12 and mutant #9 were better than others under the conditions of high osmotolerance. Therefore, these two mutants were chosen for further analysis.

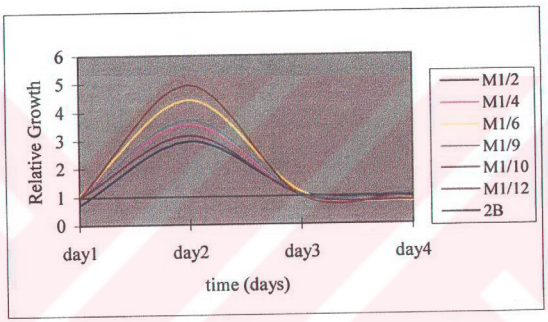
Table 3.5 Growth of *E.coli* strains carrying wild type and mutant P5CS gene in 2mM DHPro, 0.8 M NaCl and 1M Mannitol containing medium The growth rate of each mutant was divided to that of wild type to calculate the relative growth.

Bacterial Strain	2mM DHP	0,8M NaCl	1M Mannitol
M1/2	1,70	1,1	1,35
M1/4	1,24	1,44	1,21
M1/6	1,59	1,18	1,25
M1/7	1,54	1,3	1,19
M1/9	1,62	1,2	1,25
M1/10	1,59	1,22	1,13
M1/12	1,59	1,28	1,29
2B (Control)	1	1	1

(A)



(B)



(C)

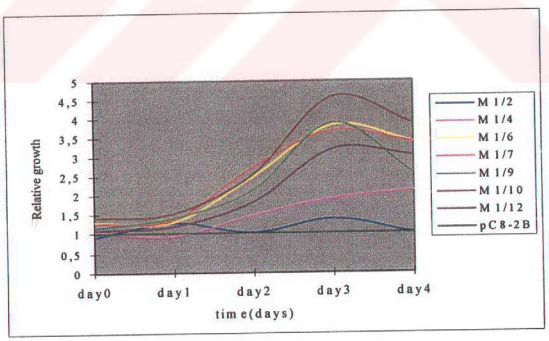


Figure 3.1 Relative growth of *E.coli* strains carrying wild type and mutant P5CS gene in M63 (A), 0.8 M (B) and 1 M NaCl (C) containing M63 medium. Growth were followed for 4 days at 28 °C. The growth rate of each mutant was divided to that of wild type to calculate relative growth.

3.2.2. PCR Mutation.

The error frequency (0.4 %) of Taq polymerase was used to obtain mutants in ATP5CS gene during PCR amplification. Different concentrations of dATP, dGTP, dCTP and dTTP and $MnCl_2$ were used in order to increase the mutation frequency. $MnCl_2$ did not work efficiently compared to $MgCl_2$. Therefore, $MgCl_2$ was used for further PCR analyses. Also, using nucleotides at different concentrations, 4 mM dNTP concentration was used. To increase the measured error rate of Taq DNA polymerase (10^{-5} to 10^{-4} errors per nucleotide synthesised) the amplification cycles were reduced to 25. The amplified fragment (Figure 3.2) was ligated to pBSK which is then used to transform auxotrophic cells.

Two colonies grew in the first selection. When their plasmids were isolated and back transformed to CSH26, these two strains (carrying plasmid) did not grow in selection media. Therefore it was decided that the origin of mutation was bacterial chromosome(reversion of auxotrophy). As a result no mutant gene could be obtained by employing PCR mutagenesis.

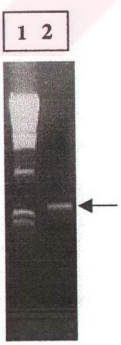


Figure 3.2 Amplified AtP5CS cDNA (2.6 kb) from Polymerase Chain Reaction. Lane 1: Hind III marker, Lane 2: Amplified P5CS (arrow).

3.3 Analysis of Mutated P5CS

At the end of the mutation experiments P5CS gene from the most promising strains were sequenced in order to determine the mutated regions. The sequence analysis of the mutant #12 showed that there were two point mutation (Figure 3.3). These mutations conferred change at 54^{th} amino acid from lysine to glutamic acid and at 172^{nd} position from isoleucine to phenylalanine. First mutation was very close to ATP binding site of the enzyme. The second mutation was close to putative leucine zipper site. These two mutations were at the glutamyl-kinase coding part of the gene. On the other hand, a higher degree of mutation was observed in mutant #9 (Figure 3.4). The mutations were just at the beginning of the gene. This high mutation might be due to artefacts during sequencing.

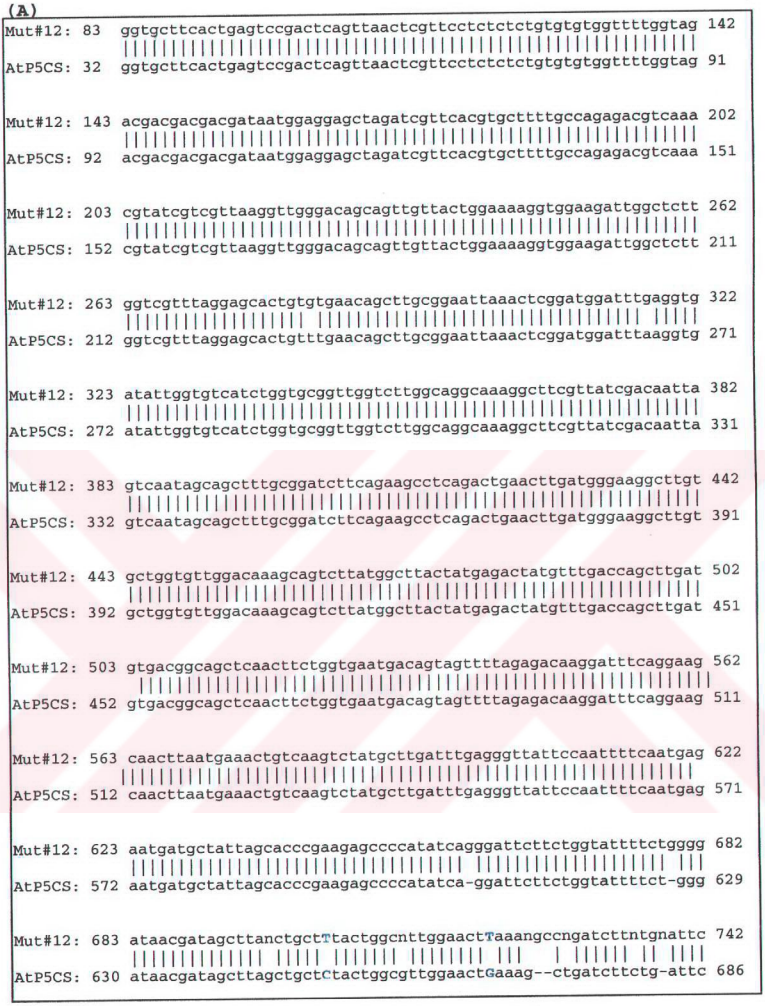


Figure 3.3 Comparison of the nucleotide sequence alignment (A) and amino acid alignment (B) of mutant #12 and AtP5CS gene.

(B)

```
Mut#12: 76 MEELDRSRAFARDVKRIXXXXXXXXXXXXXXXXXXXXXXXXCEQLAELNSDGFEVILVSS 255
           MEELDRSRAFARDVKRI                                EQLAELNSDGF+VILVSS
AtP5CS: 1  MEELDRSRAFARDVKRIVVKVGTAVVTGKGGRLALGRLGALFEQLAELNSDGFKVILVSS 60


Mut#12: 256 GAVXXXXXXXXXXXXVNSSFADLQKPQTELDGKACAGVGQSSLMAYYETMFDQLDVTAAQ 435
            GAV             VNSSFADLQKPQTELDGKACAGVGQSSLMAYYETMFDQLDVTAAQ
AtP5CS: 61  GAVGLGRQRLRYRQLVNSSFADLQKPQTELDGKACAGVGQSSLMAYYETMFDQLDVTAAQ 120

Mut#12:436  LLVNDSSFRDKDFRKQLNETVKSMLDLRVIPIFNENDAISTRRAPYQ---GFFW 588
            LLVNDSSFRDKDFRKQLNETVKSMLDLRVIPIFNENDAISTRRAPYQ   G FW
AtP5CS:121  LLVNDSSFRDKDFRKQLNETVKSMLDLRVIPIFNENDAISTRRAPYQDSSGIFW 174
```

Figure 3.3 (Continued) Comparison of nucleotide sequence alignment (A) and amino acid alignment (B) of mutant 12 and AtP5CS gene. 2 mutations occurred at the 54th and 172nd amino acids.

(A)

```
Mut#9 : 161 ataatggaCgagctagatcgntcacTtgcttttgccacnagactgtcaancgnatcntcn 220
            |||||||| |||||||||||| |||| |||||||||||  |||| ||||| || ||| ||
AtP5CS: 96  ataatggaGgagctagatcgttcacGtgcttttgcca-gagac-gtcaaacgtatcgtcg 153

Mut#9 : 221 GtanngGngggacagcnnttgtGGctngnnaaggtggaagattgnctctnggncgtttag 280
            ||  |  |||||||| ||||  || |  |||||||||||||| |||| || |||||||
AtP5CS: 154 TtaaggTtgggacagcagttgtTActggaaaaggtggaagattggctcttggtcgtttag 213

Mut#9 : 281 gancnctgtgtnaaca 296
            || | |||||| ||||
AtP5CS: 214 gagcactgtgtgaaca 229
```

(B)

```
Mut#9:      4   MDELDRSLAFATRLSXXSSXGGTAXVAXXGGRLXLGRLGXL 126

Wild type: 1    MELDRSRAFARDVKRIVVKVGTAVVTGKGGRLALGRLGAL  41
```

Figure 3.4 Comparison of nucleotide sequence alignment (A) and amino acid alignment (B) of mutant 9 and AtP5CS gene. Six base mutations caused 8 amino acid changes on the enzyme.

3.4. Partial Cloning of Proline Dehydrogenase

In proline dehydrogenase (Pdh) study, we aimed to isolate partial sequences of proline dehydrogenase gene from tobacco λ ZAP library in order to obtain antisense plant transformation vectors to develop transgenic plants with reduced proline dehydrogenase activity. This may provide an increase in proline concentration. For this aim, a PCR based cloning strategy was followed. A Pdh specific primer was designed from an already published proline dehydrogenase sequence of *Arabidopsis thaliana*. A Tobacco cDNA λZAP library was obtained (Figure 3.5). First PCR analysis with T3 and Pdh1 primers gave several bands (Figure 3.6). When the second PCR was performed, two bands were obtained having 0.597 kb and 0.276 kb molecular weight (Figure 3.7). Sequence analysis of these fragments was given in Figure 3.8 and Figure 3.9. The sequences did not match with known sequence of Proline dehydrogenase in database.

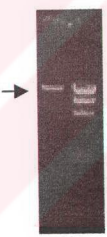


Figure 3.5. λ ZAPII DNA carrying tobacco cDNA. The cDNA library of tobacco was amplified and purified from λZAP II (arrow).

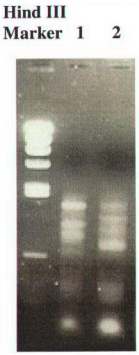


Figure 3.6 Amplified fragments fromλ ZAP cDNA library of tobacco using Pdh1 and T3 primer.Two different PCR results (1) 1 ng template DNA, (2) 5 ng template DNA

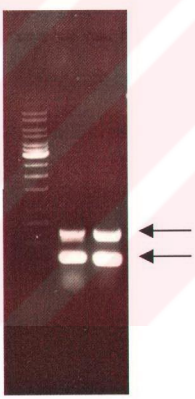


Figure 3.7 Two amplified fragments fromλ ZAP cDNA library of tobacco using Pdh1 and SK primer

GNTTTGAATCCTTTGCCTTGACCTCCCCGCTTGGCGgCCGCTCTAGAACTA
GTCCAAAGGGCATGTACTTGCACACTTCAAGGTACccATCAagAAATGGGA
AGTTGGTACATGAAAGGATCACTTTGGGGCAAACACTCTGATTGCTTATG
ATTATGAAGTCCACACCACTCCCAAAGAACCTGGTTTATCCAAAAAGGTA
CATATGAATAGCAAAGTGCGAGCCTTGGTGCAAGAAAGTGGGGCTAAGG
ATACTAAAAGGCTGAAGAAGAGGTNGGCTGAAGTGGAGACTGAAAGAN
ATGCTCTNGGANCTGAGCTaGCAAAGGAgAAGGAGAAGAATGATtGCaTTc
TTcATGACATGTTAAAACTNCTTCAAACTAATAACCTGGTNCTTCTCAGCC
TTAAACCTTGCTAAACCTAGTTANGACCAACCATTNACCCNGNNTGAGAT
TTTTTTANTTNTNNNTTGNTCAANNNNCC

Figure 3.8 Sequence Analysis of short fragment that was amplified by Pdh specific primers.

AAAACCTgGAGCTCCCCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCC
CCGGGCTGCAGGAATTCCCAGATACAGTGTCAGCATCATCAGTTTCATAC
GATTCGCTATGAGTGGCTCCATTCCCTGGTTGCTTCAAATCTTTTACCATA
TTGGATTCCTGAGCAGTGCCATTGTAAAAACTACATGGCTGTGGGAGATG
TTCATATCCTGGAGCAGCATGAAGAACCATCTGTGAAAGAGATCCAGGA
AGATAAAACCGATAAGCTAATGGTTCTGAAGAAATGGGTTTTGTTTCTCT
CCCAAATAAATGCCCTGCAAGTACATGCCCTTTGGACTAGTGGATCCCCC
GGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGG
GGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCA
CTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCA
ACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCG
AAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGG
CGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTG
GTTACGCgCAGCGTGGACCGCTACACTTGCCAGCGCCCTAACGCCCGCTC
CTTTCGCTTTCTTCCCTTNCTTTCTTCGCCACGTTCGCCGGCTTTCCCCGTC
AAGCTCTTAAATCGGGGGGCTTCCCTTTAAGGGTTCCgATTTAATGCT

Figure 3.9 Sequence Analysis of long fragment that was amplified by specific Pdh primer

3.5 Construction of Transformation Vectors

Four different transformation vectors were constructed. AtP5CS gene was cloned in sense orientation in two derivatives (pROK2 and pROK8) of the binary vector pBIN19. pROK2 contains the strong, nominally constitutive, 35S promoter from cauliflower mosaic virus and nos terminator. pROK8 contains light regulated promoter from *rbcS* gene encoding the small subunit of ribulose–1,5-biphosphate carboxylase and *nos* terminator. The mutated P5CS gene fragments (Mut#9 and #12) were also ligated to pROK plasmids. The constructed vectors and their brief description are given in Table 3.6.

Table 3.6 Definition and the molecular weight of the constructed plasmids.

Plasmid Name	Definition	Mwt
PR2-P1	Wild type AtP5CS driven by 35S promoter	12.6kb
PR8-P1	Wild type AtP5CS driven by Rubisco small subunit promoter	12.6 kb
PR2-M9	Mutant#9 AtP5CS driven by 35S promoter	12.6kb
PR8-M9	Mutant#9 AtP5CS driven by Rubisco small subunit promoter	12.6kb
PR2-M12	Mutant#12 AtP5CS driven by 35S promoter	12.6kb
PR8-M12	Mutant#12 AtP5CS driven by Rubisco small subunit promoter	12.6 kb

3.5.1 Wild Type AtP5CS

AtP5CS gene was removed from pBSK and ligated to ROK2 (Figure 3.10) and ROK 8 (Figure 3.11) binary vectors between Kpn and SacI sites. The integration was tested with restriction enzyme digestion. When the BamHI and EcoRI enzymes were used for the digestion 2000 and 2100 bp fragments were isolated from ligated plasmids, respectively. These plasmids were further used for transformation of tobacco leaves.

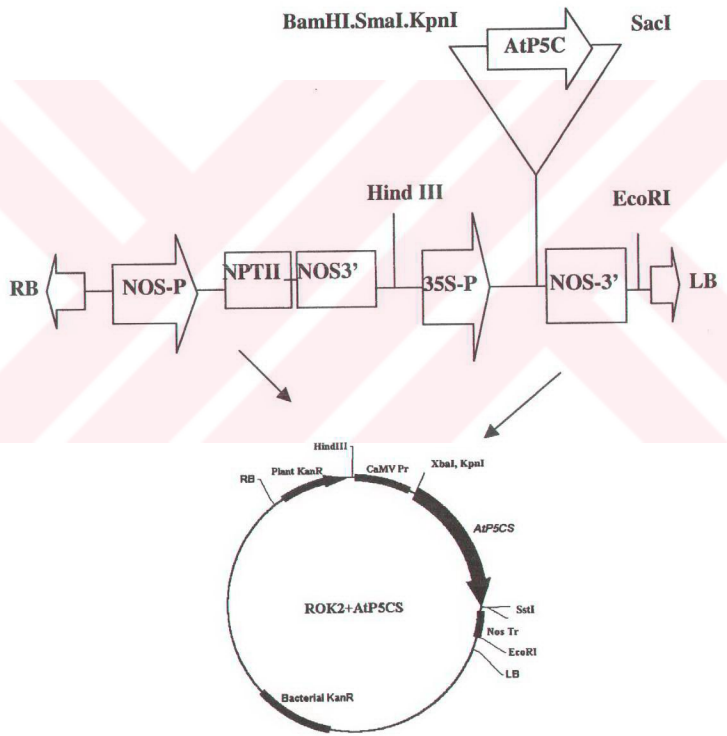


Figure 3.10 Map of the plasmid pR2-p1 used for producing transgenic plants. At P5CS cDNA was placed between CaMV35 S promoter and *nos* terminator regions. The vector also contains NPTII coding region that was used for selection of transgenic plants on kanamycin.

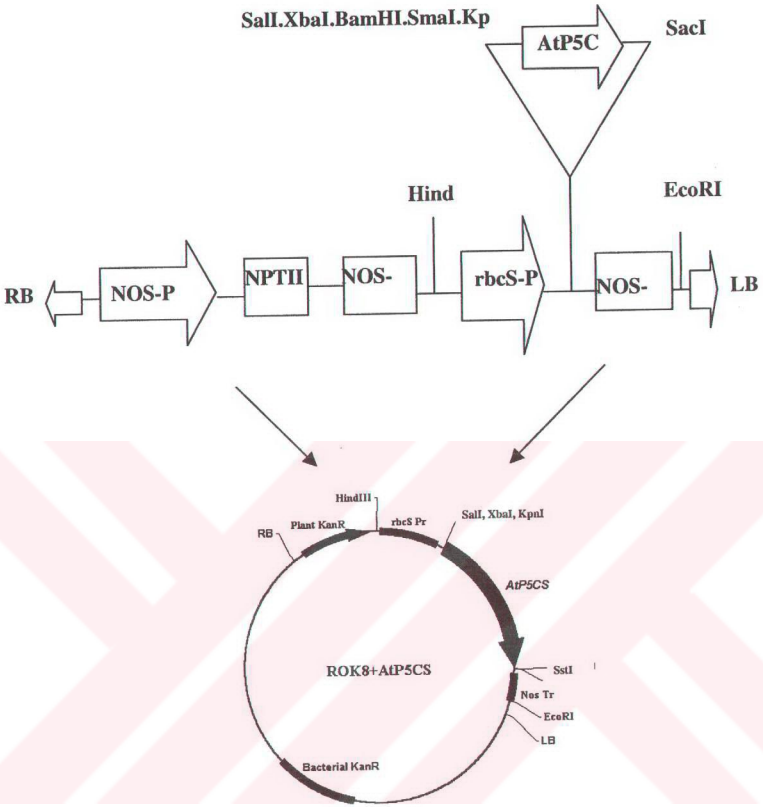


Figure 3.11 Map of plasmid pR8-p1 used for producing transgenic plants. At P5CS cDNA was placed between Rubisco small subunit promoter and *nos* regions. The vector also contains NPTII coding region that was used for selection of transgenic plants on kanamycin.

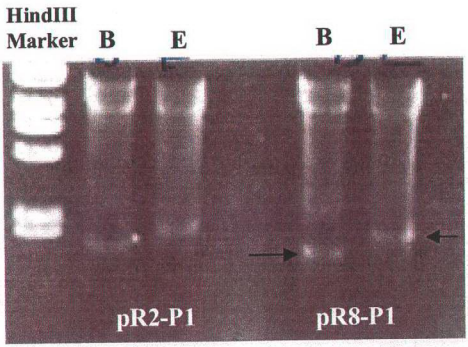


Figure 3.12 Digestion of pR2-P1 and pR8-P1plasmids containing AtP5CS gene. Plasmids from positive colony were isolated and digested with BamHI (B) and EcoRI (E) enzymes releasing the expected fragments with the sizes 2000 and 2100 bp, respectively (arrows).

3.5.2 Mutated AtP5CS

Mutant AtP5CS CDNA #12 and #9 were ligated to ROK2 and ROK8 binary vectors using the same restriction enzymes as used above. The construction was verified by restriction enzyme digestion (Figure3.13).

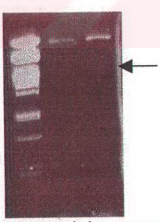


Figure 3.13 Digestion of plasmids containing mutated AtP5CS gene in Kpn and SacI site. Plasmid from positive colony was isolated and digested with Kpn and SacI enzymes to remove P5CS cDNA fragment (2.6 kb) from ROK 2 and ROK8 plasmid (arrow).

3.6. Transformation Studies

To obtain proline overproducer transgenic plants, two different sets of transformation studies were carried out. In the first one, wild type AtP5CS gene was transferred to tobacco plants under CaMV35S promoter and rubisco small subunit promoter. In the second one, two mutant AtP5CS gene (#9 and #12) were transferred to tobacco under control of the same promoters.

3.6.1. Transformation Studies with Wild Type AtP5CS Gene

Transformation studies were carried out by using two different binary vectors; pR2-P1 and pR8-P1. These binary vectors were first transformed to *Agrobacterium tumefaciens* GV3101 strain via electroporation. Plasmids from the positive colonies were isolated and after digestion with restriction enzymes, the *Agrobacterium* cells carrying the binary vectors were verified by restriction enzyme digestion (Figure 3.14).

3.6.1.1. Development of T_0 and T_1 Transgenic Plants

The leaves used for *Agrobacterium* mediated transformation were approximately of same age and appearance as for control and treated ones in all experiments. The callus formation occurred in kanamycin (75mg/L) containing selective medium after 10 days (Figure 3.15). Shoots started to emerge after 2-3 weeks. When shoots emerged, they were cut from their bases and transferred to root inducing media containing 75mg/L kanamycin (Figure 3.16). Roots started to emerge after 1 week and at the end of a month plants were ready to be transferred to soil (Figure 3.17). Five lines of independent putative transgenic plants from pR2-P1 and four independent lines of putative transgenic from pR8-P1 were obtained. Representative data are given in Figure 3.18. Prior to flowering plants were covered with plastic bags to prevent cross-pollination. F1 generation was obtained from all putative transgenic lines.

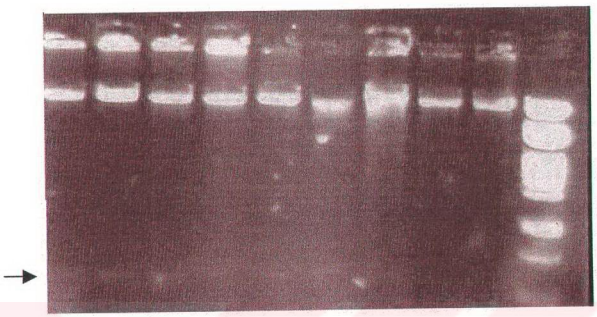


Figure 3.14. Digestion of the plasmids after electroporation. Plasmids from positive colonies were isolated and digested with HindIII enzyme releasing a expected fragment with the size of 0.7 kb (arrow).

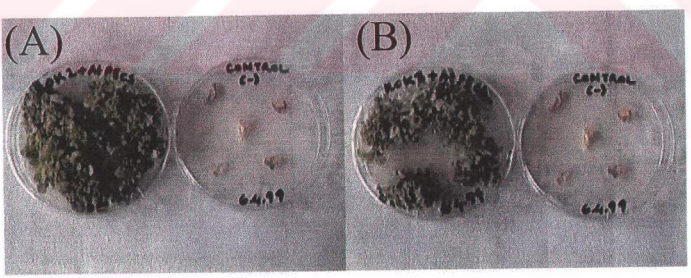


Figure 3.15 Callus development after transformation to tobacco leaf discs.
A: GV3101:: pR2-P1, **B**: GV3101::pR8-P1

Figure 3.16 Development of plantlets on 75mg/L kanamycin containing MSC media after transformation. Photographs demonstrate 2 weeks growth after transfer to rooting medium.

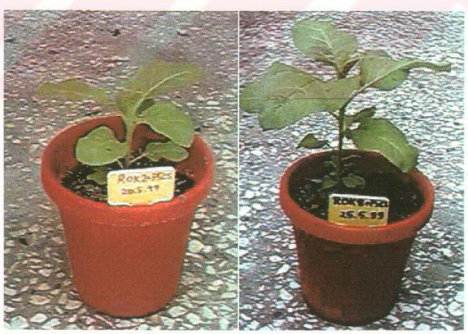


Figure 3.17 The growth of putative transgenics containing pR2-P1 and pR8-P1 plasmids in soil. Photographs demonstrate 2 weeks of growth after transfer to soil.

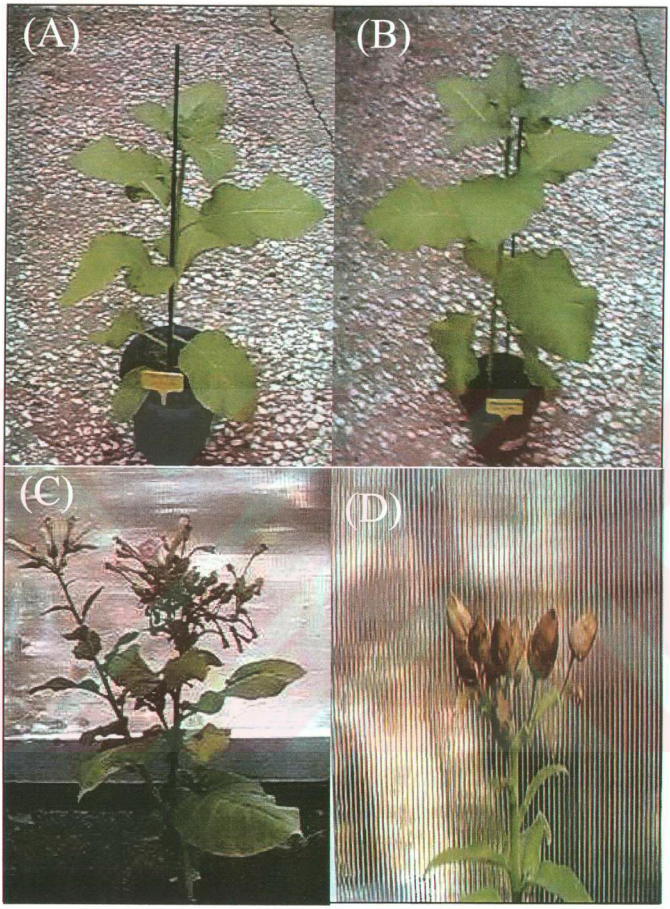


Figure 3.18 The growth of putative transgenic plants under green house conditions.
A: 8 weeks, **B**: 9 weeks after transformation, **C**. Flowering putative transgenics after 11 weeks, **D**: Mature capsules.

To obtain T_1 generation of transgenic plants, seedlings were germinated in 75 mg/L kanamycin containing medium (Figure 3.19), and transferred to selective rooting media. Then rooted plants were transferred to soil and grown to maturity under greenhouse conditions (Figure 3.20 a,b). After flowering and capsule formation (Figure 3.20 c,d), F1 seeds were collected for further analysis.

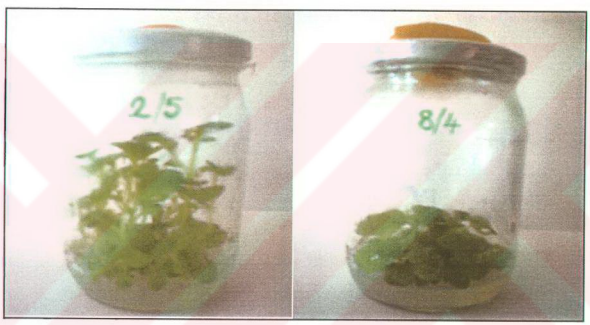


Figure 3.19 Micropropagation results for T1 progeny. Seeds were collected from T_0 plants germinated in MS media containing kanamycin (75 mg/l).

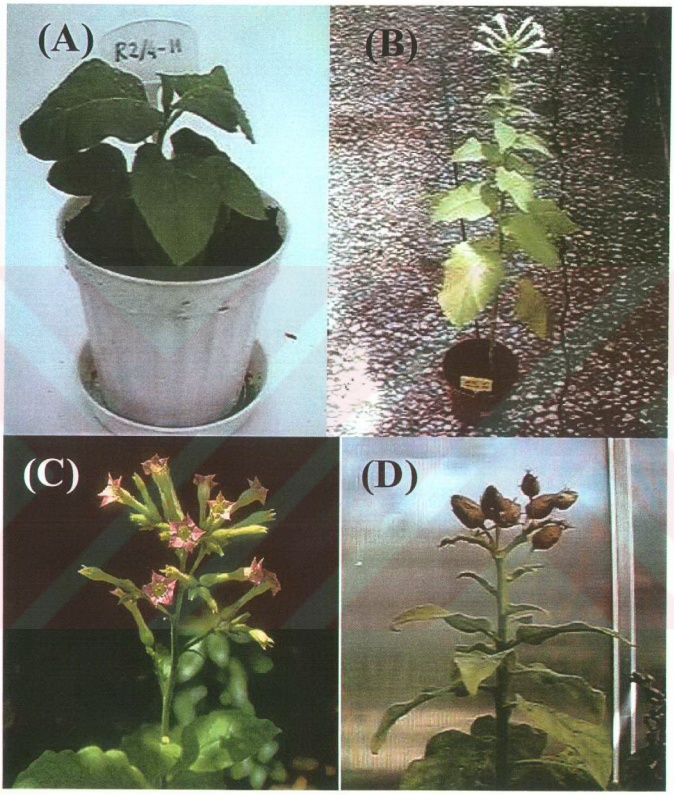


Figure 3.20 Development of T_1 progeny under green house conditions. **(A)** T_1 plantlets were transferred to soil, **(B)** grown in green house, **(C)**set flowers, and (D) formed capsules.

3.6.1.2. Analysis of T_0 and T_1 Transgenic Plants

After transformation and regeneration of transgenic plants, T_1 and T_2 progeny were analysed with respect to:

i) the presence of transformed genes. Germination of seeds and regeneration of seedlings in kanamycin selection media and PCR analysis.

ii) the performance of the transgenic plants under osmotic stress conditions. These included proline, MDA, capsule number, wet and dry weight measurements of roots.

Procedures for analyses are outlined in Figure 3.21.

3.6.1.2.1. Analysis of T_0 transgenic plants

Although selective media was used at different stages of the regeneration and propagation experiments; to be sure about positive plants, further analysis was performed to verify the integration of TDNA to plant chromosome. For this purpose Polymerase Chain Reaction was performed for *nptII* gene. A 717 bp fragment was expected to be amplified by using primers specific to npt II gene as described in Appendix F.

From the results presented in Figure 3.22 and Figure 3.23, it can be seen that in all transgenic plants, the expected amplification was achieved indicating the presence of the transferred *nptII* gene in the genome of the transgenic plants.

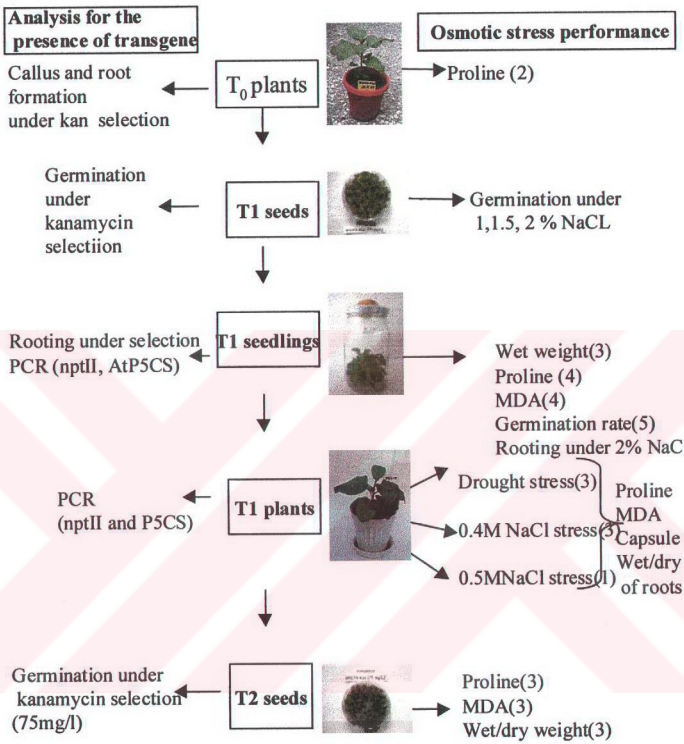


Figure 3.21 Overall scheme for the transformation and analyses of transgenic plants and progeny that were transformed with wild type AtP5CS. Numbers in parenthesis indicate the number of independent experiments.

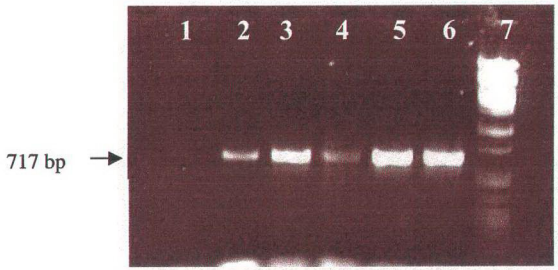


Figure 3.22 PCR analysis of 4 independent transgenic R8 plants using *nptII* primers. The samples used in PCR; (1) The genomic DNA of Control plant, (2-5) The genomic DNA of 4 independent transgenic plants, (6) Vector control, (7) λPstI Marker.

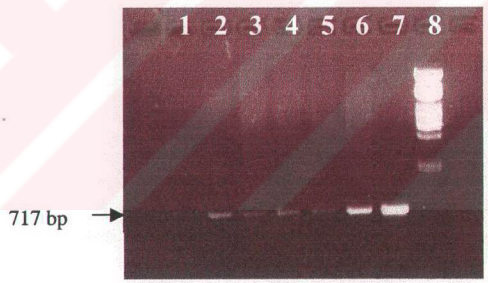


Figure 3.23 PCR Analysis of 5 independent transgenic R8 plants using *nptII* primers. The samples used in PCR; (1) The genomic DNA of control plant, (2-6) The genomic DNA of 5 independent transgenic plants, (7) Vector control, (8) λPstI Marker.

3.6.1.2.2 Analysis of Proline Content of T_0 Plants

The proline levels of the T_0 transgenic plants under normal growth conditions indicated that there was increase with respect to control (Figure 3.24).

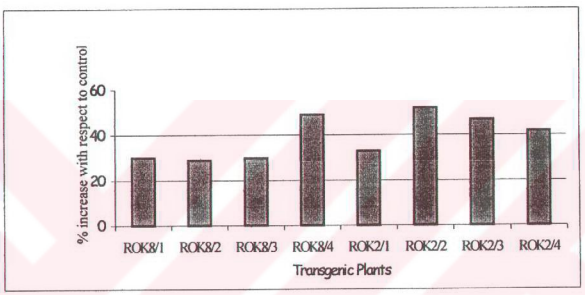


Figure 3.24 Proline results of the T_0 plants under normal growth conditions. The values represent percent increase with respect to control (untransformed) plants. Proline content of the control plant was 429 mg/ gram fresh weight.

3.6.1.2.3. Analysis of T_1 Transgenic Plants

Seeds were obtained from all T_0 transgenic plants and further used to evaluate the inheritance pattern and osmotolerance in T_1 generation. To evaluate the segregation of transformed genes, seeds were germinated in selective media (75 mg/L kanamycin) (Figure 3.25).

To obtain a phenotypic ratio for resistant and nonresistant seedlings of a given line, counted number of seeds from each transgenic plant were germinated and grown on selective medium and the survivors were counted. For each line the experiment was repeated three times by using 30 seeds in each trial. The cumulative results for 8 transgenic lines are given in Table 3.7.

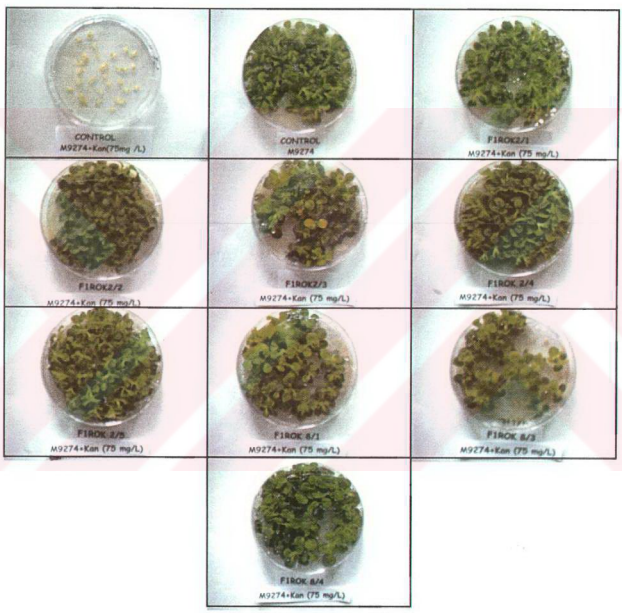


Figure 3.25 Germination results of T1 progeny transgenic plants. Seeds of untransformed (control) plants were germinated either on non-selective medium, (+) control; or on selective medium, (-) control. All seeds of transgenics were surface sterilised and germinated on selective medium (75-mg/L kanamycin).

Table 3.7 Mendelian Inheritance Analysis of T_1 progeny.

N.A. represents the test can not be applied on this line, since there was no growth.

	Total seed	Total germ	X^2	$P(X^2_{0\text{-}05,1})$	Decision
T1R2/1	90	70	0,370	< 0,05	Do not reject
T1R2/2	90	68	0,0148	<0,05	Do not reject
T1R2/3	90	72	1,2	<0,05	Do not reject
T1R2/4	90	70	0,37	<0,05	Do not reject
T1R2/5	90	83	14,23	>0,05	Reject
T1R8/1	90	76	4,28	>0,05	Reject
T1R8/2	90	0	270	N.A.	N.A.
T1R8/3	90	63	1,2	<0,05	Do not reject
T1R8/4	90	76	4,28	>0,05	Reject

The three line 2/5, 8/1, 8/4 rejected the 3:1 hypothesis while the others obey 3:1 rule. For the other T_1 lines this ratio can not be accepted, however although in most cases the segregation of the traits analogous to the segregation of single copy dominant trait in transformation studies non Mendelian inheritance pattern can be observed.

3.5.1.2.3.1. Germination in 1% and 1.5% NaCl Containing Media

To evaluate the osmotolerance of the transgenic progeny, a series of analysis were carried out on T_1 seeds and seedlings originated from this material. To test the osmotolerance of the transgenic lines, the seeds were germinated in media containing 1% NaCl and 1.5% NaCl (Figure 3.26). Uniform seed germination for all groups of plants was observed in medium containing no NaCl. With 2% NaCl concentration, rate of seed germination was very low for transgenic lines, with no germination in control seeds. The differences observed with 1% and 1.5% NaCl were however highly significant ($p<0.05$). Germination numbers showed that the transgenic lines have higher germination rate than control lines in medium containing 1 and 1.5% NaCl. With 1.5% NaCl concentration, transgenic line 8/4 exhibited the highest germination rate to an extent of 21% compared to average of 5% in control line. On the other hand, germination rate of two transgenic lines was less than that in the wild type (Figure 3.27).

Figure 3.26 Germination of seeds in medium M9274 containing 1% NaCl after 4 weeks. Seeds were surface sterilised and germinated in media containing 1% NaCl. After 4 weeks photographs were taken.

Growth of seedlings in terms of fresh weight (Figure 3.28) also showed that all transgenic lines exhibited higher growth than control plants. Seedling growth in terms of root proliferation was observed in the transgenic plants whereas only few control seedlings exhibited root initiation and elongation.

When the proline content of the transgenic seedlings was measured under 1% NaCl selection pressure, some of the transgenic lines accumulated significantly higher proline content than wild type plants ($p<0.05$) (Figure 3.29). The R8 plants (AtP5CS gene is under the control of rubisco small subunit promoter) had higher proline levels than R2 (AtP5CS gene is under control of 35S promoter) and control plants.

Under stress conditions, accumulation of free radicals is a commonly observed phenomena. These free radicals might damage various molecules in the cell (like DNA and proteins) and cause lipid peroxidation. One of the end products of lipid peroxidation is malondialdehyde (MDA). Since it has been proposed that proline may act as a free radical scavenger to protect plants from damage by oxidative stress caused during osmotic stress (Alia *et al.* 1993), measurement of MDA levels in plants give an idea about the free radical damage that occurred in the cell. Therefore, we measured the effect of NaCl on MDA levels in NaCl stressed seedlings (Figure 3.30). A significantly lower injury was found in most of the transgenic plants (between 5% to 25%) when compared with the injury that occurred in control plants (45%).

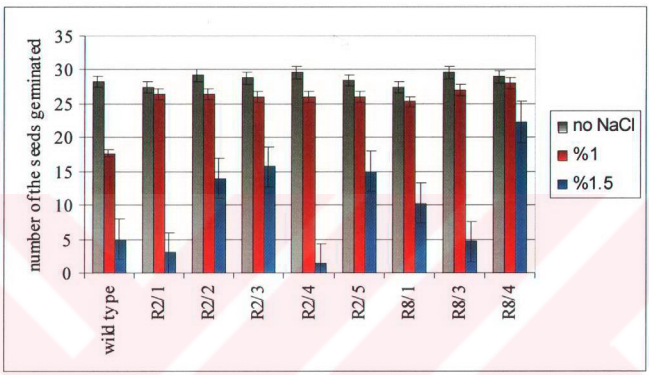


Figure 3.27 Germination number of seeds of the transgenic lines on, media containing no NaCl, 1% and 1.5 % NaCl. Seeds were surface sterilised and germinated in MS media containing forementioned concentrations of NaCl. Numbers of germinated seedlings were counted after 4 weeks. Data shown are representation of three independent experiments and vertical bars represent SEM.

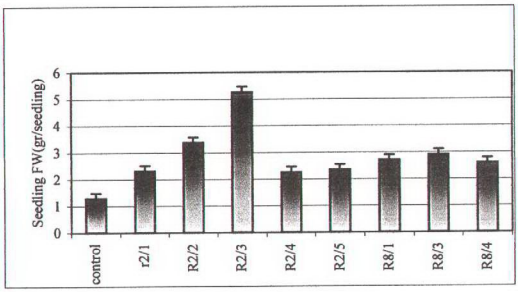


Figure 3.28 Seedling growth in transgenic tobacco plants treated with 1% NaCl. Values represented are the average of three independent experiments each containing about 100 seeds and vertical bars represent SEM.

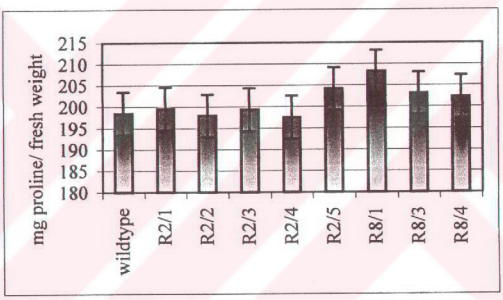


Figure 3.29 Proline content of seedlings germinated in M9274 medium containing 1% NaCl. Values represented are average of three independent experiments each containing about 100 seeds and vertical bars represent SEM.

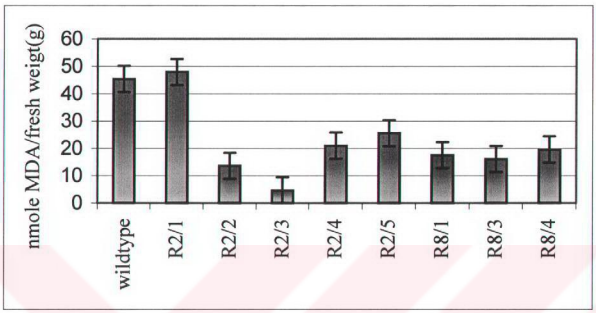


Figure 3.30 MDA content of seedlings germinated in M9274 medium containing 1 % NaCl. Seeds were germinated and grown in medium containing 1 % NaCl, after 4 weeks seedlings were harvested and MDA analysis was performed as described in materials ad methods. Values represented are the average of three independent experiments each containing about 100 seeds and vertical bars represent SEM

3.6.1.2.2.2. Growth Response Under 200 mM NaCl Stress

To determine changes in proline content at seedling level, four weeks old seedlings of transgenic lines and control plants were subjected to 200 mM NaCl stress in a liquid culture. As can be seen from Figure 3.31, transgenic lines exhibited 5 to 130 % higher proline content when compared to control plants.

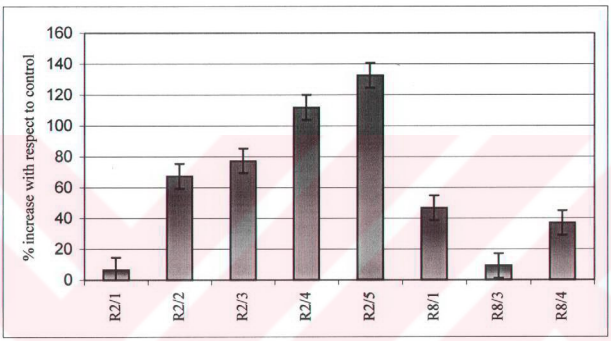


Figure 3.31 Proline analysis of the seedlings in media containing 200mM NaCl. Seedlings were kept in liquid culture for 5 days and proline content was determined and given as percentage of control. Proline content of control plant was 252 mg/gram of fresh weight. Values presented are the average of four independent samples and vertical bars represents SEM.

3.6.1.2.2.3. Response to Salt Stress After Subculturing

To see the response of plantlets to NaCl containing media for root initiation and shoot growth, the subcultured seedlings were grown in 1 and 2% NaCl containing media. Figure 3.32 shows the growth performance of wild type and of transgenic plants under stress conditions. Apparently, both control and transgenic plants exhibited a similar growth trend under 1% NaCl stress. However, at 2% NaCl concentration performance of transgenic lines was much better than that of control (Figure 3.33).

Figure 3.32 Growth response of transgenic and control seedlings in rooting medium containing 1% and 2% NaCl. 4 weeks old seedlings of control and transgenic lines were transferred to rooting media containing above mentioned concentrations of NaCl. Plants were cultured at 24°C, 16 hour photocycle for 5 weeks and photographed.

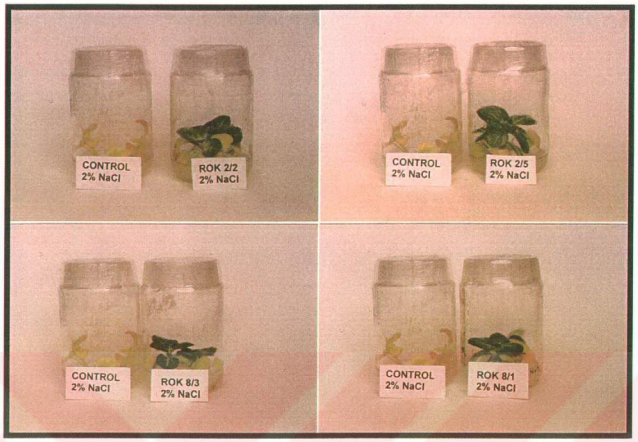


Figure 3.33 Performance of wild type and best responding transgenic lines under 2% NaCl stress. For experimental details see legend of Figure 3.32.

3.6.1.2.2.4. Analysis of Osmotolerance Under Green House Conditions

To evaluate the osmotolerance of transgenic lines under green house conditions, series of analyses were carried out. For this purpose, seedlings of trangenic lines were micropropagated in jars and transferred to soil. From each transgenic line 10-15 plants were transferred to soil and grown to 4-6 leaf stages in green house before giving stress treatments.

For evaluation of osmotolerance, three different stress applications were employed. These include 0.4 M and 0.5 M NaCl treatment and imitation of drought conditions. Details of the stress treatments are described in materials and methods. Non transformed plants served as control in all experiments. Furthermore, plants of approximately similar developmental stage and morphology were chosen before applying stress treatments (Figure 3.34).

Figure 3.34 Developmental stage of the plants at the beginning of stress application.

After the stress treatments, proline content, MDA content, capsule number and root wet/dry weight records were taken. With the exception of 0.5 M NaCl stress three independent set of experiments were carried out. In each set at least 3-5 plants from each transgenic line were used. Therefore, the presented data is an average response of 9-15 plants of each line.

3.6.1.2.2.5. 0.4 M NaCl Treatment

Before applying salt stress, the wild type plants were treated with 0.4 M NaCl in order to evaluate the growth response of control plants under stress conditions. As indicated in Figure 3.35 when wild type plant was watered with 0.4 M NaCl every two days, the growth completely stopped; whereas, unstressed plants exhibited a normal growth pattern.

(A)

(B)

Figure 3.35 Effect of 0.4 M NaCl stress on wild type tobacco. Salt stressed plant is at the right side of the photographs. A. After 2 weeks, B. After 12 weeks.

For the stress treatments, the transgenic lines were watered every two days with 0.4 M NaCl solution from the bottom of pot. Unstressed plants were similarly watered at interval of 2 days. Representative data from the experiments is given in Figure 3.36. As can be seen from the data, control and transgenic lines exhibited a more or less similar growth trend under normal growth conditions. On the other hand, the stressed plants showed great difference in shoot growth (Figure 3.37).

Figure 3.36 Effect of salt stress on representative transgenic and control plants after 0.4 M NaCl treatment. In all figures (from left to right) 1st and 3rd plants represent control and transgenic plants respectively that were grown under normal growth conditions. 2nd and 4th plants represent control and transgenic plants respectively that were grown under 0.4 M NaCl stress.

Figure 3.37 Comparison of representatives of transgenic lines with control plant after 0.4 M NaCl stress application. Plants were photographed after 8 weeks of stress treatment.

At the end of stress treatment and capsule formation, plants were removed from pots and the roots were used for further analysis. Root morphology of unstressed and stressed plants is given in Figure 3.38. The unstressed control and trasgenic plants had more or less similar root growth and elongation. However in the presence of NaCl stress, compared to controls, transgenic lines exhibited a much better performance with respect to root development (Figure 3.39).

Figure 3.38 Effect of 0.4 M NaCl stress on root development in transgenic and control plants. Plants were grown to maturity under control and stress conditions. After capsule formation the plants were removed from the pots and roots were washed and photographed.

Figure 3.39 Comparison of root development in transgenic and control plants at the end of 0.4 M NaCl treatment. For experimental details see legend of Figure 3.38.

For quantification of the data wet and dry weight of the roots were measured (Figure 3.40). The statistical analysis revealed that, in unstressed plants, there was no significant difference with respect to wet weight and dry weight of the roots. When 0.4 M NaCl was applied, there was a significant difference ($p<0.05$) between root weights of control and transgenic plants.

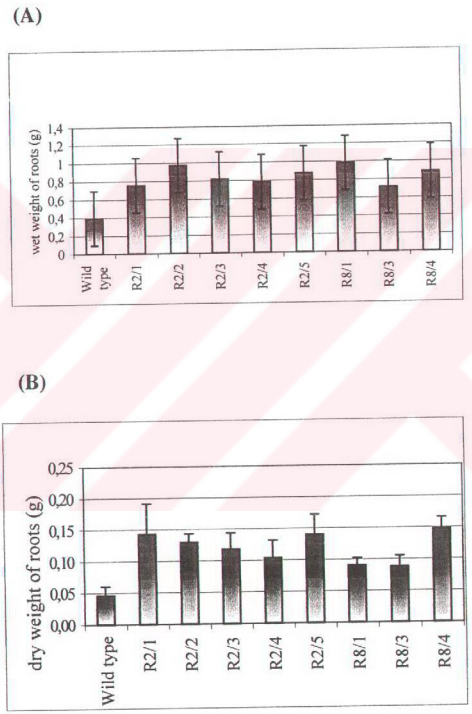


Figure 3.40 Wet and dry weight of roots after 0.4 M NaCl stress. Values are average of three independent experiments and vertical bars represent SEM. (A) Wet weight, (B) Dry weight.

To test the casual relationship between proline and MDA contents under 0.4 M NaCl stress, leaf samples were collected at the end of the stress treatment. Except lines 2/4, 8/1 and 8/3 the proline levels of transgenic plants were higher than control (Figure 3.41). On the other hand, in unstressed plants there was no difference in the proline content.

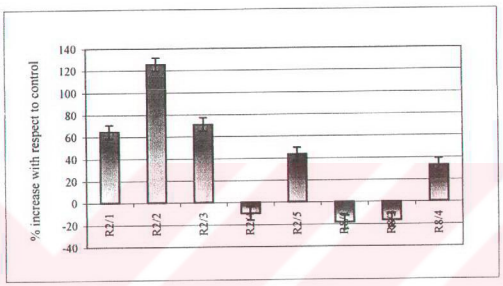


Figure3.41 Percent increase in proline content of transgenic lines at the end of 0.4 M NaCl stress. At the end of the stress treatments proline contents were determined on leaf tissues of control and transgenic plants and values are represented as % of control. Proline content of the control plant was 952 mg/fresh weight tissue.

MDA analysis of the transgenic lines showed that there was a significant difference ($p<0.05$) between control and transgenic lines whereas, as expected a lower concentration of MDA was observed in transgenic lines (Figure 3.42). In R2 transgenic plants (AtP5CS gene driven by 35S promoter), lines 1, 2 and 5 showed higher MDA content than the wild type. On the other hand, other transgenic lines had lower MDA amount, corresponding to lower extent of lipid peroxidation, thus protecting from the free radical damage.

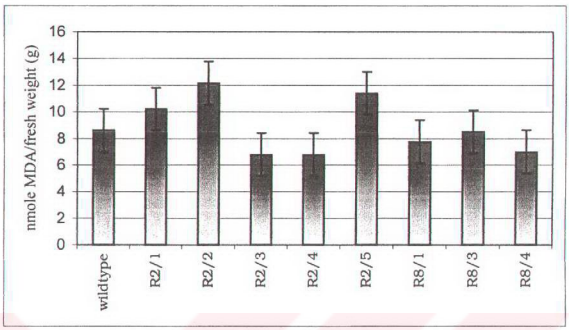


Figure 3.42 MDA analysis of leaves from 0.4M NaCl applied plants at the end of the stress. After 8 weeks of stress treatments leaves were harvested and asssayed for MDA content. Results are average of 3 experiments and vertical bars represent SEM.

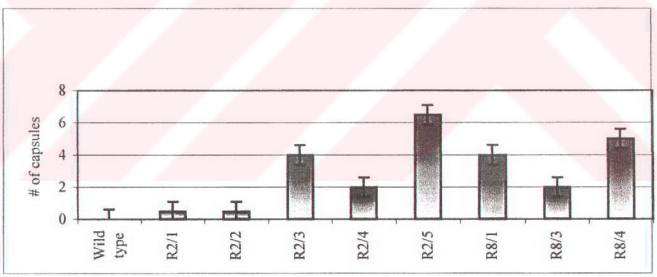


Figure 3.43 Effect of 0.4 M NaCl stress on capsule number. Values presented are the average of three independent experiments and vertical bars represent SEM.

3.6.1.2.2.6. 0.5 M NaCl Treatment

The effect of 0.5 M NaCl on growth of the plants was also tested. The stress application was performed as addition of 100 ml of 0.5 M NaCl solution directly to the soil of the plant in two days intervals. As it can be seen from Figure 3.44, unstressed control and transgenic plants showed no difference with respect to shoot growth when 100 ml of water was given every two days. Figure 3.45 shows the comparison between the stressed transgenic lines with stressed control plants. Since the difference was not significant, this test was not repeated in the later experiments.

Figure 3.44 Effect of salt stress on representative transgenic and control plants after 0.5 M NaCl treatment. In all figures 1st and 3rd plants represent control and transgenic plants that were grown under growth conditions. 2nd and 4th plants plants represent control and transgenic plants that were grown under 0.5 M NaCl stress.

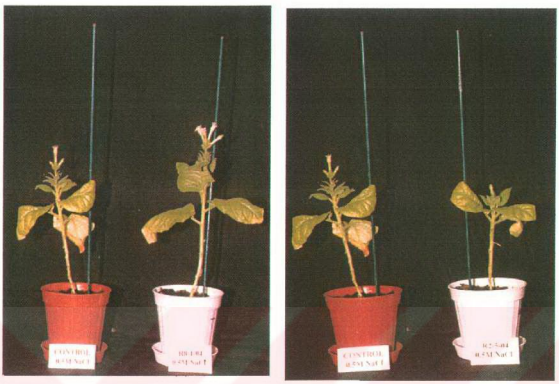


Figure 3.45 Effect of 0.5 M salt stress on representative transgenic and control plants after 0.5 M NaCl treatment. Plants were photographed after 8 weeks of stress treatment.

Effect of 0.5 M NaCl stress on root development was also tested, where no or minute phenotypic difference was observed (Figure 3.46). When the wet and dry weight of the roots of the unstressed and stressed plants was compared, it was clearly observed that there was no significant difference. Quantification of difference in root growth by wet and dry weight measurements under 0.5 M NaCl stress showed that R8 transgenic lines apperantly exhibited a better root development when compared to R2 lines (Figure 3.47).

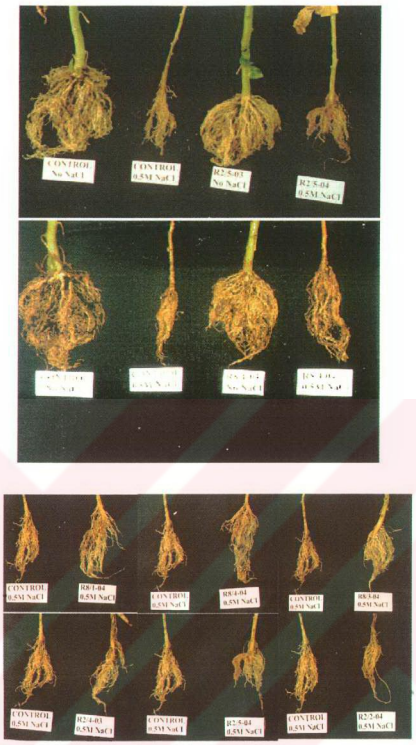
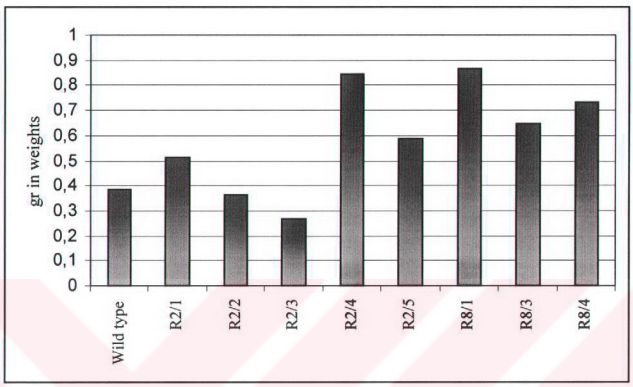


Figure 3.46 Effect of 0.5 M NaCl stress on (A) roots of the transgenics and control plants and comparison of root development. Plants were grown to maturity under control and stress conditions. After capsule formation the plants were removed from the pots and roots were washed and photographed.

The proline and MDA assays of the plants that were subjected to 0.5 M NaCl stress were not significantly different between the lines (Figure 3.48 and 3.49). The measured proline content for each line showed that the rbcS promoter controlled transgene carrying plants (R8 lines) had higher proline content than other transgenic lines (R2) and the wild type plants.

(A)



(B)

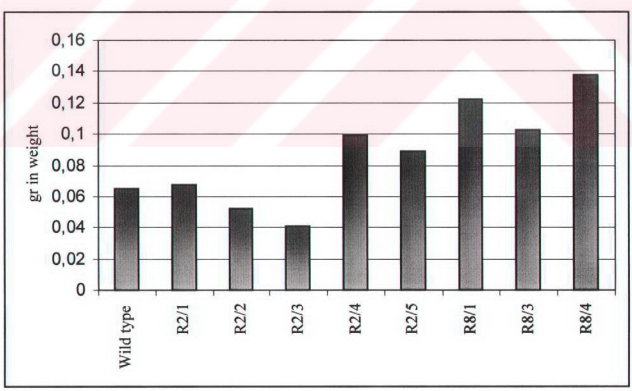


Figure 3.47 Wet and dry weight measurements of roots after 0.5 M NaCl stress.
(A) Wet weight, (B) Dry weight.

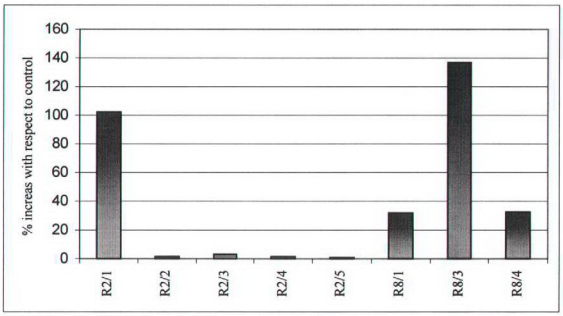


Figure3.48 Proline Analysis of leaf tissues from 0.5 M NaCl applied plants at the end of the stress. After 8 weeks of stress treatment leaves were harvested and asssayed for proline content and expressed as % of control. Proline content of the control plant was 930 mg/gr fresh weight.

MDA masurements showed that the malondialdehyde amount was same with wild type except the lines R2/2 and R8/4 where higher amount of MDA was recorded (Figure 3.49). When the capsule number was counted, R8 transgenic plants had higher capsule number than control and R2 transgenic lines (Figure 3.50).

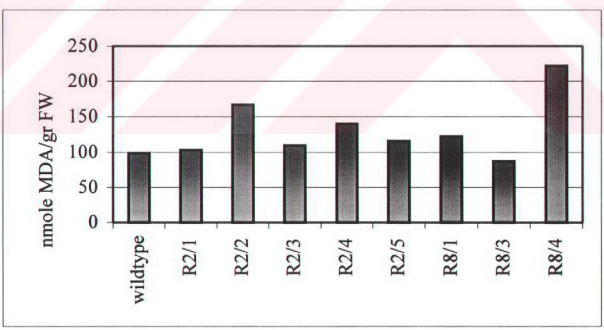


Figure3.49 MDA analysis of leaf tissue from 0.5M NaCl applied plants at the end of the stress After 8 weeks of stress treatments leaves were harvested and asssayed for MDA content.

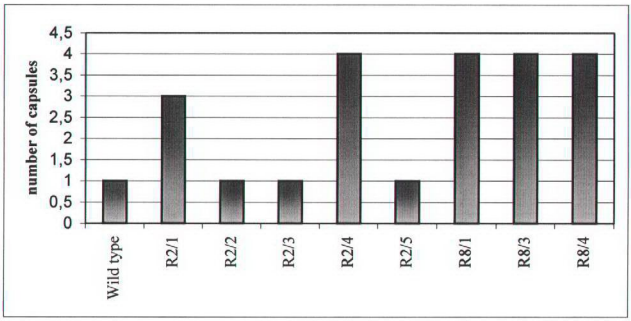


Figure 3.50 Effect of 0.5 M NaCl stress on capsule number of plants. Plants were subjected to 0.5 M NaCl stress until capsule formation (c.a. 2 months). Then capsule of each plant was recorded.

3.6.1.2.2.7 Drought Stress Applications

To investigate the influence of P5CS transgene in drought tolerance, we examined the P5CS transformed plants for their tolerance to water stress. To impose drought stress, plants were watered once a week until capsule formation. Unstressed plants were watered every 2 days. The phenotypic changes of the shoots of the plants showed that, there was no significant difference between transgenic lines and wild type plants under normal growth conditions (watered every 2 days) (Figure 3.51 and Figure 3.52).

Figure 3.51 Effect of drought stress on growth of representative transgenic and control plants. In the figures, 1st and 3rd plants represent control and transgenic plants that were grown under normal conditions and 2nd and 4th plants represent control and transgenic plants that were grown under drought stress, respectively. Plants were watered 24 hours before photography.

Figure 3.52 The comparison of the representatives for transgenic lines with control plant under drought stress application for 8 weeks. Plants were photographed after 24 hour of normal watering. From left to right 1st plant indicate the wild type and 2nd plant indicate transgenics that were grown under drought stress conditions.

Phenotypic changes in the roots of transgenic plants were also investigated (Figure 3.53 and 3.54). As in the above case, there was no big difference between wild type and transgenic lines. The wet and dry weight analysis of the roots, also gave similar results, indicating that there was no significant difference between the plants (Figure 3.55).

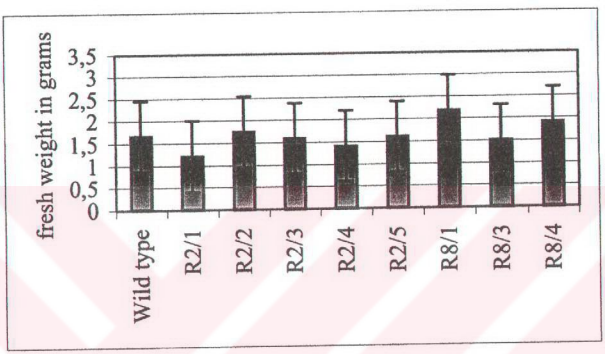
Figure 3.53 Effect of drought stress on roots of the transgenics and control plant. Root photographs were taken after removal of the soil with water.

Figure 3.54 Comparison of roots of representative transgenics with control plants after drought treatment. Root photographs were taken after removal of the soil with water.

When free proline content of the transgenics were measured it was found that some transgenics accumulate more proline than control plant (Figure 3.56). When compared with others, proline accumulation was higher in R8 transgenic lines. There was thirty to sixty percent increase in proline accumulation with respect to wild type.

MDA amount was similar or lower with respect to wild type plants, though no significant difference was observed (Figure 3.57). When the capsule number was counted on the stressed plants, except line 8/1, all lines apparently developed higher or similar number of capsules compared to control (Figure 3.58).

(A)



(B)

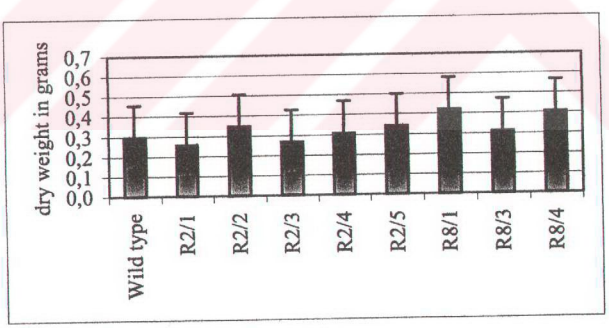


Figure 3.55 Wet and dry weight of roots after drought stress. Values are the average of three independent experiments. Vertical bars represent SEM. (A) Wet weight (B) Dry weight.

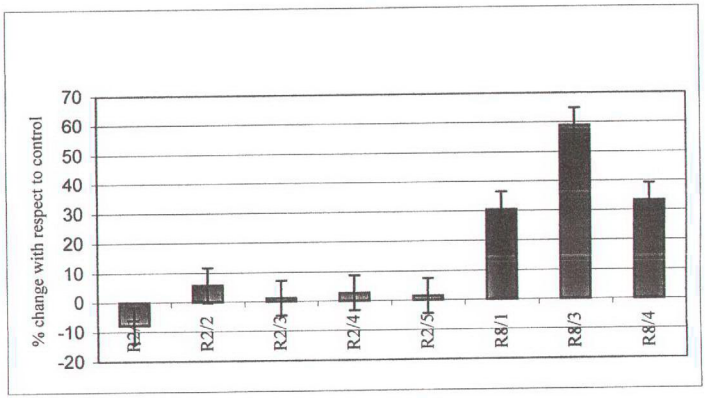


Figure 3.56 Proline analysis of leaf tissues from drought stressed plants. After 8 weeks of stress treatment leaves were harvested and assayed for proline content and expressed as % of control. Proline content of the control plant was 1017 mg/gr fresh weight. Values are the average of three independent set of experiments. Vertical bars represent SEM.

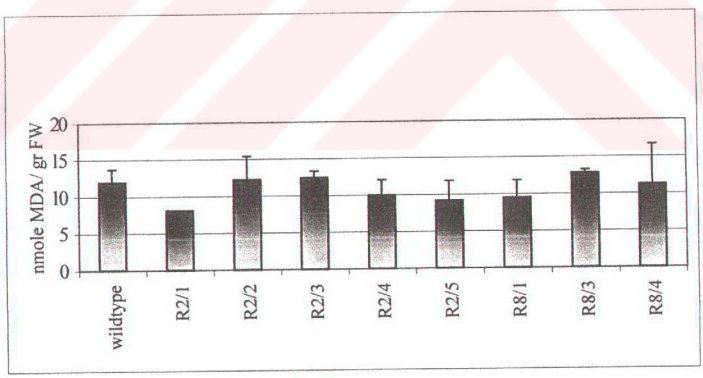


Figure 3.57 MDA analysis of leaf tissues from drought stressed plants. After 8 weeks of stress treatments leaves were harvested and assayed for MDA content. Values are the average of three independent sets of experiments. Vertical bars represent SEM.

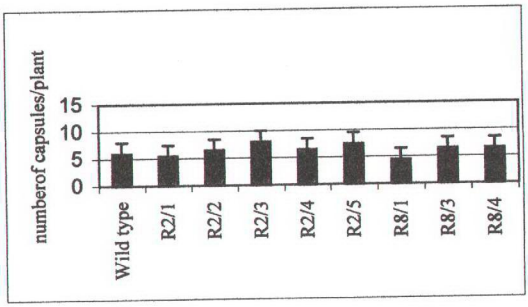


Figure 3.58 Capsule number of transgenic and wild type plants after drought stress application. Plants were subjected to drought stress until capsule formation (c.a. 2 months). Then capsule of each plant was recorded. Values are the average of 3 independent sets of experiments. Vertical bars represent SEM.

To compare the total response of the R series transgenic lines under all conditions and applied tests, the cumulative result of all experiments were taken (Table 3.8). The results showed that R8 lines had better response than R2 lines. R8/4 line is the best osmotolerant under all stress conditions. R2/5 line is the best line in R2 lines. Wild type plant was the most effected one under all stress conditions. R series trangenic plants were ranked as R8/4, R8/1, R8/3, R2/5, R2/2, R2/4,R2/3,R2/1 and wild type plant.

Table 3.8 Cumulative results with respect to osmotolerance of the R series transgenic plants under 1% and 200mM NaCl at seedling level, 0.4M and 0.5M NaCl and drought in greenhouse conditions. Abbreviations indicate, MDA; Malondialdehyde, Pr; proline, WW;Wet weight, DW; Dry weight, Cap#; Capsule number, GR; Germination rate, 200 mM; 200mM NaCl treatment

	1% NaCl seedling			0.4M NaCl						0.5M NaCl					Drought					200 mM		
	MDA	WW	prolin	GR	cap #	WW	DW	Pr	MDA	cap #	WW	DW	Pr	MDA	cap #	WW	DW	Pr	MDA	Pro	Total	RANK
Cont	7	8	7	6	5	7	8	6	5	3	7	7	8	2	4	4	7	8	6	9	124	9
R2/1	8	4	5	8	4	7	6	3	7	2	6	6	2	3	5	8	9	9	1	8	111	8
R2/2	2	2	8	4	4	3	4	1	7	3	8	8	6	8	3	3	3	4	7	4	92	5
R2/3	1	5	6	2	3	7	7	2	3	3	9	9	5	4	1	5	8	7	8	3	98	7
R2/4	5	7	9	9	3	4	3	7	2	1	2	4	6	7	3	7	6	5	4	2	96	6
R2/5	6	6	5	3	2	5	1	4	8	3	5	5	7	5	2	4	4	6	2	1	84	4
R8/1	5	5	1	5	3	1	5	9	4	1	1	2	4	6	6	1	1	3	3	5	71	2
R8/3	3	1	3	7	4	6	3	8	6	1	4	3	1	1	3	6	5	1	9	7	82	3
R8/4	4	3	4	1	1	2	2	5	1	1	3	1	3	9	3	2	2	2	5	6	60	1

3.6.1.2.3. Molecular Analysis of T1 Plants

For detection of the transgene in the genome of the transgenic tobacco lines the *nptII* gene was amplified with appropriate primers from genomic DNA preparations. The results showed that a *nptII* fragment was found in all transgenic lines, while there was no signal in wild type plant (Figure 3.59).

When genomic DNA of the transgenic lines were amplified with P5CS specific primers, a fragment was obtained at the expected size of 2.6 kb (Figure 3.60) This result further confirms the stable integration of both transgenes in the transgenic progenies.

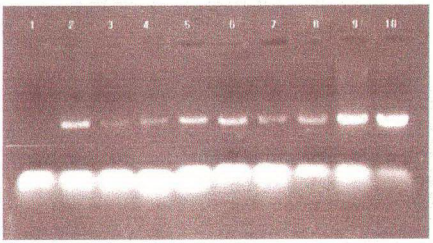


Figure 3.59 PCR analysis of T_1 plants using *nptII* primers. Samples used in PCR; (1) Genomic DNA of control plant (2-10) Genomic DNA of 8 independent transgenic plants: (from left to right) R2/1,R2/2,R2/3,R2/4,R2/5,R8/1,R8/3,R8/4.

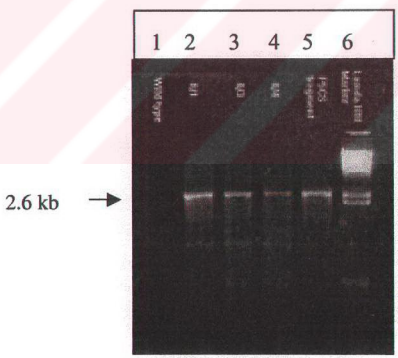


Figure 3.60 PCR Analysis of T_1 progeny of R8 transgenic lines using P5CS primers. Samples used in PCR; (1) Genomic DNA of Control plant, (2-4) Genomic DNA of transgenic plants (from left to right R8/1, R8/3, R8/4), (5) Vector control, (6) λHindIII Marker.

3.6.1.3. Analysis of T_2 Progeny of Transgenic Plants

Two plants from each transgenic line were allowed to grow to maturity to obtain T_2 progeny. Seeds were obtained from all T_1 transgenic plants and further used to evaluate the inheritance pattern and osmotolerance in T_2 generation. To evaluate the inheritance of the transformed genes, seeds were germinated in selective media (75 mg/L kanamycin). To obtain a phenotypic ratio for the resistant and non resistant seedlings of a given transgenic plant, counted number of seeds from each line were germinated and grown on selective medium and the survivors were counted. For each line the experiment was repeated three times by using 30 seeds in each trial. The cumulative results for 8 transgenic lines are represented in Table 3.9.

Table 3.9 Results for the segregation of the transgene *npt II* among the T_2 progeny.

	Total seed	Total germ	X^2	P	% survival	Decision
T1R2/1	90	59	4,28	>0,05	66	Reject
T1R2/2	90	85	18,1	>0,05	94	Reject
T1R2/3	90	82	12,4	>0,05	91	Reject
T1R2/4	90	79	7,8	>0,05	88	Reject
T1R2/5	90	57	6,5	>0,05	63	Reject
T1R8/1	90	82	12,4	>0,05	91	Reject
T1R8/3	90	59	4,28	>0,05	66	Reject
T1R8/4	90	66	0,13	<0,05	73	Do not reject

The ratio for segregation of the introduced trait, was 1:1 for 3 of the transgenic lines, R2/1, R2/5 and R8/3. R8/4 line had 3:1 ratio. The others had 1:0 ratio. Assuming that the first generations were heterozygous dominants, the progenies should be homozygous recessives, heterozygous dominant and homozygous dominant. Since only resistant individuals were selected during propagation the progenies could be either homozygous dominants or heterozygous dominants.

For T_2 generation, germination potential in the media supplemented with 1.5% NaCl was also tested. In non-stressed media they showed a similar behaviour as control, whereas in media containing 1.5% NaCl, the T_2 seedlings of the 2/1, 2/5, 8/3, and 8/4 exhibited a significantly higher ($p<0.05$) germination rate with respect to controls (Figure 3.61).

When the proline accumulation in T_2 seedlings were measured under 1% NaCl stress, it was found that except R8/1, all transgenics had similar or less amount of proline accumulation with respect to control. The proline contents also correlates with the MDA measurements (Figure 3.62 and Figure 3.63). The R2/4 line that exhibited the least proline accumulation, showed higher injury thus MDA content under stress conditions. R8 lines (driven by *rbcS* promoter) had better proline accumulation and less injury according to MDA contents

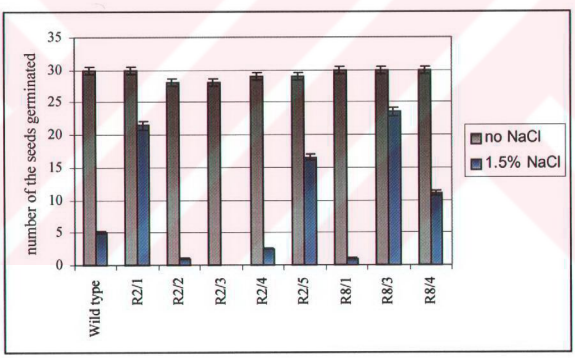


Figure 3.61 Germination test for T_2 seeds in the media supplemented with no NaCl and 1.5% NaCl. Seeds were surface sterilised and germinated in M9275 medium containing a forementioned NaCl concentration. After 4 weeks, numbers of germinated seedlings were counted. The data are average of three independent experiments and vertical bars represent SEM.

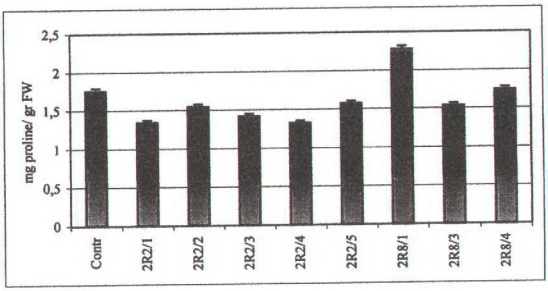


Figure 3.62 Proline content of T_2 seedlings after germination and growth for four weeks in M9274 medium containing 1% NaCl. At the end of stress treatment proline content were determined on leaf tissues of control and transgenic plants. Values are the average of three indeendent experiments. Vertical bars represent SEM.

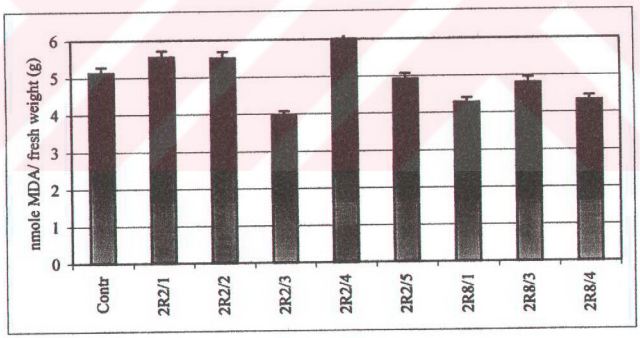


Figure 3.63 MDA content of T_2 seedlings after germination and growth for four weeks in M9274 medium containing 1% NaCl. At the end of the stress treatment MDA content were determined on leaf tissues of control and transgenic plants. Values are the average of three indeendent experiments. Vertical bars represent SEM.

The fresh weight analysis under 1% NaCl stress revealed that compared to control the transgenic lines R8/4 and R2/5 had 2 and 3 fold increase in fresh weight, respectively (Figure 3.64).

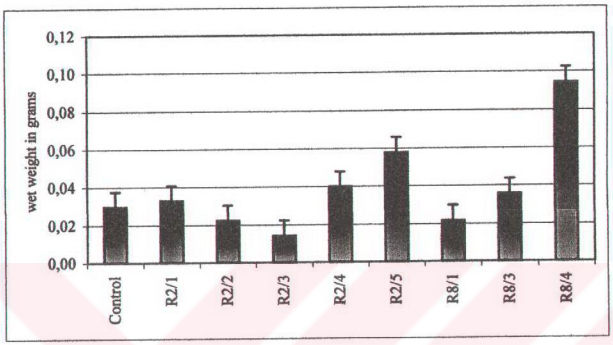


Figure 3.64 Fresh weight of seeedlings under 1% NaCl. Known number of seeds were germinated and grown in M9274 media supplemented with 1% NaCl. At the end of 4 weeks, number of seedlings were recorded and weighted. Values represent average seedling weight per plate. Vertical bars represent SEM.

3.7. Transformation Studies With Mutated AtP5CS Gene

As explained previously, two promising mutated clones were identified from mutation studies. These mutated AtP5CS (designated as #9 and #12) genes were cloned into ROK2 and ROK8 plasmids leading to 4 different binary vectors.

After mobilisation into *Agrobacterium* cells, the mutated AtP5CS genes were transferred to tobacco cells by leaf disc transformation and callus was obtained in selective medium (50 mg/l kanamycin) (Figure 3.65). Small shoots, which emerged, from these calli were transferred to selective rooting medium (Figure 3.66).

Figure 3.65 The callus formation of plants transformed with binary vectors containing mutated AtP5CS gene. Photograph shows 4 weeks of development in callus and shoot induction medium.

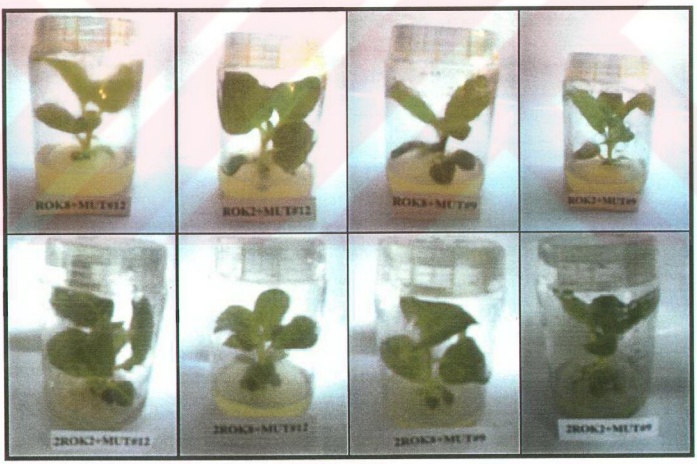


Figure 3.66 Shoot and root development of M series transgenic plants in media containing kanamycin (75 mg/l). Photograph shows 2 weeks of development in rooting medium.

Leaves from some of randomly selected transgenic lines, were subjected to chlorophenol red (CPR) color indication test. The main idea of this approach is based on change in the color of pH indicator CPR which has a red color at pH 6. The living plant tissue dividing on selective medium decreases pH around itself. At lower pH values, the indicator changes its color from red to yellow, so the medium of the surviver becomes yellow. On the other hand, tissues that can not survive on the selective medium could not decrease the pH thus, there would not be any color change in the medium. Results of the test indicated that all examined lines carried *nptII* gene, since they can reduce the color of the medium to yellow while no color change was encountered in control plates (Figure 3.67).

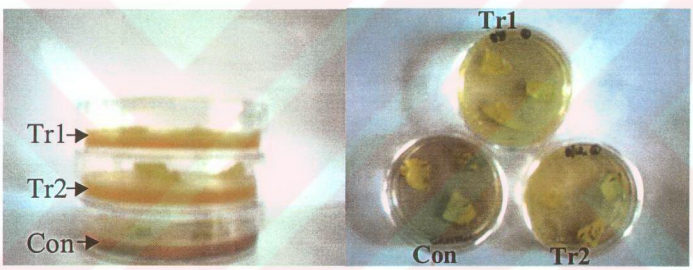


Figure 3.67 Chlorophenol red (CPR) indication test. The leaf discs were placed on media containing 15 mg/l CPR and 75mg/l kanamycin. After 3 weeks incubation randomly selected two transgenic lines (Tr1 and Tr2) converted the medium to yellow while control (Con) remains red.

From transformation experiments, for each binary vector more than 40 independent putative transgenic lines were regenerated. These plants were designated as M series transgenics. Some of these lines were further analysed with respect to their proline content. As can be seen from Figure 3.68, some transgenic lines showed an 8 fold increase in the proline contents compared to control.

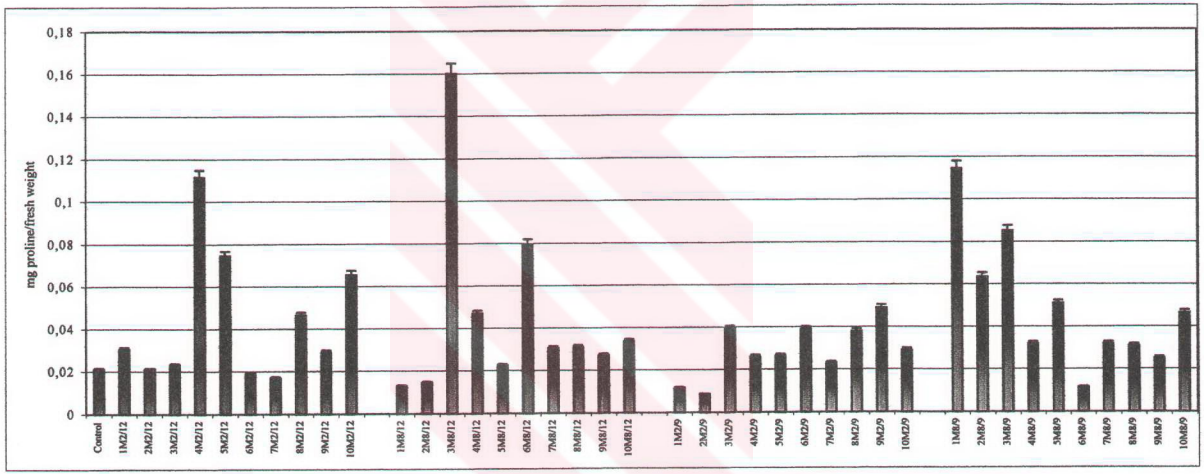


Figure 3.68 Proline analysis of putative transgenic plants that were transformed with mutated AtP5Cs genes. After the plantles were transferred to selective (75 mg/l kanamycin) rooting media, leaf samples of similar developmental stages from randomly selected lines were subjected to proline assay. M2 and M8 refer to ROK2 (35S) and ROK8 (rscS) binary vectors respectively, whereas M2/12 and M2/9 indicated the mutated genes. The numbers before M (7M2/12) refer to the number of independent transformant. For each line, values represent average of three independent experiments conducted on the same plant. Vertical bars represent SEM.

Since most of the M series putative transgenic plants were still at rooting stage at the time of preparation of the thesis, they could not be used for further analysis. Neverthless, some of the lines were transferred to soil and exhibited a normal morphology and growth pattern (Figure 3.69).

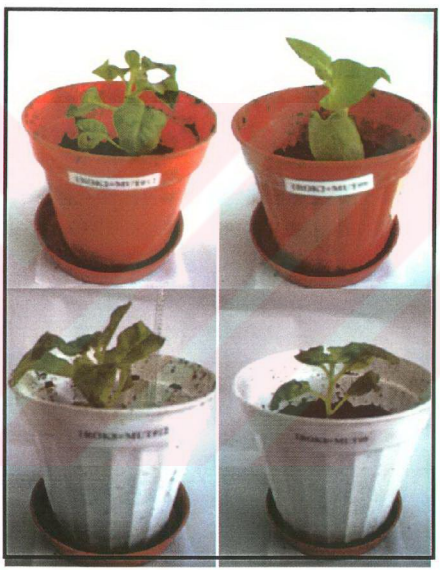


Figure 3.69 Growth of putative M series transgenics in soil. Photographs demonstrate 1-week growth after the plants were transferred to soil.

CHAPTER IV

DISCUSSION

Many studies have focused on manipulating the accumulation of diverse compatible osmolytes because of their relatively simple biochemical traits and because of current advantages in biotechnology, aiming to analyse the osmolytes' biological functions in stress tolerance and to breed stress tolerant crops. Proline is one of the most common osmolyte and in this study we have studied its potential role in development of stress tolerant transgenic tobacco plant.

There are three approaches to increase the plant tolerance to osmotic stress by proline. The first one is the over expression of proline by transfer of P5CS gene under control of constitutive promoters. In this study we transformed *Arabidopsis thaliana* P5CS gene to tobacco in order to test the transgenic plants' performance under both drought and salt stress conditions. As a second strategy, studies were conducted on isolation of feedback insensitive mutant form of the AtP5CS gene by random mutagenesis followed by vector construction and transformation of tobacco plants. The last strategy was the isolation of coding sequences of proline dehydrogenase gene from a tobacco cDNA library and construction of antisense vectors for further transformation studies.

4.1 Production of feedback inhibition resistant mutants

It is possible to select mutant colonies carrying mutated P5CS gene that can grow on toxic analogue DHPro and NaCl and thus to obtain a mutant gene, which can lead overproduction of proline. In proline deficient media, 3,4 Dehydroxyproline

blocks the synthesis of proline in auxotroph bacteria. But if the concentration of proline reaches to a high level (which indicates the loss of feedback inhibition), proline antagonise this toxic analogue. Therefore, as an initial effort the lethal concentrations was evaluated for 3,4 Dehydroxyproline and NaCl, for the selection of bacteria carrying the feedback insensitive mutant of *A.thaliana* P5CS gene.

Proline overproducing mutants were isolated from *Salmonella typhimurium* by selecting derivatives that acquired resistance to the toxic proline analogue L-azetidine-2-carboxylic acid. One of the mutants carrying the allele called proB74, exhibited a particularly marked ability to tolerate osmotic stress (Csonka 1981). Lack of feedback inhibition allows the cellular proline levels to increase 30 times more than wild type in minimal media and approximately 400 times more in minimal media containing 0.65 M NaCl (Csonka 1981). Upon adaptation to high concentrations of NaCl, plant cells accumulate as much as 300 mM proline (Binzel *et al.* 1987). According to Csonka *et al.* (1981), mutant enzyme has apparently normal catalytic activity. Therefore, it was decided that amino acid substution may affect only the binding site for proline.

The analysis of the isolated mutants showed that only 7 of them having better growth than control. Further tests with these 7 colonies showed that only 2 of them had better growth than control. The overproducing mutation results in an impairment of the growth rate of its hosts in media of higher osmolarity. During the back transformation of this plasmid, AtP5CS gene was passed throught other strain and it was verified that the mutation comes from the mutation of P5CS gene but not from a chromosomal mutation.

In this study two different methods for *in vitro* mutagenesis was showed that mutator bacteria was more effective than PCR method. The mutation frequency was too low in PCR method and therefore, no mutants could be obtained.

In mutant 12, two mutations from lysine to glutamic acid and isoleucine from phenyl alanine in kinase part of the enzyme provided inhibition of negative feedback by proline. In mutant 9 there were 8 amino acid change between 1st and 26th amino

acid at kinase part of the enzyme. The prelimnary results showed that proline production was higher than control plants.

Various proline analogs (i.e. L-azetidine–2-carboxylate and 3,4-dehydroxyproline) have been used to select proline overproducing mutants of *E.coli* (Csonka *et al.* 1988, Ruslow *et al.* 1984) , *Serratia marcescens* (Omori *et al.* 1992) and *Streptococcus thermophilus* (Massarelli *et al.* 2000). All these mutants were shown to contain single mutations within proB and as a consequence, a glutamyl kinase activity resistant to feed back regulation by proline. Our results demonstrate that while one of the mutants (mut 12) having two point mutation, the other (Mut#9) had more than one mutation at kinase encoding part of the gene.

In the literature, there is only one data for the mutagenesis of a plant gene for proline overproduction via mutation on the glutamyl kinase part of the P5CS gene. A single base mutation was created with the site directed mutagenesis at the amino acid residue 129 from alanine to phenyalanine (Zhang et al. 1995). It was shown that this single base mutation resulted in a significant reduction of proline feedback inhibition. Although most plants AtP5CS genes conserved their feedback inhibition sites (Szabados *et al.* 1998), there are other studies in bacteria in which mutation sites were different. In *E.coli* proB74 mutation resulted in a change of aspartate to an asparagine at amino acid 107 of the protein product (Csonka 1982). While Rushlow *et al.* (1984) determined that another proB mutation (designated as DHP^r) that resulted in a loss of allosteric inhibition of proline of *E.coli* glutamyl kinase. Mutation was due to a substution of an alanine for a glutamate at amino acid residue 143. Therefore, even though both DHP^r and proB74 mutation caused a loss of allosteric inhibition of γ-glutamyl kinase, in another study *S.thermophilus* proline overproducing mutant had a replacement of aspartic acid to glycine at position 192 of the γ-glutamyl kinase. As it is evident from the above, different amino acid substutions might cause a loss in feedback inhibition of the AtP5CS. Therefore, the mutations obtained in this study can confer a loss of allosteric inhibition although the mutation sites were different than previously published data.

Moreover, this is the first study for production of a mutant plant gene using bacterial mutation and selection by in vitro mutagenesis. Our study demonstrate that a mutation in P5CS gene of *Arabidopsis thaliana* can confer tolerance to NaCl, mannitol and DHPro stress in *E.coli* auxotroph. This was verified by growth analysis of mutated genes in different growth media. The performance of the mutation can also be verified by using enzyme analysis.

4.2 Transformation to tobacco plants

Due to the importance of creating drought- and salt- tolerant crops, proline overproduction strategy is being pursued to genetically engineer increased osmoprotection in plants. The potential for increasing osmotolerance by genetic manipulation of proline metabolism had been studied by other researchers(Kavi-Kishor *et al.* , 1995, Hu *et al.* 1992). Since proline biosynthesis during stress has been demonstrated to be controlled by P5CS activity (Delauney and Verma 1993, Hu *et al.* 1992), attemps to incraese osmotolerance by overexpression of proline was centered on P5CS enzyme.

Up to date, transgenic plants engineered for proline overproduction had been obtained in tobacco (Kavi-Kishor *et al.* 1985), rice (Zhu *et al.* 1988) and *Arabidopsis thaliana* (Nanjo *et al.* 1999). In two of these studies a mothbean originated P5CS gene was used (Kavi Kishor *et al.* 1985, Zhu *et al.* 1988). In the most recent study (Hong *et al.* 2000), a proline feedback insensitive mutant of mothbean P5CS gene was utilised. In the study of Nanjo *et al.* (1999), proline overproduction was achieved by antisense blokage of proline dehydrogenase enzyme activity. Other than the above mentioned studies there is no other literature data concerning engineered osmotolerance via proline accumulation. In our studies we have utilised P5CS gene originated from *A. thaliana.* Therefore, in this respect, to the best of our knowledge our experimental data demonstrates the first example of a transgenic plant engineered with an *A. thaliana* originated P5CS gene.

In this study, overexpression of AtP5CS gene in tobacco was performed under control of two different promoters; CaMV35S and *rbcS* promoters. CaMV35S

promoter is a constitutive promoter and rbcS is the light inducible promoter. From independent transgenic lines, 5 lines were under control of 35S promoter and 4 lines were under control of *rbcS* promoter. In our study these independent primary transformants (T_0) produced higher levels of proline than control plants. Among the transgenic lines the accumulation of proline was compared, it was observed that light inducible gene experession provides a higher accumulation of proline under stress conditions than constitutive expression.

Kavi Kishor *et al.* (1995) have reported that elevated proline biosynthesis levels caused by overexpression of mothbean P5CS in transgenic tobacco plants confer improved tolerance to salt stress. Their results provide clear evidence for a positive correlation between the accumulation of proline and osmotolerance in plants. However it is still argued whether proline accumulation is effective in tolerance to abiotic stress (Bohnert *et al.* 1999). Mothbean P5CS gene was also transformed to rice under the control of stress inducible promoter (Zhu *et al.* 1998). Expression of the P5CS transgene led to stress induced overproduction of P5CS enzyme and proline accumulation in transgenic rice plants. T1 generation rice plants showed an increased biomass under salt stress conditions.

In the literature various procedures are available in order to test the osmotolerance of transgenic plants engineered for proline overproduction. Most of these protocols were conducted at seedling level or at initial stages of the plant development. Other sets of analysis were conducted at later developmental stages (green house experiments) of transgenic plants after application of salt and drought stress conditions. Generally the recorded values indicate, morphological appearance, proline content, root wet/dry weight and capsule number. However, there is no study that includes all of these tests at the same time. In some other studies, green house experiments were also conducted, when plants are already developing in pots.

In our experiments we tried to analyse the osmotolerance of the transgenic plants and their progeny both at seedling level, initial stages of development and at later developmental stages by employing all of the above mentioned protocols.

In our study, the physiological and biochemical analysis of transgenic lines under salt and drought conditions showed that, both at seedling and plantlet level transgenic lines showed better response than wild type plant. Furthermore P5CS gene was transmitted to T1 generation and this was proved by kanamycin selection and PCR analysis.

4.2.1. Effect of Salt Stress on Transgenic Seedlings

In order to measure the effect of salt stress on germination at seedling level, T_1 seeds were germinated in media containing 1 and 1.5% NaCl. This provided quick and easy treatment to analyse the level and protective effect of proline under stress conditions. Seedling weight, germination number and MDA analysis were also investigated. Results showed that most of the transgenics had lower injury than control and the seed germination and weight was higher than control. Analysis of transgenic seedlings was also used for the analysis of antisense proline dehydrogenase constructs and antisense P5CS constructs (Nanjo *et al.* 1999) in *A.thalaiana* plants. In these studies the effect of transgene on proline accumulation was detected at seedling level and these studies confirmed that the effect of stress and response of transgenic lines to stress conditions can be detected at seedling level. Although there is need for test under field conditions, our results also showed that the response can be detected at the seedling level.

4.2.2. Salt Stress Tolerance of Transgenic Plants

To elucidate the role of proline in salt stress, we examined the transgenic tobacco plants for their tolerance to salt stress treatment. The transgenic plants, which showed constitutive accumulation of proline, tolerated salt treatment (0.4M) and showed better growth and rooting response than wild type plant. This supports the previous data that a Vigna P5CS transformed plants accumulated more proline and salt tolerant (Kavi-Kishor *et al.* 1995). But the physiological tests under 0.4M NaCl stress, led us to realize that proline biosynthesis is important in the stress response.

The age of the plant appears to be critical for the adaptation to salinity stress (Tarczynski *et al.* 1993). It is also known that salt stress can severley affect developmental traits such as flowering, and seed setting and there is a time–limited capacity for the plants to adjust to a changing environment. Therefore, 6 leaf stage plants were used at the beginning of the stress treatment in order to obtain a reliable data. Under stress conditions, our data demonstrated a significant reduction in the capsule numbers both in the transgenic and control plants. However compared to controls where no or minute capsule formation was observed, transgenic lines produced a significantly higher number of capsules. In our experiments this parameter appeared to be one of the most relevant data to give osmoprotection in transgenic plants. In previous studies conducted with AtP5CS transgenic plants such a data was not recorded, which enables us to compare our experimental data with others. Neverthless, our data clearly demonstrate that in future studies, capsule number would be also accepted as an important indicator of stress tolerance and adaptation.

Accumulation of proline in plants, under stress may offer multiple benefits to the cell. It was showed that free radicals are formed during osmotic stress, as measured by an increase in the MDA (a product of lipid peroxidation) production (Hong *et al.* 2000). These radicals can react with many cellular constituents, including DNA, proteins, and lipids, leading to radical chain processes, peroxidation, membrane leakage and production of toxic compounds (Davies 1995). MDA, a lipid peroxidation product, has been widely used to measure the levels of free radicals in cells (Kunert and Ederer, 1985).

We found that under 0.4 M NaCl conditions, the MDA levels increased with the 0.4M NaCl concentration in tobacco leaves. This also correlates with the earlier observations of Alia *et al.* (1993) on the production of free radicals under salt stress. The increase in proline level as a result of free radical generation was indicated by treating tobacco cells with plumnabgin, a known free radical generator. Other studies also demonstrated that proline has a role in scavening free radicals in cells exposed to salinity. Our results showed that there is correlation between proline accumulation and MDA levels under both salt and drought conditions. The transgenic lines having higher proline content showed low amount of MDA levels. Therefore it can be

concluded that increased resistance to oxidative stress in AtP5CS carrying transgenic lines can be due to some indirect metabolic or physiological consequence of the accumulation of proline.

Root growth is very sensitive to water conditions, and accordingly, a significant difference in root wet and dry weight was noticed between control and P5CS transgenic plants. This data also correlates with the results Kavi-Kishor *et al.* (1985). It was shown that roots of transgenic plants were 40% longer and had 2 fold greater biomass and and there was significant difference between control and transgenic plants' stem development under saline-stressed conditions. These results suggest that proline accumulation in plants enhances biomass production and facilitates flower development under stress conditions. In our experiments we observed similar results where the transgene accumulation provided increase both in root weight and stem development. This observation well correlates with the data that had been obtained on capsule numbers, where better pattern of flowering under stress conditions eventually lead formation of higher numbers of capsules in the transgenic lines.

The application of different salt treatment showed that under 0.5M NaCl treatment the differences were not significant when compared to 0.4 M NaCl treatment. This can be explained with two mechanisms. First the application of stress is different. While 0.4 M NaCl application is from the bottom of the pot, 0.5 M NaCl solution was applied directly to the soil. This may affect the response of plant to stress. Second, in all related studies salt treatment was applied between 0.2 and 0.4 M NaCl. Therefore, the concentration is very important for the stress development.

4.2.3. Drought Stress Tolerance of Transgenic Plants

The application of drought stress is very important for development stress response. The stage of the plant is very important because of the time-limited capacity of plants to adapt changing environment. The limitations in the studies on response of transgenics overexpressing osmolytes to water deficit are; the drought stress has not been qualified either as soil water deficit or in terms of plant water

relations. The transgene must be examined under various stress intensities at various growth stages.

When transgenic and control plants were exposed to drought conditions, transgenic plants showed increased response to water deficit. Stress-inducible production of P5CS transgene resulted in overproduction of P5CS enzyme in transgenic tobacco plants along with increased proline accumulation. The similar behaviour was observed in transgenic rice plants under water stress (Zhu *et al.* 1998).

The effect of increase proline level on wet and dry weight of root, MDA level and capsule number were also similar as in the salt stress conditions. Overexpression of P5CS transgene resulted in increase in proline accumulation. Increased levels of proline may contribute, at least in part, to an enhanced biomass production, as reflected as higher level of fresh and dry root weight, of transgenic tobacco plants under drought stress conditions.

When the response of plants under drought and salt stress was compared. It was found that during onset of both stresses there was an increase in proline accumulation but only salt stress resulted in a decrease in MDA levels. Long exposure of NaCl resulted in increased proline than those observed under drought stress conditions. Osmotic potential may also be influenced by accumulation of the salt. It has been demonstrated that with water deficit resulting from saline environment, plant cells accumulate three kinds of osmotica ; small organic solutes, glycine rich proteins and controlled amount of salt (Chiang *et al.* 1995, Mattiori *et al.* 1997). In fact, tobacco was shown to be highly drought resistant compared to other species previously studied under same condition (Liga and Vartainan 1999). Therefore salt stress may be more effective than drought stress for development of tolerance in transgenic plants with P5CS transgene.

4.3. Analysis of T_2 Transgenic Plants

The attempts to produce stress tolerant transgenic plants are limited to laboratory experiments. The inheritence of the transgenes in succeeding generations of progeny has to be analysed.

In this study, the analysis of T_2 generation showed that under stress conditions germination of the seeds and growth of seedlings were better than wild type tobacco. Although there is need for genetic proof, the physiological and biochemical tests can be used to test inheritance of the gene. Our results showed that the proline accumulation, fresh weight and MDA analysis of T_2 plants was consistent providing tolerance to 1% NaCl stress. The germination number and fresh weight analysis do not correlate with each other. Because all germinated seeds do not continue to grow. Therefore, even the germination number is low, the average fresh weight of the seedlings can be higher.

4.4. Partial Cloning of Proline Dehydrogenase

It is well known that plants accumulate proline during osmotic stress and reduce free proline levels after stress. Proline dehydrogenase seems to play a crucial role in degradation of proline in plants after termination of stress. Molecular characterisation of proline dehydrogenase is very important to understand the molecular mechanism that regulates proline concentration in plants.

Proline oxidation donates electrons to the respiratory electron transport chain and thus may provide energy that faciliates recovery from stress. Proline degradation also produces glutamate, which could act as a source of nitrogen for synthesis of other amino acids. The roles of proline degradation in plants can be more precisely explored by reducing/eliminating proline dehydrogenase activity in antisense or negative dominant mutants. The availibility of plant proline dehydrogenase cDNA will be helpful for this kind of experiments.

It was reported that the efficient accumulation of proline can be achieved by the suppression of proline degradation in antisense *Arabidopsis thaliana* proline dehydrogenase transgenic arabidopsis plants (Nanjo *et al.* 1999). Their study showed that over-accumulated transgenic plants in antisense proline dehydrogenase plants conferred a specific resistance to damage due to salt stress. This is also consistent with study on antisense glutamine synthetase that reduces the proline level and renders transgenic plants more sensitive to salt treatment (Brugiere *et al.* 1999).

In this study, a partial proline dehydrogenase gene from tobacco cDNA library was isolated in order to construct an antisense plasmid to reduce the proline dehydrogenase activity. Two fragments were isolated using specific pdh primer. But the sequence did not showed homology with the known sequences (Kiyosue *et al.* 1996, Peng *et al.* 1996). *A. thaliana* cDNA clone has 23.6% homology with yeast put1 gene and 34.5% homology with Drosophilia sluggish-A gene. The homolog sequence was used for primer synthesis. Since the homology with the known sequences was also not high. This can be the reason of the low homology of our amplified fragments.The amplified fragments also had a homology with adaptin-like protein in *Arabidopsis.* Therefore, further analysis and transformation studies is necessary to verify the similarity of the isolated sequences.

4.5. Transformation of Mutant AtP5CS to Tobacco

Biosynthesis of proline is controlled by several mechanisms in many organisms. In bacteria and yeast, two enzymes, Glutamyl kinase and GSA dehydrogenase are synthesized as seperate peptides. Feedback inhibition from proline occurs on glutamyl kinase enzyme. But in plants and animals, a bifunctional P5CS gene encodes γ-glutamyl kinase and GSA dehydrogenase. Plant P5CS is subject to allosteric regulation by proline (Hu *et al.* 1992, Zhang *et al.* 1995) while in human, P5CS is regulated by ornithine (Hu *et al.*1999).

If the feedback regulation of the wild type P5CS is not completely lost during stress, then expression of the mutant P5CS will result in the synthesis of more proline than expression of the wild type P5CS transgene. The site directed feedback

insensitive mutant of the *Vigna aconitifolia* was transformed to tobacco plants. The transgenic plants expressing P5CS129A (site directed *Vigna* mutant) accumulated about 2-fold more proline than the plants expressing *V. aconitifolia* non mutated gene (Hong *et al.* 2000).

Furhermore, previously conducted mutagenesis studies on P5CS gene utilised a site directed mutagenesis approach (Hong *et al.* 2000). However in the present study feedback insensitive mutant P5CS genes were obtained by employment of a random mutagenesis approach. Again in that respect our experimental data presents the first findings in the literature.

In our study two mutants obtained from random mutagenesis showed different mutation properties. The Mut#12 had two point mutation while the mut#9 had more than two mutations. All mutation sites were in kinase part of the enzyme. The P5CS cDNA having mutation was ligated to binary vector and transferred to tobacco plants. The proline analysis from transformed regenerants showed that some plants have higher proline content than wild type tobacco plant and transgenic plants transformed with wild type P5CS transgene.

The results of the Hong *et al.* (2000) showed that feedback regulation of P5CS in plants is not completely eliminated under stress. Thus, proline synthesis in plants under stress is regulated not only by transcriptional activation of P5CS (Hu *et al.* 1992, Garcia-Rios *et al.* 1997, Zhang *et al.* 1997) but also by feedback regulation by the end product of the pathway.

It was demonstrated that the feedback regulation property of P5CS can be changed to different degrees by modification of different amino acid residues. Zhang et al 1995, reported site directed mutagenesis to substitute each of the six amino acid residues between positions 126 and 131 of *Vigna* P5CS peptide. Only two of them found to have a different degree of effect than others. Substitution of phenylalanine at 129 with alanine produced most effective mutation for the *Vigna* P5CS gene. While other mutation, producing relatively moderate change in feedback inhibition.

In our study although, there were different mutation sites at different parts of the gene, for both mutants the mutation was on the kinase part of the gene. This confirms earlier observations on the feedback interaction site which is believed to be on the kinase part.

Since accumulation of proline in plants under stress is a result of the reciprocal regulation of two pathways: increased expression of proline synthetic enzyme (P5CS) and repressed activity of proline degradation (Delauney and Verma, 1993, Peng *et al.* 1996) all of these results will help us to improve crop tolerance to abiotic stresses.

CHAPTER V

CONCLUSION

In this study we conducted experiments towards engineering tobacco plants against salt and drought stress by transferring a wild type or randomly mutated *Arabidopsis thaliana* originated coding sequences of a gene (P5CS) which is responsible for the synthesis of the key enzyme (pyrolline-5- carboxylate synthetase) of proline biosynthetic pathways in plants. Moreover, a PCR based cloning strategy was followed to isolate partial cloning sequences of proline dehydrogenase from λZAP cDNA library of tobacco to be used for construction of proline dehydrogenase antisense plant transformation vectors.

Random mutagenesis work using mutator bacteria yielded two mutated forms of AtP5CS which were apperantly insensitive to proline feedback inhibition. Sequence analysis revealed that all of the mutations were at the sequences coding for the kinase part of the enzyme. Pdh cloning work revealed two fragments of 276 and 597 base pair. However, both of the cloned sequences did not exhibit sequence similarity with the known sequences of Pdh.

Transformation studies were conducted with both wild type and mutated AtP5CS genes under the control of two different promoters, 35S Cauliflower Mosaic virus (CaMV35S) promoter (R2 series transgenic lines); a constitutive one, and Rubisco small subunit (*rbcS*) promoter (R8 series transgenic lines); a light inducible one.

Eight independent transgenic lines were obtained from transgenic plants that were transformed with the wild type gene (R series) whereas more than 100 independent lines (M series) were obtained from transformation studies conducted with mutated AtP5CS gene (M series).

From R series transgenic plants T_0, T_1, T_2 generations were obtained. Molecular analysis revealed that all transgenic plants and their T_1-T_2 progeny carried the transgenes.

A series of analysis at seedling (T_0, T_1, and T_2), early developmental stages and later developmental stages of T_0 and T_1 transgenic plants under normal salt/drought stress conditions were carried out in order to asses the osmotolerance levels. These analyses includes, proline and MDA content, germination rate, seedling weight, root wet/dry weight and capsule number measurements.

According to the cumulative results of the above mentioned analysis, R series transgenic plants were ranked as R8/4, R8/1, R8/3, R2/5, R2/2, R2/4, R2/3, R2/1, with respect to their osmotolerance.

Proline assays conducted on T_0 plantlets of M series of transgenic plants under normal conditions revealed that some lines were able to accumulate up to 8 fold more proline content with respect to controls.

The studies to be conducted in future would include:

(i) further analysis of M series transgenic lines and progeny with respect to their osmotolerance under salt/drought conditions,
(ii) further attempts towards isolation of tobacco Pdh gene
(iii) generation of double transformants with feedback insensitive mutant AtP5CS gene and antisense Pdh gene.

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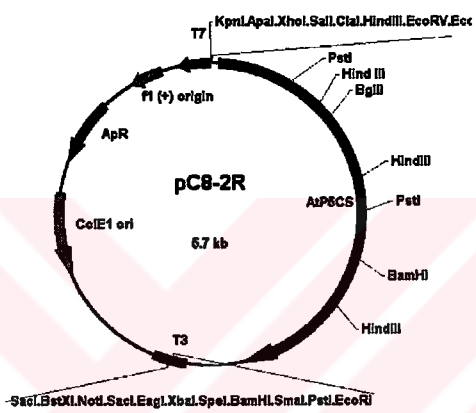
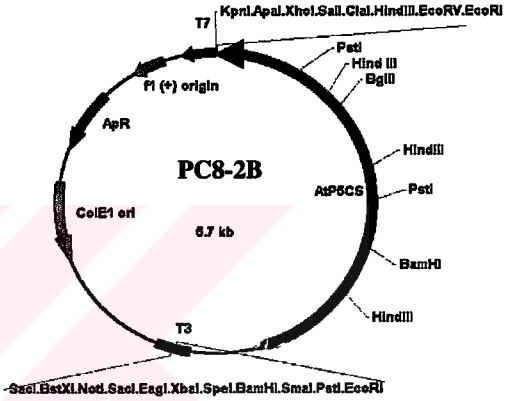
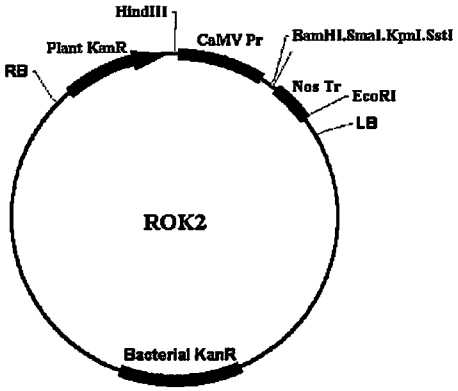
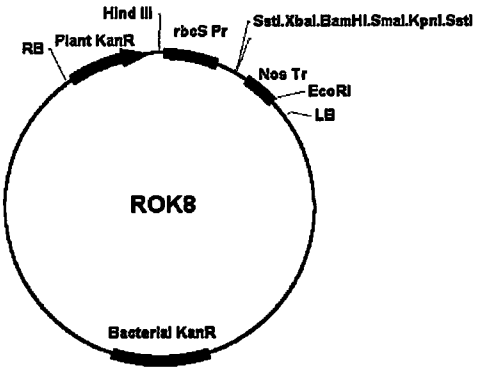
APPENDIX A

PLASMID MAPS









APPENDIX B

BACTERIAL CULTURE MEDIA

LB (1 lt) : (pH: 7.4)

Yeast Extract	5 gr
Tyrptone	10 gr.
NaCl	10 gr
1n NaOH	1 ml
Agar	15 g/lt

YEB Medium (1 lt): (pH: 7.2)

Sucrose	5 gr
Peptone	5 gr
Beef Extract	1 gr
Yeast Extract	1 gr
Agar	15 g/lt

M63 Medium: (pH: 7.2)

KH_2PO_4	0.1M
KOH	0.075 M
$(NH_4)_2SO_4$	0.015 M
$MgSO_4.7H_2O$	0.16 mM
$FeSO_4.7H_2O$	1.8μM
Glucose	10mM
Agar	1.5%
Thiamine	0.05 mM

0.2 μM all amino acids (except proline)

SOB Medium (1 lt): (pH:7.0)

Tyrptone	20 gr
Yeast Extract	5 gr
NaCl	0.5 gr
0.250 M KCl	10ml
2 M MgCl2	5 ml (Add after autoclave)

2XYT Medium (1 lt): (pH:7.0)

Tyrptone	16 gr
Yeast Extract	10 gr
NaCl	5 gr
1 M NaOH	1 ml
Agar	1.4 % for plates
	0.7% for top

SM solution: (1 lt)

NaCl	5.8 gr
$MgSO_4.7H_2O$	2 gr
1M Tris-HCl (pH 7.5)	50 ml
2% gelatine	5 ml

YM Medium: (1 lt) (pH:7.0)

Yeast Extract	0.4 gr
Mannitol	10 gr
NaCl	0.1 gr
$MgSO_4.7H_2O$	2 gr
$KH_2PO_4.3H_2O$	0.5 gr
Agar	15 gr

APPENDIX C

COMPOSITIONS OF PLANT TISSUE CULTURE MEDIA

From Sigma Plant Cell Cultures	**M9274**	**M0404**
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
Cubric Sulfate. $5H_2O$	0,025	0,025
Na_2EDTA	37,26	37,26
Ferrous Sulfate./H_2O	27,8	27,8
Magnesium Sulfate	180,7	180,7
Magnesium Sulfate.H_2O	16,9	16,9
MolybdicAcid(Sodium salt).$2H_2O$	0,25	0,25
Potassium Iodide	0,83	0,83
Potassium Nitrate	1900	1900
Potassium Phosphate Monobasic	170	170
Zinc Sulfate.$7H_2O$	8,6	8,6
Organics		
Agar	8000	-
Glycine	2	-
Myoinositol	100	100
Nicotinic Acid	0,5	1
Pyrodoxine.HCl	0,5	1
Sucrose	30000	-
Thiamine.HCl	0,1	10
Grams of powder to prepare 1liter	42,4	4,4

APPENDIX D

PLASMID ISOLATION SOLUTIONS

Sol O:

0.5M NaCl

0.1% Sarcosyl in TE buffer

Sol I:

50 mM Glucose

25 mM Tris.Cl(pH:8)

10mM EDTA (pH:8)

Sol II

0.2N NaOH(freshly diluted from 10N stock)

1% SDS

Sol III

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

APPENDIX E

PRIMERS

Primers used during PCR amplification:

***NptII* primers**

5' GAGGCTATTCCCGGCTATGACT3'
3'ATCGGGAGCGGCGATACCCTA 5'

P5CS primers

5' GACGACGATAATGGAGGA 3'
3' ATCCCAATACTAACGTGG 5'

Pdh1 primer

5' TTAAGTACTAGTTCCAAA5GGCAT5AACTTGC 3'

T3 primer
5' ATTAACCCTCACTAAAG 3'

T7 primer
5' AATACGACTCACTATAG 3'

SK primer
5' TCTAGAACTAGTGGATC 3'

APPENDIX F

AtP5CS COMPLETE SEQUENCE

: **Arabidopsis thaliana mRNA for delta1-pyrroline-5-carboxylate synthase, complete cds**
D32138
LOCUS ATHATP5CS 2571 bp mRNA
DEFINITION Arabidopsis thaliana mRNA for delta1-pyrroline-5-carboxylate synthase, complete cds.
KEYWORDS ATP5CS; delta1-pyrroline-5-carboxylate synthetase.
SOURCE Arabidopsis thaliana (strain:Columbia) cDNA to mRNA.
ORGANISM Arabidopsis thaliana
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;Spermatophyta; Magnoliophyta; eudicotyledons; core eudicots;Rosidae; eurosids II; Brassicales; Brassicaceae; Arabidopsis.
REFERENCE 1 (bases 1 to 2571)
AUTHORS Yoshiba,Y., Kiyosue,T., Katagiri,T., Ueda,H., Mizoguchi,T., Yamaguchi-Shinozaki,K., Wada,K., Harada,Y. and Shinozaki,K.
TITLE Correlation between the induction of a gene for delta 1-pyrroline-5-carboxylate synthetase and the accumulation of proline in Arabidopsis thaliana under osmotic stress
JOURNAL Plant J. 7 (5), 751-760 (1995)

/**translation**="MEELDRSRAFARDVKRIVVKVGTAVVTGKGGRLALGRLGALFEQ

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TRRAPYQDSSGIFWDNDSLAALLALELKADLLILLSDVEGLYTGPPSDPNSKLIHTFV

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GTLFHQDARLWAPITDSNARDMAVAARESSRKLQALSSEDRKKILLDIADALEANVTT

IKAENELDVASAQEAGLEESMVARLVMTPGKISSLAASVRKLADMEEPIGRVLKKTEV

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VITDAIPETVGGKLIGFVTSREEIPDLLKLDDVIDLVIPRGSNKLVTQIKNTTKIPVL

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VEGLLTTRWIMRGKGQVVDGDNGIVYTHQDIPIQA"

polyA_signal 2446..2451
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polyA_site 2571

P5CS nucleotide sequence:

```
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       61 tcgttcctct ctctgtgtgt ggttttggta gacgacgacg acgataatgg aggagctaga
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      181 tgttactgga aaaggtggaa gattggctct tggtcgttta ggagcactgt ttgaacagct
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     1921 agttgtagaa gacgtttatg gtgctataga tcacattcac cgacatggga gtgcacacac
     1981 agactgcatt gtgacagagg atcacgaagt tgcagagcta ttccttcgcc aagtggatag
     2041 cgctgctgtg ttccacaacg ccagcacaag attctcagat ggtttccgat ttggacttgg
     2101 tgcagaggtg ggggtaagca cgggcaggat ctatgctcgt ggtccagtcg gggtcgaagg
     2161 attacttaca acgagatgga taatgagagg aaaaggacaa gttgtcgacg gagacaatgg
     2221 aattgtttac acccatcagg acattcccat ccaagcttaa acaagacttc cgagtgtgtg
     2281 tttgtgtatt tggttgagac ttgaggagag acacagagga ggatgggctt ttttgtttcc
     2341 tctctgctta gtactcatat cctatcatta ttattattac tactacttat tattgaaacc
     2401 ctcgcttatg tagtggtttt gatttagggt taggattgca ccaaaaataa gatccacttt
     2461 accacttagt cttgctcata agtacgatga agaacattta attagcttct cttcttgtca
     2521 ttgtaagcta cctacacatt tctgatcttt atcaagatac tactactttt c
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VITA

Personal

Name, Surname	Füsun (İnci) EYİDOĞAN
Birth Date and Place	August , 11th, 1971, İskenderun Turkey
Nationality	Turkish
Marital Status	Married , 1 child
Current Address:	Department of Biology Middle East Technical University Inonu Bulvari 06531 Ankara Turkey
Phone:	90 312 2105171-73
Fax:	90 312 2101289
e-mail :	biofusun@metu.edu.tr, fusuneyidogan@yahoo.com

Education

1988-1992	Middle East Technical University M.E.T.U, Ankara, Turkey Bachelor of Science in Biology (Cumulative Grade Point Average : 3.15/4) ,
1992-1995	Graduate School of Natural & Applied Sciences, M.E.T.U Master of Science in Biotechnology (MSc., CGPA 3.60/4)
1995-2001	Graduate School of Natural & Applied Sciences, M.E.T.U Ph. D. in Biotechnology

Professional Experiences and Previous Employment

1994-2001	Research and Teaching Assistant in Plant Tissue Culture, Plant Biology, Biochemistry, Molecular and Genetics, M.E.T.U

Languages

English (fluent), Turkish (native)

Professional Skills

- Recombinant DNA techniques including cloning, vector construction, analysis of gene expression
- In vitro mutagenesis
- Plant transformation systems (direct DNA uptake, vacuum and agrobacterium mediated transformation)
- Microscopy (light, fluorescent)
- Enzyme activity assay (SOD) and Polyacrylamide gel electrophoresis (1D, 2D)

Other Skills

Competent with all common Microsoft-DOS based software including word processor, data base, graphics, molecular biology programs (nucleic acid/protein sequence retrieval and analysis) and INTERNET/WWW information services.

Fellowships, Awards and Technical Courses attended

UNESCO-ITC course (11 months) 1997-1998

MED-CAMPUS, a short course on 'Enzymes as industrial tools', July,1993, İzmir-TURKEY

TUBİTAK-Joint Ph.D Project fellowship in Szeged-HUNGARY 1997-1998

Field of Interest

- Crop improvement by transgenic technologies; genetic modifications against stress factors

Details of Research Projects

Main projects :

- Isolation of feedback insensitive mutants of pyrolline-5-carboxylate synthetase gene
- Isolation and characterization of the tobacco proline dehydrogenase gene

Others :

Characterization of superoxide dismutase isoenzymes under different stress conditions in different wheat varieties

Determination of the effects of synthetic hormone(Polystimulin A-6) on PS-II activity under drought stress conditions in wheat varieties

Vacuum transformation to *Arabidopsis thaliana*

Related Publications

- **İnci, Füsun.** "Characterization of superoxide dismutase isoenzymes in Turkish wheat varieties under different stress conditions" Master of Science Thesis (October 1995) Middle East Technical University, Ankara, TURKEY
- **İnci F.**, Öktem H.A., Yücel M. (1998) "Effect of water deficit conditions on superoxide dismutase isoenzyme activities in wheat" Cereal Research Communications,Vol 26 (3) 1998, .
- Weinheimer M.A., **İnci F.**, Öktem H., Mavituna M.A., Özkan F., Yücel M.(1994) "Physiological and biochemical responses of wheat cultivars to drought stress " International symposium on Biotechnology for sustainable Development, Faisalabad, Pakistan. Proceeding Book
- Özkan F., Öktem H.A., Mavituna M.A., **İnci F.**, Soydan A., Weinheimer A.M., Yücel M.(1994) "Physiological and biochemical responses of wheat varieties to heat stress " International symposium on Biotechnology for sustainable Development, Faisalabad, Pakistan. Proceeding Book
- Öktem H.A., Mahboobi H., Mavituna M., **İnci F.**, Yücel M. "Identification of heat shock proteins in lentil (1998) " LENS Newsletter Vol 25 pp 1-2.
- Öktem H.A., **Eyidogan (İnci) F.**, Ertuğrul (Setenci) F., Yücel M.,Jenes B., Toldi O., ' Marker gene delivery to Mature Wheat Embryos via Particle Bombardent' (1999) Tr. J. of Botany, Vol 23, pp 303-308.

- Öktem H.A., Mahmoudian M., **Eyidogan (İnci) F.**, Yücel M., 'Gus gene delivery and expression in lentil cotyledonary nodes using particle bombardment'(1999) LENS Newsletter, Vol 26, p1-2.
- **Eyidoğan (İnci) F.**, Öktem H.A., Yücel M "Wheat superoxide dismutase isoenzyme activity under salt stress conditions" Submitted to Journal of Cereal Science.

Selected Presentations and meeting abstracts

- Özalp V.C., **İnci F.**, Naqvi S.M.S., Özkan F., Öktem H.A., Göktürk H.A., Yücel M. (1992) "Effect of air pollutant SO_2 on photosynthetic activity in different wheat varieties" Journal of Biochemistry(Turkish Biochemical Society) Vol XVII,pp B-12.
- **İnci F.**, Öktem H.A., Yücel M.(1993) "Characterization of superoxide dismutase Isoenzymes in wheat varieties under stress conditions" The Med-Campus Programme of the European Commision and Agence Pour Les Reseaux Transmediterranees, Lecture Notes, Enzymes as Industrial Tools,İzmir, Turkey.
- **İnci F.**, Öktem H.A., Yücel M. "Identification of superoxide dismutase isoenzymes in leaf and root tissues of Turkish wheat varieties under stress conditions " 4th International Congress of Plant Molecular Biology June 1994, Amsterdam, The Netherlands.
- **İnci F.**, Weinheimer A.M., Allahverdiev S., Özkan F., Öktem H.A., Yücel M. (1994) "The effects of polystimulin A-6 on Photosystem II activity of Turkish Wheat Genotypes under slowly developing drought stress" Turkish Journal of Biochemistry,Vol 19.
- **Eyidoğan F.**, Szabados L., Yücel M, Öktem H.A. (2000) ' Transformation of tobacco with AtP5CS gene : Vector Construction and proline analysis of F_0 transgenic plants' Second Balkan Botanical Congress,14-18 May 2000, İstanbul-Turkey.