EVALUATION OF SALT TOLERANCE IN *STO* TRANSFORMED *ARABIDOPSIS THALIANA* AND *NICOTIANA TABACUM* PLANTS

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

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
THE DEPARTMENT OF BIOLOGY

JANUARY 2004

Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

EVALUATION OF SALT TOLERANCE IN *STO* TRANSFORMED *ARABIDOPSIS THALIANA* AND *NICOTIANA TABACUM* PLANTS

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January 2004, 181 pages

Salinity is one of the limiting factors of crop development. Together with causing water loss from plant tissues, salinity also leads to ion toxicity. Under salt stress, increase in Ca^{+2} concentration in cytosol can decrease the deleterious effects of stress. The binding of Ca^{+2} to calmodulin initiates a signaling cascade involving the activation of certain transcription factors like STO and STZ. This signal transduction pathway regulates transport of proteins that control net Na^{+} influx across the plasma membrane and compartmentalization into the vacuole.

Previously *Arabidopsis* STO was identified as a repressor of the yeast calcineurin mutation. Genetical and molecular characterization of STO; a putative transcription factor that takes role in salt stress tolerance can provide a better understanding in the mechanism of salt tolerance and development of resistance in higher plants.

The aim of the present study was to amplify and clone the *Arabidopsis thaliana sto* gene in plant transformation vectors and use them for the transformation of *Nicotiana tabacum* and *Arabidopsis thaliana* plants via *Agrobacterium tumefaciens* mediated gene transfer systems. T_0 and T_1 progeny of transgenic plants carrying *sto* were analysed for the stable integration of transgenes, segregetion patterns, expression of the gene and their tolerance to salt stress. The results of the study showed that all transgenic *Nicotiana tabacum* lines are differentially expressing a transcript that is lacking in control plants and most transgenic lines exhibited higher germination percentages and fresh weights, lower MDA contents under salt stress. On the other hand overexpression of *sto* in *Arabidopsis* plants did not provide an advantage to transgenic plants under salt stress, however the anti-sense expression of *sto* caused decreased germination percentages even under normal conditions.

According to the *sto* expression analysis of wild type *Arabidopsis* plants, *sto* was shown to be induced under certain stress conditions like cold and sucrose, whereas it remained constant in salt treatment. External application of plant growth regulators had no clear effect on *sto* expression, with the exception of slight induction of expression with ABA and ethylene treatments.

Key Words: *sto*, *Arabidopsis thaliana*, *Nicotiana tabacum,* Transgenic plants, Salt stress, MDA.

ÖZ

STO GENİ AKTARILMIŞ *ARABIDOPSIS THALIANA* VE *NICOTIANA TABACUM* BİTKİLERİNDE TUZ TOLERANSININ DEĞERLENDİRİLMESİ

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Tuz stresi bitki gelişimini ve verimini etkileyen en önemli faktörlerden biridir. Tuzlu ortamda yaşayan bitkilerde su kaybının yanı sıra iyon toksisitesi de görülmektedir. Bu koşullar altında sitozoldeki Ca^{+2} miktarındaki artışın stresin etkilerini azalttığı bilinmektedir. Ca^{+2}'un kalmoduline bağlanması ile başlayan sinyal iletim ağı yardımı ile STO ve STZ gibi bazı transkripsyon faktörlerinin aktif hale geldiği, bu sinyal iletim ağının da hücre zarından geçen net Na^{+2} miktarını kontrol ettiği ve fazla miktarın kofullarda depolanmasını sağlandığı düşünülmektedir.

Önceki çalışmalarda *Arabidopsis thaliana* STO proteini ile desteklenen maya kalsineurin mutantlarının tuzlu ortamlarda yaşayabildiği gösterilmiştir. Bir transkripsyon faktörü olduğu düşünülen STO'nun genetik ve moleküler karakterizasyonu bitkilerde strese direnç mekanizmalarının daha iyi anlaşılmasını sağlayacaktır.

Bu çalışmanın amacı *Arabidopsis thaliana sto* geninin amplifikasyonu, genin bitki transformasyon vektörlerinde klonlanması ve bu vektörlerin *Agrobacterium tumefaciens* kullanılarak *Nicotiana tabacum* ve *Arabidopsis thaliana*'ya transformasyonunda kullanılmasıdır. *Sto* genini taşıyan T_0 ve T_1 transgenik bitkileri genin bitki genomuna integrasyonu, açılımı, ifadesi ve tuz stresine toleransları yönünden incelenmiştir. Elde edilen sonuçlara göre tüm transgenik tütün bitkileri kontrol bitkilerinde bulunmayan ve değişik oranlarda ifade edilen bir transkripte sahip olup, bitkilerin çoğunda tuz stresi altında yüksek çimlenme yüzdeleri ve yaş ağırlıklar ile düşük MDA miktarları tesbit edilmiştir. Bunun yanında *sto* geninin *Arabidopsis* bitkilerinde yüksek miktarlarda ifadesinin bitkilere tuz stresi altında bir avantaj sağlamadığı gözlenmiş, fakat genin anti-sense yönde ifadesinin normal ortamlarda bile çimlenme yüzdelerini önemli ölçüde düşürdüğü bulunmuştur.

Gen aktarılmamış *Arabidopsis* bitkilerinde yapılan ifade analizleri sonucuna göre, *sto* ifadesinin soğuk ve sükroz gibi stres koşullarında arttığı, bunun yanında tuz uygulaması sonrasında değişmediği tesbit edilmiştir. Dışardan bitki büyüme düzenleyicilerinin uygulanmasının *sto* ifadesine belirgin bir etkisi olmamakla birlikte ABA ve etilen uygulamaları az da olsa genin ifadesini arttırmıştır.

Anahtar Kelimeler: *sto*, *Arabidopsis thaliana*, *Nicotiana tabacum,* Transgenik bitkiler, tuz stresi, MDA.

To my family for all their support and understanding

ACKNOWLEDGEMENTS

I would like express my deep appreciation to my supervisor Prof. Dr. Meral Yücel and co-supervisor Prof. Dr. Hüseyin Avni Öktem for their encouragement and guidance throughout my research. Without their understanding and support, this study would not have been completed. I also would like to thank them for being the leaders of our group that is working with a great harmony and more than that being like a big family.

I would like to thank to Dr. Laszlo Szabados for his contributions that initiated my interest to the subject and his suggestions that affected my scientific point of view. The experience I obtained in his group will always help me in my future career. I also would like to thank all my friends in Szeged for their valuable help and friendship I would never forget.

I would like to thank the members of my thesis examining committee, Prof. Dr. Zümrüt Ögel and Assoc. Prof. Dr. Sertaç Önde for their suggestions and constructive criticism during the study.

I cannot pass without mentioning the great help of Assist. Prof. Füsun (İnci) Eyidoğan whom I always admired and who stands as a model for me. I would like to thank her for being there whenever I needed suggestions and help.

During my research in the lab and writing the thesis, Tufan Öz had significant help and support. I would like to thank him for his understanding and friendship. I believe he is and he will always be a great scientist who can create a friendly and relaxing atmosphere in his lab.

Very special thanks to all my lab-mates; Betül, Ebru, Didem, Özgür, Hamdi, Işın, İrem, Serpil, Simin, Tahir, Taner, Tahir, Tarek, Ufuk for their friendship and collaborations.

I cannot ignore the valuable love and care of Ayhan Aydın who gave me motivation and hope to finish this work, and who has always been with me.

Finally I am thankful to my family especially I would like to send all my love and ultimate appreciation to my mother Bedia Selçuk and to my sister Burcu Selçuk for their deep trust, love and motivation at every stage of my life.

This work is supported by the research fund: BAP-2003-01-08-05.

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ABREVIATIONS

ABA……..	Abscisic Acid
AQP……..	Aquaporin
Ap…….	Ampicillin resistance gene
At……..	*Arabidopsis thaliana*
ATP……..	Adenosine triphosphate
BA…….	Benzyl aminopurine
BastaR……..	Basta resistance gene
CaM……..	Calmodulin
CaMV35S…….	Cauliflower mosaic virus 35 S promoter
CDPK…….....	Calcium dependent protein kinase
CN…….	Calcineurin

Cpm……..	Counts per minute
CTAB…….…...	Hexadecyl-trimethyl ammonium bromide
DAG…….….	Diacylglycerol
DRE……	Dehydration responsive element
IP_3……..	Inositol triphosphate
LB……..	Luria-Bertani Broth
LEA……...	Late embryogenesis abundant
MAPK……...	Mitogen activated protein kinase
MDA…….….	Malondialdehyde
MS…….….	Murashige and Skoog
MOPS……	3-N-[Morpholino] propane sulfonic acid
NAA……...	Naphtalene acetic acid
NaCl……...	Sodium chloride
OD…….	Optical density

PCR……	Polymerase chain reaction
PPT……	Phosphinotricin
STO……....	Salt tolerance
RFP……	Red fluorescence protein
ROS……	Reactive oxygen species
SEM……....	Standard error of mean
SOS…….	Salt overly sensitive
STO-S…….	STO-Sense
STO-AS……..	STO-Antisense
STZ…….	Salt tolerance zinc finger
T-DNA……...	Transferred DNA
TAE……	Tris Acetate EDTA
TBE……	Tris Borate EDTA
TE……...	Tris-EDTA
Ti……..…	Tumour inducing
Ubq…….	Ubiqutine
YEB……....	Yeast extract broth

CHAPTER I

INTRODUCTION

1.1. Environmental Stress Factors

Environmental stresses are of two main types; biotic stresses including infection or competition by other organisms, and abiotic stresses (Table 1.1). In the natural environment, plants often grow under unfavorable conditions, such as drought, salinity, chilling, freezing, high temperature, flooding, or strong light. These conditions are known collectively as abiotic stresses, and any of them can delay growth and development, reduce productivity and, in extreme cases, cause the plant to die.

The fates of cellular components under stress conditions depend on the balance between rate of damage and rate of repair. When the rate of repair of a given component is more rapid than the rate of damage, no damage becomes apparent. This situation is probably the norm in plants under non-stress conditions. As the level of stress increases, the balance tips gradually toward damage. When the rate of repair is slower than the rate of damage, damage becomes apparent. This is probably the situation in plants under stress (Chen and Murata, 2002).

Table 1.1 Environmental Stresses

A. Biotic: Infection and/or competition by other organisms
B. Abiotic (Physicochemical stress):
Light: High intensity, low intensity
Temperature: High, low (chilling, freezing)
Water: Deficit (drought), excess (flooding)
Radiation: IR, visible,UV, ionizing (X-ray and γ-ray)
Chemical:Salts, ions, gases, herbicides, heavy metals
Mechanical factors: Wind, pressure

1.2. Salt Stress

Salt stress is certainly one of the most serious environmental factors limiting the productivity of crop plants. Today, approximately 20% of the worlds' cultivated lands and nearly half of all irrigated lands are affected by salinity (Rhoades and Loveday, 1990).

Salinity imposes two stresses on plant tissues. Osmotic stress; a water deficit resulting from the relatively high solute concentrations of the soil and ion imbalance; altered K^+/Na^+ ratios and Na^+ and Cl^- ion concentrations that are inimical to plants. As a consequence of these primary effects, secondary stresses such as oxidative damage often occur.

Plants can be divided into two groups according to their salinity resistance. The vegetation adapted to saline habitats is called halophytic, and the vegetation that inhabits "non-saline" habitats is called glycophytic (Waisel, 1972). Most crops are salt sensitive or hypersensitive plants (glycophytes) in contrast to halophytes, which are native flora of saline environments.

1.2.1. Physiological and Biochemical Aspects of Salt Stress

The integrity of cellular membranes, the activities of various enzymes, nutrient acquisition and function of photosynthetic apparatus are all known to be prone to the toxic effects of high 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 resources (e.g. building blocks and energy) to combat stress. In nature, the extent of salt or drought tolerance often appears to be inversely related to growth rate (Zhu, 2001a).

The ability to accumulate or excrete selective ions, the control of ion absorption and translocation to the shoots, selective fluid transport from the xylem to other parts of the plant, ion accumulation upon osmotic adaptation, ion intracellular compartmentation, organic solute accumulation, macromolecules protection, membrane lipids homeostasis, and function of membrane systems in saline environments are important cellular strategies, which may confer plant salt tolerance (Franco and Melo, 2000).

Understanding the effect of salt stress on the physiological or biochemical processes of cells, can provide important information needed for selection of salt tolerant plants and provide basic understanding of the effect of salinity on growth and development of plants (Mansour *et al*., 2003). Figure 1.1 shows the stress tolerance and response mechanisms of a plant under stress.

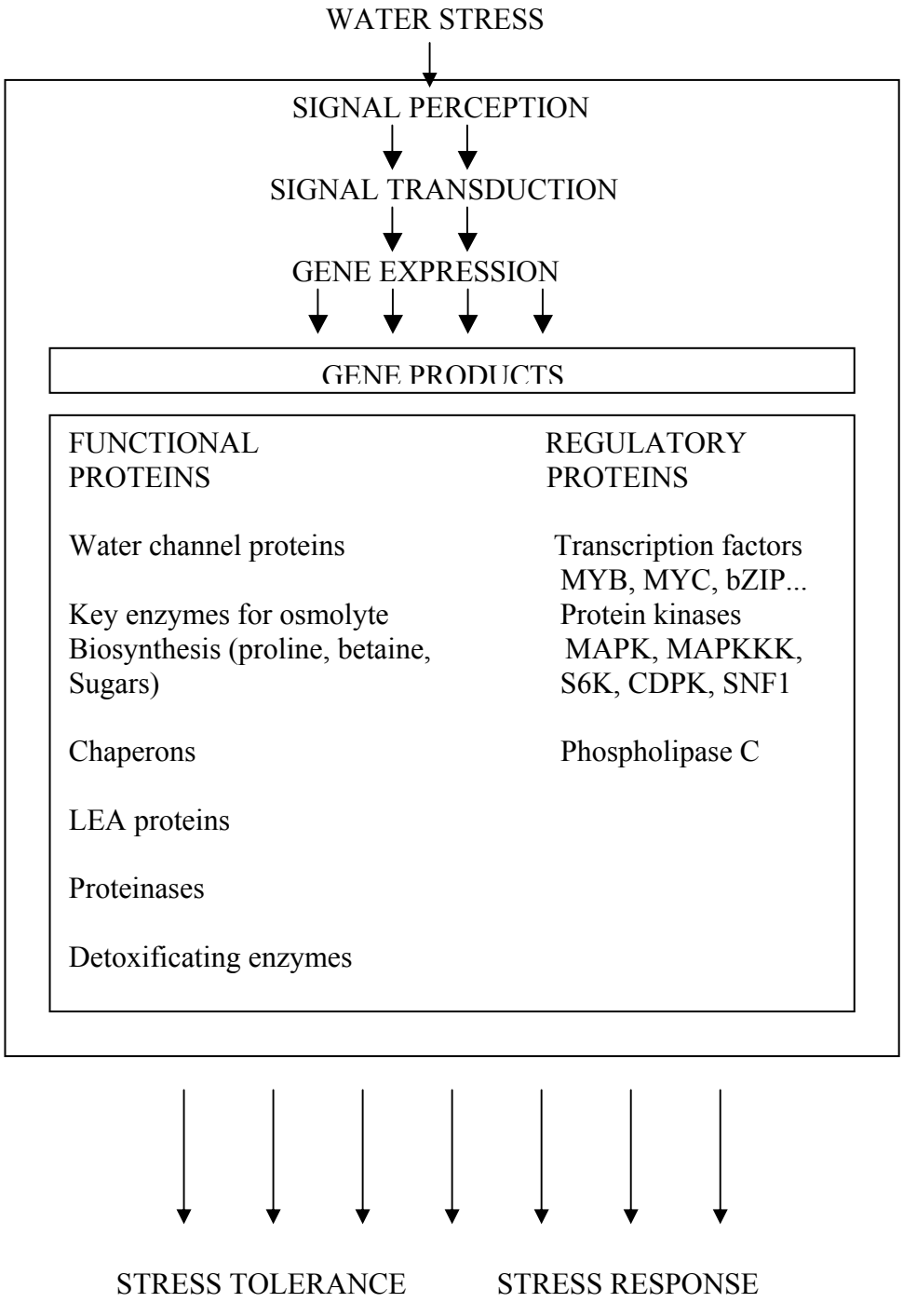


Figure 1.1 Stress tolerance and response mechanisms under salt tress.

1.2.2. Effector Determinants of Salt Tolerance

Effector molecules for salt stress tolerance are metabolites, proteins or components of biochemical pathways that lead to adaptation. Stress adaptation effectors are categorized as those that mediate ion homeostasis, osmolyte biosynthesis, toxic radical scavenging, water transport and transducers of long-distance response coordination. The stress regulated-genes that encode stress-tolerance effectors are listed in Table 1.2.

Table 1.2 Osmotic regulated genes that encode proteins with function in stress tolerance downstream of signaling cascades.

Components	**Suggested mechanism and/or metabolic function**	**Gene/Protein**
Proteins	**Protein stability**	
LEA/dehydrins	Unknown function (protein stability); control of desiccation; preventing the generation of or detoxifying ROS; inhibiting OH^- production; increases ROS scavenging enzymes	HVA1, various classes
ROS scavenging		Fe-SOD, Mn-SOD, GP, PHGPX,ASX,Catalase, Gst/Ppx
Chaperons	Heat-/cold/-salt-shock proteins; protein folding	Hsp, Csp, Ssp, *DnaJ*
Carbohydrates	**Osmolytes and/or compatible solutes**	
Polyols	Inhibiting OH^- production, osmotic adjustment, redox control	*Mtld, Imtl, Stldh*
Fructan	Osmoprotection	*SacB*
Trehalose	Osmoprotection ; (signaling?)	*Tps; Tpp,* trehalase
Quaternary N-compounds	**Osmoprotection**	
Glycine betaine	Protein protection, one-carbon sink	*Coda* BADH CMO
Dimethyl sulfonium compounds	Protein protection; pathogen defense	
Amino acid/ derivatives	**Osmotic adjustment (& possibly other functions)**	
Proline	Substrate for mitochondrial respiration; redox control; nitrogen balance/storage, transport	P5CS/P5CR POX, ProT2
Ectoine	Osmoprotectant, (signaling?)	*EctA, B, C* (operon)

Table 1.2 Osmotic regulated genes that encode proteins with function in stress tolerance downstream of signaling cascades (continued).

Components	**Suggested mechanism and/or metabolic function**	**Gene/Protein**
Inadvertent Na+ uptake	**Control over potassium uptake**	
K^+-transporters	High affinity K^+ uptake; possibly significant contribution to sodium uptake	*Hkt1, Hak1*
K^+-channels	Low affinity or dual affinity K^+ uptake; minimal contribution to sodium uptake Sodium stimulation of potassium uptake	*Akt1, Akt2*
Sodium partitioning	**Tissue/cell-specific deposition of sodium**	
Na^+/H^+ transport or antiport	Vacuolar storage of Na^+ as an osmoticum and/or plasma membrane Na^+ exclusion/export	Na^+/H^+ antiporters
H^+-ATPase	Establishing proton gradients	
Na^+/*myo*-inositol transport	Long-distance phloem and xylem transport	ITR-family
Water relations	**Control over water flux into and out of cells**	
Water channel proteins (AQP, MIP)	Membrane cycling controlling presence and amount; posttranslational modifications	γ-TIP MIP-A, -B, -F RD28 SITIP, SIMIP
	Controlling transcript amounts; expected functions in tolerance for homologues that transported other small metabolites (glycerol, urea, polyols)	Fps1p

1.2.2.1. Ion Homeostasis

Salt stress disrupts plant ion homeostasis, resulting in excess toxic Na^+ in the cytoplasm and a deficiency of essential ions such as K^+. When salinity results from an excess of NaCl, which is by far the most common type of salt stress, the increased intracellular concentration of Na^+ and Cl^- ions is deleterious to cellular systems (Serrano *et al*., 1999a). In addition, the homeostasis of not only Na^+ and Cl^-, but also K^+ and Ca^{+2} ions is disturbed (Hasegawa *et al*., 2000a, Rodriguez-Navarro, 2000). This disruption of

homeostasis occurs at both the cellular and the whole plant levels. Drastic changes in ion and water homeostasis lead to molecular damage, growth arrest and even death.

Plant survival and growth under salt stress will depend on adaptations that re-establish ionic homeostasis, thereby reducing the duration of cellular exposure to ionic imbalance. To protect actively growing and metabolizing cells, plants regulate ion movement into tissues (Flowers and Yeo, 1992, Munns, 1993). Various ion transporters function to limit Na^+ entry into and exit out of plant cells, to regulate Na^+ compartmentation in the vacuole, and to selectively import K^+ over Na^+ into plant cells. Many of the salt stress induced genes function in ionic homeostasis; these include e.g., plasma membrane Na^+/H^+ antiporters for Na^+ extrusion, vacuolar Na^+/H^+ antiporters for Na^+ compartmentation in the vacuole and high-affinity K^+ transporters for K^+ acquisition.

1.2.2.1.1. Na^+ Influx and Efflux Across The Plasma Membrane

The alteration of ion ratios in the plant is due to the influx of sodium through pathways that function in the acquisition of potassium. The stealth of sodium entry is due to the similarity between the hydrated ionic radii of sodium and potassium, which makes difficult the discrimination between the ions by transport proteins. This discrimination problem is also the basis for Na^+ toxicity. During salt stress in plant cells there is a decrease in K^+ uptake and an increase in Na^+ flux. Electrophysiological studies have shown that the so-called "voltage-insensitive monovalent-cation channels" (VIC) are responsible for the bulk of Na^+ influx into plant cells (White, 1999).

Na^+ efflux is important in maintaining a low Na^+ concentration in the cytoplasm. Unlike other organisms plant cells do not appear to contain Na^+ATPases. By contrast, Na^+-H^+ antiport activities have been detected in

plasma-membrane-enriched membrane vesicles. In plants, the main mechanism for Na^+ extrusion is mediated by the plasma membrane H^+-ATPase (Sussman, 1994). The H^+-ATPase uses the energy of ATP hydrolysis to pump H^+ out of the cell, generating an electrochemical H^+ gradient. This proton-motive force generated by the H^+-ATPase operates the Na^+/H^+ antiporters, which couple the movement of H^+ into the cell along its electrochemical gradient to the extrusion of Na^+ against its electrochemical gradient (Blumwald *et al*., 2000).

1.2.2.1.2. Na^+ and Cl^- Vacuolar Compartmentation

Many plants respond to high salt levels by sequestering ions within the vacuole. This process is mediated by a vacuolar Na^+/H^+ antiporter that uses the proton-motif force to concentrate ions against a gradient. By increasing the ion concentration in vacuole, the vacuolar Na^+/H^+ antiporters also function in osmotic homeostasis (Zhu, 2002). Na^+ compartmentation is an economical means of preventing Na^+ toxicity in the cytosol. The compartmentalization of Na^+ (and Cl^-) into the vacuole allows the plants to use NaCl as an osmoticum, maintaining an osmotic potential that drives water into the cells.

1.2.2.2. Osmolyte Biosynthesis

One way many plants and other organisms cope with osmotic stress is to synthesize and accumulate compounds termed osmoprotectants (or compatible solutes). These are small, electrically neutral molecules that are nontoxic at molar concentrations and stabilize proteins and membranes against the denaturating effect of high concentrations of salts and other harmful solutes (Yancey, 1994). The compatible solutes that accumulated

differ among plant species and can include betaines and related compounds; polyols and sugars, such as mannitol, sorbitol, and trehalose; and amino acids, such as glutamic acid and proline (Rhodes and Hanson, 1993, McNeil *et al*., 1999, Serrano, 1996). In plant cells, osmoprotectants are typically confined mainly in cytosol, chloroplasts and other cytoplasmic compartments that together occupy 20% or less of the volume of mature cells.

Compatible solutes are typically hydrophilic, which suggests they could replace water at the surface of proteins, protein complexes, or membranes, thus acting as osmoprotectants and non-enzymatically as low-molecular-weight chaperons. The physicochemical basis of this protective effect involves the exclusion of osmoprotectant molecules from the hydration sphere of proteins (Timasheff, 1992). This creates a situation in which native protein structures are thermodynamically favored because they present the least possible surface area to the water. In contrast, salts enter the hydration sphere and interact directly with protein surfaces, favoring unfolding. In dry or saline environments osmoprotectants can therefore serve both to raise cellular osmotic pressure and to protect cell constituents (Rontein *et al*., 2002).

Osmoprotectants are thought to mediate osmotic adjustment, protecting sub-cellular structures and oxidative damage by their free radical scavenging capacity (Hong *et al*., 1992, Smirnoff, 1993, Hare *et al*., 1998). Thus, genes regulating the accumulation of these organic compounds can be considered as salt tolerance determinants.

1.2.2.2.1. Betaines

Betaines are quaternary ammonium compounds in which the nitrogen atom is fully methylated. The most common betaines in plants include glycine betaine, proline betaine, β-alanine betaine, choline-*O*-sulfate and 3-dimethylsulfoniopropionate (Rhodes and Hanson, 1993, McNeil *et al*., 1999).

Glycine betaine appears to be critical determinant of stress tolerance. Its accumulation is induced under stress conditions, with its concentration being correlated with the level of tolerance. Possible roles for glycine betaine in stress tolerance include stabilization of complex proteins and membranes *in vivo*, protection of the transcriptional and translational machinery, maintaining the highly ordered state of membranes, and intervention by glycine betaine as a molecular chaperone in the refolding of enzymes (Papageorgiou and Murata, 1995, Sakamato and Murata, 2000, Sakamato and Murata, 2001, Sakamato and Murata, 2002). The metabolic engineering of plants to endow them with the ability to synthesize glycine betaine might appear to be an effective method for improving stress tolerance.

1.2.2.2.2. Polyols and Sugars

In some plants polyol synthesis is upregulated in response to osmotic stress, leading to significant polyol accumulation. Polyols are derived from sugar phosphates by reduction and dephosphorylation. Glycerol, sorbitol, mannitol can act as osmoprotectants in response to salt stress. (Serrano, 1996, Garcia *et al*., 1997). Mannitol, sorbitol and D-ononitol synthesis have been introduced into transgenic plants (Nuccio *et al*., 1999).

Soluble sugars can also be accumulated in the leaves of many plant species under osmotic stress. Sucrose and trehalose accumulation are observed to be increasing under osmotic stress. The protective effect of trehalose remains unknown at the molecular level, but some evidences suggests that this soluble carbohydrate stabilizes membrane structures and proteins under osmotic stress (Iwahashi *et al*., 1995). This is because of the chemical stability and flexibility of trehalose when compared to other sugars.

1.2.2.2.3. Amino Acids

Amino acids can be used by higher plants as osmolytes and as a source of nitrogen and carbon. They can be translocated from other parts of the plant to stress-affected organs or released by the hydrolytic degradation of protein. The products of protein hydrolysis, such as proline, glutamic acid, and aspartic acid, can be used in *de novo* protein synthesis or as osmoprotectants. Among the amino acids proline is the most effective against salt stress. Proline is an osmoprotectant that is widely distributed among organisms. This osmoprotectant is an organic solute frequently synthesized in crop plants under salt and water stress.

It has been suggested that proline protects plant tissues against osmotic stress because it is an osmosolute, a source of nitrogen compounds, a protectant for enzymes and cellular structures (Le-Rudulier *et al*., 1984), and a scavenger for hydroxyl radicals (Smirnoff and Cumbes, 1989).

In addition to these, proline accumulation in plants may function in the storage of energy, and reducing power. Such energy, reducing power and amino nitrogen storage would be of crucial importance in the maintenance of repair processes operative after osmotic stress and in the rapid restoration of cellular homeostasis (Verbrunggen *et al*., 1996).

1.2.2.3. Water Uptake and Transport

As mentioned before high concentrations of salt impose a hyperosmotic shock by decreasing the chemical activity of water and causing loss of cell turgor. This negative effect in the plant cell is thought to be similar to the effects caused by drought (Price and Hendry, 1991).

Changes in the osmotic water permeability may be caused by reducing the probability of opening of water channels or by a change in their number. Water channel proteins might be involved in controlling the speed of water flux across cellular membranes under salt stress (Chrispeels *et al*., 1999). Species- and stress-specific changes in water permeability may be caused by aquaporin (AQP) phosphorylation (Johansson *et al*., 1996, Johansson *et al*., 1998). Drought and salt stresses regulated protein amounts for the location of AQPs in the tonoplast, internal vesicles, and plasma membrane differently, indicating the existence of signaling pathways that exert control over water flux. It is intuitively clear that water channels (or water/solute channels, "aquaglyceroporins") should have significance for water relations in stressed plants (Schaffner, 1998).

1.2.2.4 Oxidative Stress Tolerance

Plants experiencing various stress conditions synthesize reactive oxygen species (ROS) that directly damage cellular components, such as membrane lipids and inhibit photosynthesis (Price and Hendry, 1991). Increasing evidence has indicated that much of the injury to plants due to various environmental stresses is associated with oxidative damage through direct or indirect formation of ROS. The ROS, including superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen

($^1O_2$), are inevitable by-products of cell metabolism. These ROS attack lipids, proteins and nucleic acids, causing lipid peroxidation, protein denaturation and DNA mutation.

Under physiological conditions, the production and destruction of ROS is regulated well in the cell metabolism. However, under stress conditions, the formation of these radicals might be in excess of the amount present under physiological conditions, thus creating oxidative stress (Yu and Rengel, 1999).

It seems likely that ROS, which are synthesized by plants experiencing various stress conditions, directly damage. It also seems likely that ROS are scavenged by compatible solutes, resulting in the protection of plants against stress conditions (Smirnoff, 1993).

1.2.3. Regulatory Determinants of Salt Tolerance and Salt Stress Signal Transduction

The process by which plant cells sense the stress signals and transmit them to cellular machinery to activate adaptive response is referred to as signal transduction. A multitude of exogenous stimuli, like light, temperature, nutrient availability, needs to be perceived and processed simultaneously to achieve an integrated response ensuring optimal adaptation to the environment (McCarty and Chory, 2000). Despite decades of physiological and molecular effort, knowledge of how plants sense and transduce low temperature, drought and salinity signals is still very limited (Xiong and Zhu J.K., 2001).

Regulatory molecules are signal transduction pathway components that control the amount and timing of these effector molecules. Stress regulated genes that encode stress-tolerance effectors are listed in Table 1.3 and the models for stress tolerance are shown in Figure 1.2 and Figure 1.3.

Table 1.3 List of stress regulated genes (Hasegawa *et al.*, 2000).

YEAST	PLANTS					
	Sequences mined from databases with similarity to signal molecule genes	Expression is stress-controlled	Activates or interacts with other gene, gene product, or signal component	Suppresses or replaces yeast gene function	Causes phenotype in plants	Signal molecule category
SLN1 SHO1 ↓?	AtRR1-4	AtRR1 AtRR2		AtHK1		Receptor/sensor
↓ ↓ CNB / CaM ↓	AtCBL	AtCBL1 AtCP1		AtCBL1	SOS3 CNA/B	Ca^{+2} binding
Ydp1 ↓ Ssk1 ↓ Ssk2/22 → Pbs2 ↓ HOG1 ↓ ; Stell ↓ ; CNA ↓	ASK1 AtDsPTP1	MMK4 VP14 DBF2 AtMEKK1 AtMPK3 AtPK19 AtPLC1 AtCDPK2 SAMK ASK1 AtGSK1 14-3-3	AtCDPK ABI1 MP2C AtGSK1 VP14 14-3-3 AtDsPTP1 AtMPK4 AtMEKK1 ATMEKK2 MEK1	MP2C AtGSK3 PsMAPKMEK1 AtMEKK1 SAL1 DBF2 Nt/AtSLT1 HAL1 TPS1	DBF2 ABI1,2 HAL1 TPS1	(K) Kinase (P) Phosphatase (L) Lipase (R) regultor or biosynthesis of hormone/signal
Msn2 Msn4 ↓ ; Ssn1 Sko Tup1 ; Tcn1 ↓		Atmyb2 lip19 REB/CBF mlip15 Rd22BP1	Alfin1 DREB/CBF	**STO** **STZ**	Alfin1 DREB/CBF	Transcription factor
STRE	CDRE	ABRE	Myb Myc	DRE LTRE	mlipis GBOX	Cis elements

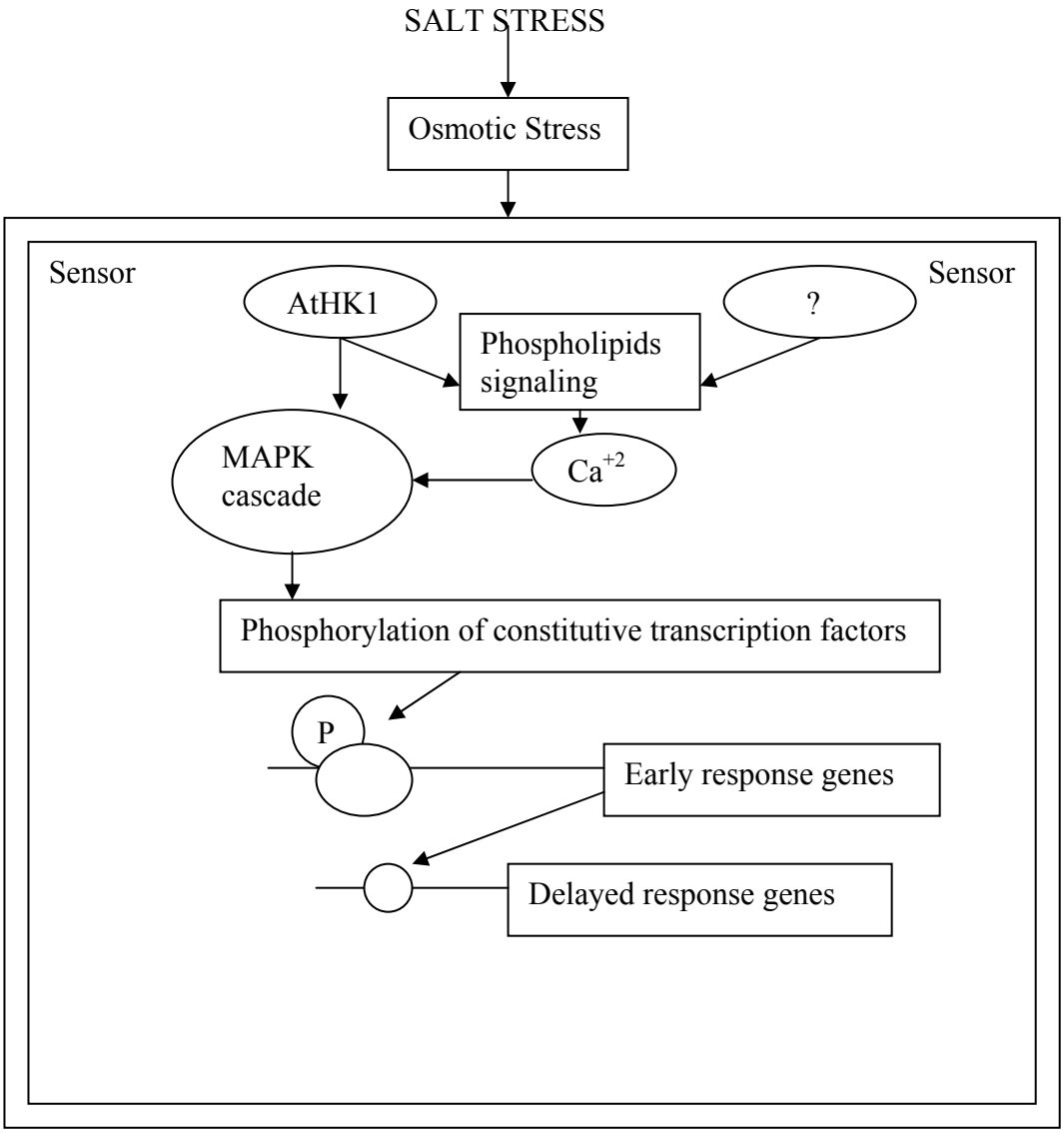


Figure 1.2 Model showing osmotic stress regulation of early-response and delayed-response genes (Zhu, 2002).

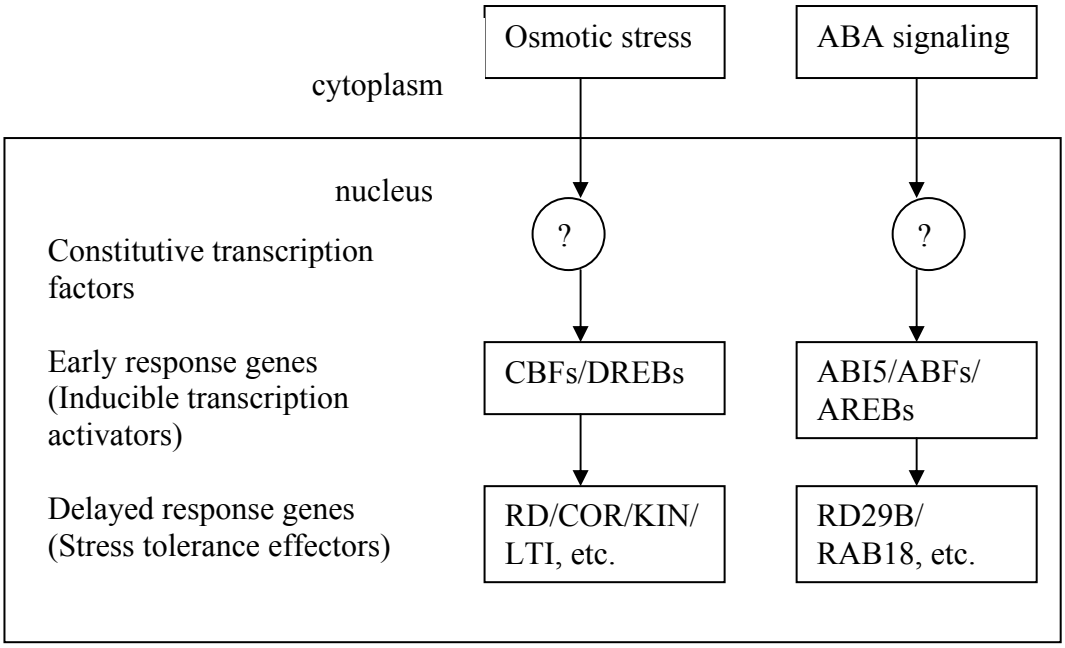


Figure 1.3 Model showing osmotic stress regulation of early-response and delayed-response genes (Zhu, 2002).

Signal transduction networks are essential to plant growth and development, allowing cells and tissues to perceive and respond to continuous changes in their environment. Multiple signaling pathways exist in plant cells, controlling important processes including hormone and light perception, pathogen defense, stress survival, cell division and circadian rhythms.

It is believed that stress-inducible genes may function in the signaling network or may play roles in stress tolerance. Environmental signals are thought to be first perceived by specific receptors that, upon activation, will initiate (or suppress) a cascade to transmit the signal intracellularly and in many cases, activate nuclear transcription factors to induce the expression of specific sets of genes.

Receptor-coupled protein phosphorylation is a common form of signal initiation. Although none of the receptors for cold, drought, salinity or the stress hormone abscisic acid in plants is determined to certainty; current knowledge indicates that receptor-like protein kinases, two component histidine kinases, as well as G-protein-associated receptors may represent the potential sensors of these signals.

Upon receiving a signal from membrane receptors, cells often utilize multiple phosphoprotein cascades to transduce and amplify the information. Protein phosphorylation and dephosphorylation are perhaps the most common intracellular signaling modes. They regulate a wide range of cellular molecules, protein localization and degradation. In plants many protein kinases and phosphatases are thought to be involved in environmental stress responses on the basis of pharmacological studies (Xiong and Zhu, 2001).

Salt stress signaling can be divided into three functional categories: ionic and osmotic stress signaling for the reestablishment of cellular homeostasis under stress conditions, detoxification signaling to control repair stress damages, and signaling to coordinate cell division and expansion to levels suitable for the particular stress conditions (Zhu, 2002). Homeostasis and detoxification signaling lead to stress tolerance and are expected to negatively regulate the growth inhibition response, i.e., to relieve growth inhibition (Figure 1.4).

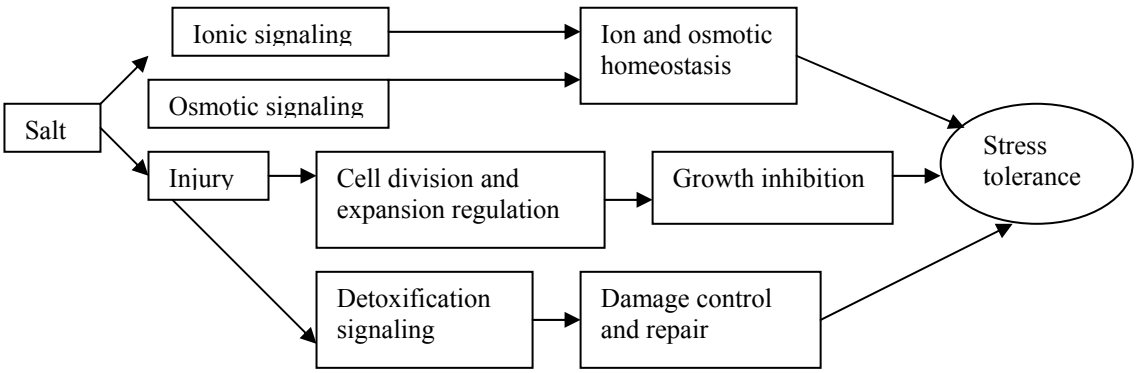


Figure 1.4. Functional demarcation of salt stress signaling pathway (Zhu, 2002)

1.2.3.1. Cross-talk Between Signaling Pathways

It has long been recognized that most aspects of plant growth and physiology are regulated by networks of signaling mechanisms rather than by linear signal transduction pathways, rendering plants particularly attractive for dissecting cross-talk and specificity mechanisms (Giraudat and Schroeder, 2001). The first step in switching on molecular responses to stress conditions is to perceive the stress as it occurs and to relay information about it through a signal transduction pathway. These pathways eventually lead to physiological changes, such as guard cell closure, or to the expression of genes and resultant modification of molecular and cellular processes (Knight and Knight, 2001).

During the past years, it has become increasingly clear that plant signaling systems do not only consist of linear pathways but rather form a complex signaling network with extensive overlaps and nodes of interconnecting its branches (Gilroy and Trewavas, 2001). One possible reason for this is that, under certain conditions, the two stresses cannot be distinguished from one another. Alternatively, each stress might require the same protective action (or at least some common elements).

In spite of considerable overlap between many abiotic stress signaling pathways, there might, in some instances, be a benefit to producing specific, inducible appropriate responses that result in a specific change suite to the particular stress conditions encountered. Specificity might occur at the point of initial stress perception itself. If specific stresses are actually sensed by dedicated receptor molecules, these molecules themselves have the potential to encode specificity of response.

1.2.3.2. ABA in Stress Signaling

Although ABA has broad functions in plant growth and development, its main function is to regulate plant water balance and osmotic stress tolerance. It also has regulatory roles in cell cycle and other cellular activities. The role of ABA in drought and salt stress is at least twofold: water balance and cellular dehydration tolerance. Whereas the role in water balance is mainly through guard cell regulation, the latter role has to do with induction of genes that encode dehydration tolerance proteins in nearly all cells (Zhu, 2002).

The role of ABA in abiotic stress signaling is not straightforward. Not all stress signaling pathways employ ABA and the intertwined relation of ABA and stress signaling is obscure. The studies up to now have suggested that ABA-dependent and ABA-independent osmotic and cold stress pathways might converge at several hitherto unexpected points. If they do, this increases opportunities for coordination between stress signals and ABA in the regulation of gene expression.

Osmotic stress induced ABA accumulation is a result of both activation of synthesis and inhibition of degradation. It is evident that ABA biosynthesis is subjected to osmotic stress regulation at multiple steps. To date, genes responsible for ABA degradation have not been identified. Nothing is known about the signaling between osmotic stress perception and the induction of ABA biosynthesis genes. Presumably, it involves calcium signaling and protein phosphorylation cascades (Zhu, 2002).

Analyses of the expression of dehydration-inducible genes in *Arabidopsis* have indicated that at least four independent signal pathways function in the induction of stress-inducible genes in response to dehydration (Shinozaki and Shinozaki-Yamaguchi, 1997). Two are abscisic acid (ABA)-dependent and two are ABA-independent (Figure 1.5).

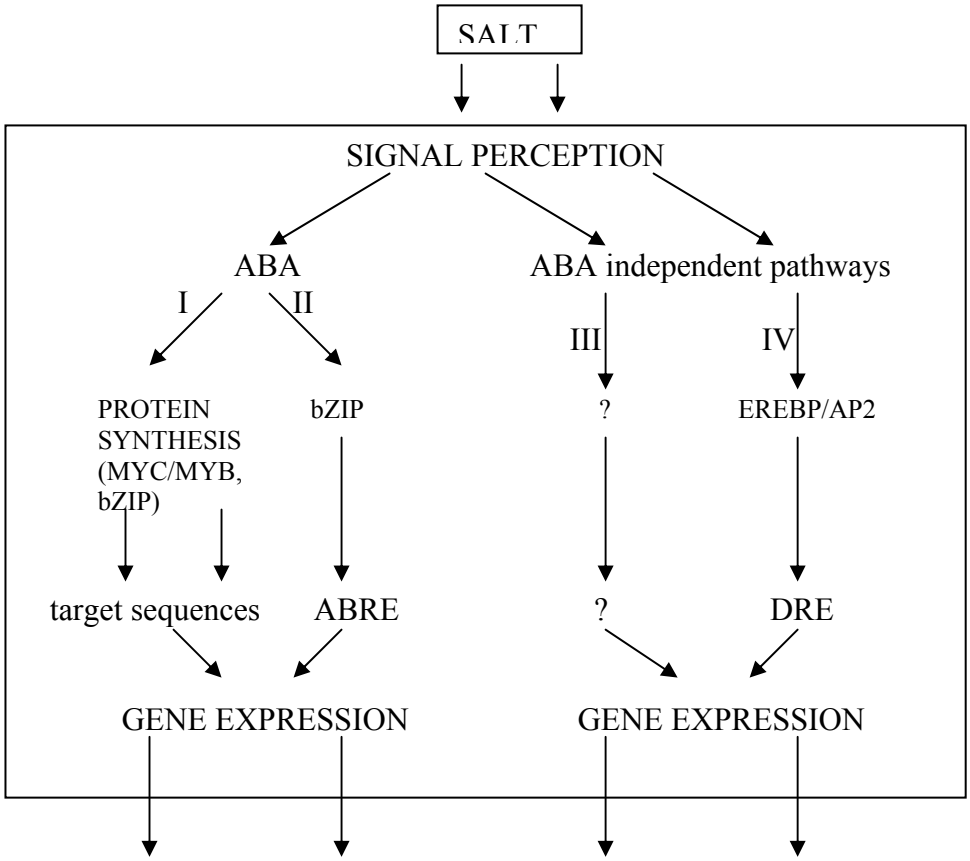


Figure 1.5. Signal transduction pathway between the perception of a salt stress signal and gene expression (Shinozaki and Yamaguchi-Shinozaki, 1997).

1.2.3.3. Phospholipids in Signal Transduction

Membrane phospholipids constitute a dynamic system that generates a multitude of signal molecules (e.g., IP_3, DAG, PA etc.) in addition to serving important structural roles during stress responses. Phospholipid based signaling is a double-edged sword: as signaling molecules are at low levels, the phospholipid messengers may activate downstream adaptive responses, whereas at high levels, phospholipid-generated products may reflect stress damage or may be damaging.

The phospholipase C (PLC) pathway has been the best characterized phospholipid signaling system. PLC catalyzes the hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP_2), generating the second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases Ca^{+2} from internal stores, whereas DAG activates protein kinase C (plants do not seem to have PKC genes). Increased Ca^{+2} concentration in cytoplasm triggers stomatal closure and lead to the expression of osmotic-stress responsive genes.

Phospholipase D cleaves membrane phospholipids to produce phosphatidic acid (PA) and free head groups. Osmotic stress activates PLD activity in suspension cells of *Chlamydomonas*, tomato and alfalfa (Zhu, 2002). In addition to PLC and PLD based lipid signaling, there are other lipid metabolizing enzymes that also respond to osmotic stress. PLA_2 cleaves phopholipids at the sn-2 position to generate lyso-phopholipids and free fatty acids. The role of PLA_2 in osmotic stress adaptation and whether it is activated in higher plants is currently unclear.

1.2.3.4. Yeast Models For Salt Stress Signal Transduction

A number of different organisms have been employed to identify processes and genes required for salt tolerance. These organisms vary from prokaryotic organisms such as *Eschericia coli*, unicellular eukaryotic organisms such as *Saccharomyces cerevisiae*, to halophytic land plants such as *Mesembryanthemum crystallinum*, and glycophytic plants such as rice, tomato or *Arabidopsis thaliana*.

Determinants of plant stress tolerance have been identified, in recent years, by functional complementation of osmotic, sensitive yeast mutants (Hasegawa *et al*., 2000b). Many of the transport determinants that mediate ion homeostasis in yeast and plants are very similar (Serrano *et al.,* 1999b). Furthermore, it is likely that the rudiments of salt responsive signal regulatory pathways controlling ion homeostasis in both organisms are analogous (Bressan *et al*., 1998). Therefore, it can be used as an excellent model system for the study of salt tolerance at cellular

level. Genetic analysis has been very successful in elucidating salt stress tolerance determinants in yeast (Toone and Jones, 1998, Serrano *et al*., 1999b). A number of salt-sensitive yeast mutants have been identified and the cloning of the corresponding genes has shed light on the nature of many genes that are essential for salt tolerance (Brewster *et al*., 1993, Mendoza *et al*., 1994). Because of the numerous similarities in signal transduction of yeast and plants, yeast can be used as a model to study and understand the mechanism of salt stress signaling in plants (Figure 1.6).

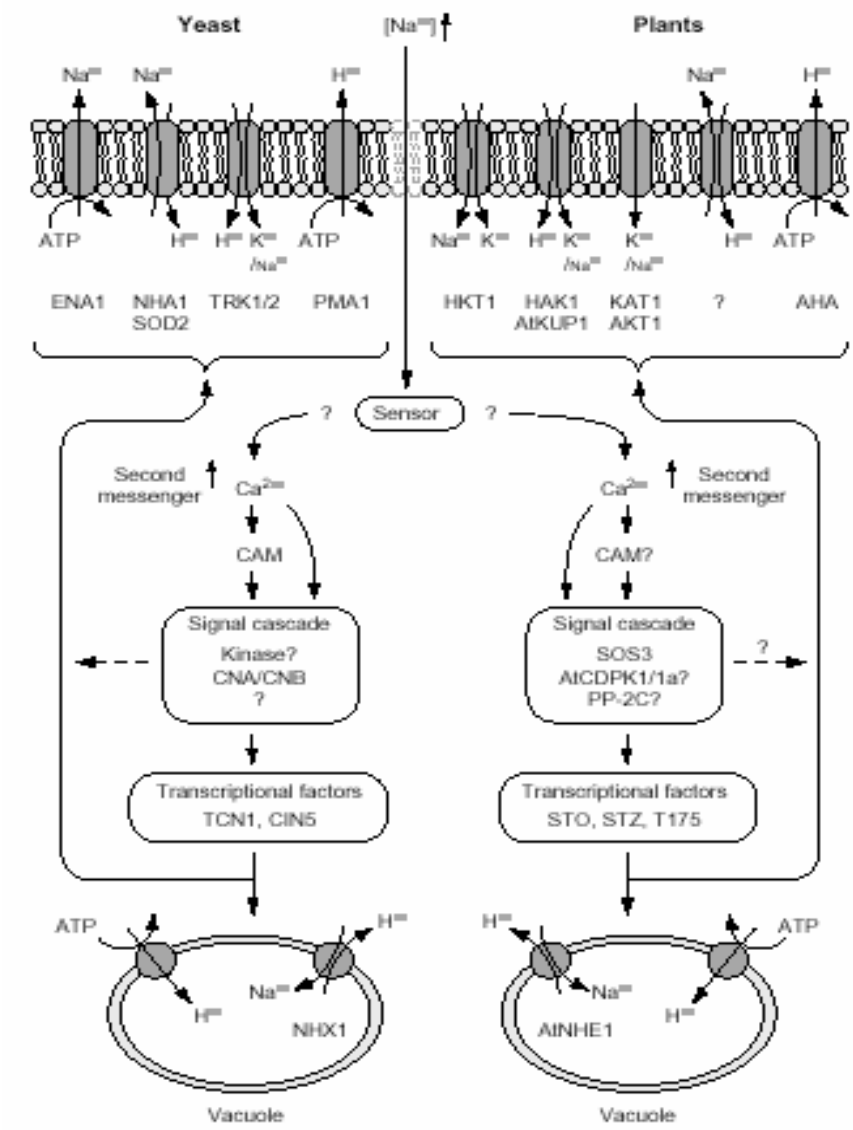


Figure 1.6 Signal transduction of yeast and plants (Bressan and Hasegawa, 1998).

1.3. Calcium

An early event in the response to many different environmental stresses is an elevation in cytosolic Ca^{+2} concentration, which is thought to be the primary stimulus-sensing event for several stresses (Sanders *et al*., 1999, Knight, 2000). No single messenger has been demonstrated to respond to more stimuli than has cytosolic free Ca^{+2}.

Although the underlying mechanism has remained largely unexplained, prevailing models for Ca^{+2} function include both membrane stabilization and signaling roles. The role of Ca^{+2} as a second messenger in many biological systems, coupled with these observations, indicates that plants are able to adjust to high salt environments by activating a signal transduction system involving Ca^{+2} (Bressan *et al*., 1998). The calcium ion acts as an intracellular second messenger in a variety of signaling pathways. In plants stimulus-induced oscillations in cytosolic free calcium encode information that is used to specify the outcome of the final response (Giraudat and Schroeder, 2001). The precise kinetics, magnitude and cellular source of stimulus-induced cytoplasmic Ca^{+2} concentration elevations have been proposed to encode information about the particular stimulus, to determine the specific end response elicited (McAinsh and Hetherington, 1998). The Ca^{+2} signal is ubiquitous in abiotic stress signaling and it is therefore an important node at which cross-talk can occur.

Cytosolic Ca^{+2} signals can be thought of as the result of two opposing functions; influx into and efflux out of the cytosol. The increase of cytoplasmic free Ca^{+2} that is induced by ABA, is an event regulated by cyclic ADP-ribose (Wu *et al*., 1997). Upon stimulation Ca^{+2} is released from intracellular storage or enters the cell via various Ca^{+2} channels (Xiong and Zhu, 2001). One mechanism of Ca^{+2} increase seems to involve inositol-1,4,5- triphosphate production and Ca^{+2} release from the vacuolar compartment (Knight *et al*., 1997, Drobak and Watkins, 2000).

Another mechanism is based on the activation of Ca^{+2} channels in the plasma membrane. Nothing is known about the mechanism of osmotic induction of these pathways, although G-proteins and phospholipase C are likely to be the mediators (Blatt, 2000).

1.3.1. Calcium Regulated Proteins

Transient changes in calcium concentration can encode information on its own, and also additional level of regulation in calcium signaling is achieved via the action of calcium-binding proteins (Allen and Schroeder, 2001, McAinsh and Hetherington, 1998, Sanders *et al*., 2002). Cytosolic Ca^{+2} concentration elevations achieve control of various processes via Ca^{+2} regulated effector proteins. These proteins include calmodulin, calcium-dependent protein kinases (CDPKs) and calcium-regulated phosphatases. The Ca^{+2} signal is perceived by protein kinases containing either a Ca^{+2} binding calmodulin like domain (CDPKs) or as in the case of SOS2, a separate Ca^{+2} binding subunit of the SOS3 type. (Harmon *et al*., 2000, Liu *et al*., 2000, Halfter *et al*., 2000, Kudla *et al*., 1999, Shi *et al*., 1999).

1.3.2. Calcineurin

Calcineurin is a Ca^{+2}- and calmodulin-dependent protein phosphatase consisting of a catalytic A subunit (CnA; 60 kDa) and a regulatory B subunit (CnB; 19 kDa). CnB has four high affinity EF-hand calcium binding sites and full activation of CnA requires calcium-CnB and calcium-calmodulin dependent complexes (Klee *et al*., 1988). Calcineurin regulates Na^{+}, K^{+} and Ca^{+2} homeostasis. Mutations in the catalytic and/or the regulatory subunit of the phosphatase calcineurin render yeast strains hypersensitive to Na^{+} and Li^{+} (Nakamura *et al*., 1993, Mendoza *et al*., 1994).

Calcineurin is required for the transcriptional induction of genes encoding Na^+ and Ca^{+2}-ATPases and a cell wall β-1,3, glucan synthase (Matheos *et al*., 1997). A downstream zinc-finger transcription factor, CRZ1/TCN1, participates in the transcriptional induction of these genes.

Calcineurin, first has been identified as a modulator of salt tolerance in yeast, because mutants lacking this phosphatase have increased sensitivity to Na^+ ions (Breuder *et al*., 1994, Mendoza *et al*., 1994, Nakamura *et al*., 1993). Calcineurin null mutants of yeast are sensitive to Na^+ and Li^+ ions, indicating that calcineurin is an essential component in the pathway regulating tolerance to these cations. One of the calcineurin null mutants (*cnb1* mutants) accumulate abnormally high levels of Li^+ ions due to the reduced expression of *ENA1/PMR2*, which codes a P-type ATPase involved in Na^+ and Li^+ efflux in yeast. And a failure of the K^+ uptake system encoded by *TRK1* to convert to the high affinity state of K^+ transport. These results suggest that calcineurin mediate NaCl tolerance in part by regulating expression of *ENA1* and activity of ion transporters (Haro *et al*., 1991, Rudolph *et al*., 1989, Gaber *et al*., 1988).

Calcineurin has been identified in a variety of eukaryotic organisms from yeast to mammals and the presence of a calcineurin-like activity has been implicated in plants.

1.3.3. Calcium Dependent Protein Kinases (CDPKs)

A family of protein kinases unique to plants, the calcium-dependent protein kinases (CDPKs) are also involved in stress response. In the case of CDPKs, which harbor a calcium-binding domain (Sanders *et al*., 2002) the calcium signal can be sensed and transmitted by a single protein.

Functional analyses of plant CDPKs has recently provided strong evidence for a crucial function of these kinases in processes like hormone and stress signaling as well as plant pathogen response (Harmon *et al*., 2000, Romeis *et al*.,

2001). Because osmotic stress elicits calcium signaling, calcium-dependent protein kinases are prime candidates that link the calcium signal to downstream responses.

1.3.4. Calmodulin

Calmodulin has been implicated in plant response to cold, mechanical stimulation and oxidative stress (Braam and Davis, 1990, Botella and Arteca, 1994, Harding, 1997). The use of different isoforms could be involved in control of specificity to biotic stresses. The transient increase in cytoplasmic Ca^{+2} concentration activates the calcineurin leading to the transcription of ENA1, which encodes the P-type ATPase that is primarily responsible for Na^{+} efflux across the plasma membrane. Hyperosmotically-induced cytoplasmic Ca^{+2} transient activates calmodulin. Calmodulin in turn activates signaling through the calcineurin pathway, which mediates ion homeostasis and salt tolerance (Yokoi *et al*., 2002).

1.3.5. SOS Signaling Pathway

A regulatory pathway of *Arabidopsis* for ionic homeostasis under salt stress was discovered through the cloning of the salt overly sensitive (SOS) genes. Genetic dissection of salt tolerance in *Arabidopsis* established the involvement of the SOS pathway in the response to the ionic aspect of the salt stress. This novel protein kinase pathway is activated by calcium signaling and regulates ion transporters, which bring about ion homeostasis. Biochemical studies identified several protein kinases that are activated by the osmotic aspect of salt and water stress (Zhu, 2001b).

Like the calcineurin pathway in yeast, calcium has been proposed as a second messenger for the SOS pathway (Zhu, 2000). The SOS signaling pathway functionally resembles the yeast calcineurin cascade that controls Na^+ influx and efflux across the plasma membrane (Bressan *et al*., 1998). Mutations in the SOS genes render *Arabidopsis* plants more sensitive to Na^+ stress. The pathway begins with SOS3, a myristoylated protein with three EF hands for calcium binding (Liu and Zhu, 1998, Ishitani *et al*., 2000). The pathway begins with SOS3, a myristoylated protein with three EF hands for calcium binding. These proteins share high sequence identities with the B-subunit of calcineurin. SOS2 represents a novel family of proteins (PKS) so far only found in plants. SOS2 contains an SNF-1-like catalytical domain and a unique regulatory domain that interacts with SOS3. Upon stimulation SOS3 interacts physically with SOS2 (Liu *et al*., 2000, Halfter *et al*., 2000). One downstream target of SOS3-SOS2 kinase complex is SOS1, which is a plasma membrane Na^+-H^+ antiporter that exports Na^+ from the cell (Shi *et al*., 2000). SOS1 has a very long tail that is predicted to be on cytoplasmic side. Membrane transporters with long cytolasmic tails have been proposed to function as sensor of the solutes they transport. SOS3 and SOS2 regulate Na^+ influx as well as vacuolar compartmentation systems because these systems are also vital for salt tolerance (Zhu, 2002).

1.3.6. MAPK Cascades

Mitogen activated protein kinase (MAPK) cascades are evolutionary conserved signaling modules with essential regulatory functions in plants as in other eukaryotes (Giraudat and Schroeder, 2001). MAPKs are promiscuous serine/threonine kinases that phosphorylate a variety of substrates, including transcription factors, protein kinases and cytoskeletal proteins (Jonak *et al*., 2002).

The MAP kinase pathways are intracellular signal modules that mediate signal transduction from the cell surface to the nucleus. They seem to be widely

used as osmolarity signaling modules. The core MAPK cascades consist of 3 kinases that are activated sequentially by an upstream kinase. The MAP kinase kinase kinase (MAPKKK) upon activation, phosphorylates a MAP kinase kinase (MAPKK) on serine and threonine residues. This dual-specificity MAPKK in turn phosphorylates a MAP kinase (MAPK) on conserved tyrosine and threonine residues. The activated MAPK can then either migrate to the nucleus to activate transcription factor directly, or activate additional signal components to regulate gene expression, cytoskeleton-associated proteins or enzyme activities, or target certain signal proteins for degradation (Xiong and Zhu, 2001).

In plants, MAPK cascades have been shown to participate in auxin and cytokinin signal transduction and cell-cycle regulation and are implicated in wound and pathogenesis responses as well as in environmental stress signal transduction.

Transcript levels for a number of protein kinases including a two-component histidine kinase; MAPKKK, MAPKK and MAPK increase in response to osmotic and other stress treatments. It is unclear whether the protein or, more importantly, the activity levels of these kinases change upon osmotic stress treatment. The input signals for these kinases can be osmotic stress (e.g., turgor changes) or derived from osmotic stress injury. The output can be osmolyte accumulation that helps establishment of osmotic homeostasis, stress damage potection, or repair mechanisms (e.g., induction of LEA/dehydrin-type stress genes).

1.4. Transcription Factors

The biology of plants is in many aspects common to that of other organisms: there are, however, a number of biological processes that are unique to plants, e.g. photosynthesis, the reproductive process, development and responses to environmental signals. In the past decade, hundreds of transcription factors have

been identified in plants that are involved in the regulation of many biological processes. Their protein structures suggest that plants have in some cases adopted preexisting prototype functional motifs and modified them for specific regulatory processes. Indeed, most of the functional motifs present in eukaryotic transcription factors have their counterparts in the plant kingdom. In the other cases, plants seem to have evolved new classes of functional motifs that are not present in other organisms. The modified and new functional motifs are considered to have coevolved with regulatory processes that are unique to plants (Takatsuji, 1998). In general, transcription factors have modular structures composed of a few functional domains for binding to target DNAs, for interaction with other proteins including other transcription factors and components of basic transcriptional machinery, and for other functions.

Transcriptional modulation has always been predicted to play a major role in the control of plant responses to salt stress. Transcription factors have been identified based on interaction with promoters of osmotic/salt stress-responsive genes. These factors participate in the activation of stress-inducible genes, and presumably lead to osmotic adaptation. Since the promoters that are controlled by these transcription factors are responsive to several environmental signals, it is not clear which transcription factors, if any, function only in salt stress responses, or if salt-specific transcriptional regulation alone is a requisite component of salt tolerance *in planta* (Liu *et al*., 1998, Sheen, 1996, Shinozaki and Yamaguchi-Shinozaki, 1996, Winicov and Bastola, 1999, Zhu *et al*., 1997).

Promoters of ABA-dependent osmotic stress-responsive genes include regulatory elements that interact with basic leucine-zipper motif (bZIP), MYB, or MYC domains in DNA binding proteins (Abe *et al*., 1997, Shen and Ho, 1995, Shinozaki and Yamaguchi-Shinozaki, 1997). Transcription factors thought to function in osmotic/salt stress gene induction, independent of ABA, include dehydration response element (DRE) binding proteins. Two gene families have been characterized, DREB1 and DREB2. Both DREB1 and DREB2 family members also have domains that bind ethylene-responsive elements (Stockinger *et al*., 1997).

Using genetic approaches (gene tagging and map-based cloning) several transcription favors have been identified that are involved in various important phenomena. In many cases, their actions at the molecular level (including target genes of respective factors) are poorly understood at present.

In the activation of abiotic stress-responsive genes in plants, it seems that there is not a general rule regarding which class of transcriptional factors activate which class of stress-responsive genes. Instead, there could be several kinds of transcriptional factors regulating one group of stress-responsive genes, or even several transcriptional factors that can cooperatively activate the same gene (Xiong and Zhu, 2001).

1.4.1. Zinc Finger Transcription Factors

Zinc finger transcription factors are among the most common transcription factors and it is estimated that *Arabidopsis* contain 85 genes that encode zinc finger transcription factors (Riechmann and Ratcliffe, 2000).

The term “zinc-finger” represents the sequence motifs in which cysteines and/or histidines coordinate a zinc atom(s) to form local peptide structures that are required for their specific functions. The zinc-finger motifs, which are classified based on the arrangement of the zinc-binding amino acids, are present in a number of transcription factors and play critical roles in interactions with other molecules. Some classes of zinc-finger motifs (e.g. TFIIIA- and GATA types) are, in most cases, parts of DNA-binding domains of transcription factors and have been shown to be directly involved in the recognition of specific DNA increases sequences. Other classes (e.g. LIM- and RING-finger types) are mostly implicated in protein-protein interactions (Takatsuji, 1998).

1.4.1.1. TFIIIA Family

In a TFIIIA-type zinc finger, two cysteines and two histidines in a conserved sequence motif ($CX_{2-4}FX_5LX_2HX_{3-5}H$) tetrahedrally coordinate a zinc atom to form a compact structure that interacts with the major groove of DNA in a sequence specific manner (Miller *et al*., 1985, Pavletich and Pabo, 1991). All TFIIIA-type zinc-finger proteins contain QALGGH sequence in zinc-finger motifs. Interestingly, this conserved sequence motif has not been reported from organisms other than plants, suggesting that this type of zinc-finger protein, which forms a major class of transcription factors in plants, might be involved in controlling the processes that are unique to plants. The only TFIIIA-type zinc-finger protein without the QALGGH sequence is PCP1 in potato (Kuhn and Frommer, 1995).

1.4.1.2. WRKY Family

A new type of Cys_2/His_2-type zinc finger (CX_4-$CX_{22-23}HX_1H$) unique to plants has been found within the conserved regions of the WRKY family of proteins. Three members of this protein family (WRKY1, 2 and 3) in parsley are implicated in the activation of *PR1* (pathogen-related 1) gene in response to fungal infection. Sequence comparisons of the proteins belonging to this family revealed that a conserved sequence (the WRKY domain) is extended to the N-terminal region of the zinc-finger motif, and there is a stretch of seven invariant amino acids (WRKYGQK) in this region. The WRKY proteins contain either one or two WRKY domains (Takatsuji, 1998).

1.4.1.3. GATA1 Family

The GATA1 family forms one of the major families of Cys_2/Cys_2-type zinc-finger transcription factors in eukaryotes. The DNA-binding domain consensus is $CX_2CX_{17}CX_2C$ and contains a zinc atom coordinated by the conserved four cysteines (Omichinski *et al*., 1993).

The gene for the first GATA1–type protein in plants (NTL1) was isolated from tobacco by polymerase chain reaction (PCR)-based methods as a plant homologous of *NIT2*, a well characterized transcription factor in the fungus *Neurospora crassa* (Daniel-Vedele and Caboche, 1993, Feng *et al*., 1993).

The CONSTANS protein that promote flowering in *Arabidopsis* has two repeats of GATA1 like zinc fingers (Putterill *et al*., 1995). The two zinc fingers of the CONSTANS protein are in $CX_2CX_{16}CX_2C$ arrangement, and the zinc-finger sequences show low but significant similarity to those in the GATA1 family. The two zinc-fingers are very similar to each other, with the most apparent conservation being in the C-terminal extension of each finger that is rich in basic amino acids. The high conservation of the basic C-terminal region is again reminiscent of GATA1, in that this region is a basic domain required for DNA binding and is highly conserved (Omichinski *et al*., 1993, Ramain *et al*, 1993).

1.4.1.4. The Dof Family

The Dof (DNA binding with one finger) family has been identified as sequence specific DNA-binding proteins that interact with the promoter sequences of several genes, whose expression is regulated tissue-specifically or in response to stress signals. The proteins of the Dof family are characterized by the presence of a conserved domain (Dof domain) including a zinc-finger-like motif, $CX_2CX_{21}CX_2C$, followed by a basic region. Dof domain like sequences are

neither present in the yeast genome nor they have been found in animals. Although it has been only a few years since this protein family was identified, several members have been implicated in the transcriptional regulation of various biological processes, including developmental and stress induced gene expression (Takatsuji, 1998).

1.4.1.5. RING-finger Family

The RING finger is an asymmetric motif, C-X_2-C- loop I-C-X-H-X_2-C-X_2-C-X_2-C-loop II-C-X-C (loops I and II are variable in length), which has been found in many regulatory proteins throughout the plant, animal, fungal, viral and protozoan kingdoms. Many RING-finger-containing proteins have putative involvement in some aspects of transcriptional regulation (Freemont, 1993, Satijn *et al*., 1997). In these proteins, the RING finger domain is considered more likely to mediate protein-protein interactions, but their direct involvement in DNA binding has not been ruled out (Borden *et al*., 1995). In plants, COP1 is the best-characterized regulatory protein containing RING-finger motif. The mutant phenotypes and several lines of evidences from transgenic studies strongly suggest that COP1 acts as a light-inactivetable repressor of photomorphogenesis development in light specific signaling pathways (McNellis *et al*., 1996, McNellis *et al*., 1994). COP1 was also found to be involved in the regulation of the genes inducible by pathogen infection, hypoxia and developmental programs (Mayer *et al*., 1996). Therefore, COP1 might act as a repressor of various kinds of gene expression that are targets for multiple signal transduction pathways and thus might play a general role as a nuclear regulatory protein.

1.4.1.6. PHD-finger Family

The PHD finger (Cys_4-His-Cys_3) is similar to the RING finger (Cys_3-His-Cys_4) in the arrangement of putative zinc-binding amino acids. The PHD finger (plant homeodomain finger) is so called because this sequence motif was originally noted in two plant proteins containing homeodomains, *Arabidopsis* HAT3.1 and maize Zmhox1a (Schindler *et al*., 1993, Bellmann and Werr, 1992). The involvement of PHD fingers in DNA binding is unclear.

1.4.1.7. The LIM family

The LIM domain contains a cysteine-rich motif of $CX_2CX_{17-19}HX_2CX_2CX_2CX_{16-20}CX_{2-3}$. Many LIM-containing proteins have been implicated in the transcriptional regulation of cell differentiation and growth regulation. Some are associated with cytoskeleton, while others are implicated in chromosome translocation. Evidences so far suggest that the LIM domain serves as the site for protein-protein interactions with itself (homodimerization), with helix-loop-helix type transcription factors or with protein kinases (Feuerstein *et al*., 1994, Kong *et al*., 1997, Johnson *et al*., 1997, Kuroda *et al*., 1996).

1.4.2. STO and STZ

In 1996, Lippuner's research group took the advantage of the salt-sensitive phenotype of yeast calcineurin mutants and the availability of an *Arabidopsis* cDNA library constructed in a yeast-*Escherichia coli* shuttle vector to develop a screen for identification of *Arabidopsis* cDNA clones that confer increased salt tolerance on yeast. This procedure was successful in identifying two genes, *sto* (salt tolerance) and *stz* (salt tolerance zinc finger), that complement the Na^+ and

Li^+ hypersensitive phenotypes of yeast calcineurin mutants (*cna1-2*△ or *cnb*△). STO and STZ have no sequence similarity to calcineurin and therefore are unlikely to be directly replacing the phosphatase activity of calcineurin in this salt tolerance cascade. STO complements all tested calcineurin-related phenotypes in calcineurin-deficient yeast, whereas STZ only reduces the Na^+ and Li^+ sensitivity of these mutant cells. Experiments with the yeast mutants indicated that STO and STZ provide a growth advantage to wild type yeast only in the presence of salt.

ENA1 encodes the major sodium and lithium efflux system in yeast and is the first repeat of a tandem array of four genes encoding almost identical proteins (Haro *et al*., 1991, Rudolph *et al*., 1989, Garciadeblas *et al*., 1993). STZ is at least partially dependent on ENA gene products for manifestation of salt tolerance, however ENA activity is not essential for STO function.

STO is similar to *Arabidopsis* CONSTANS and is predicted to be a member of a multigene family. STO shares considerable similarity with CONSTANS protein in its zinc-finger sequence. The STO clones encode a 27.6 kDa hydrophilic protein of 249 amino acids with a calculated pI of 5.4. *Arabidopsis* CONSTANS (CO) protein showed the greatest similarity to STO (Putterill *et al*., 1995). Similarity was confined to two regions, which have been hypothesized to represent zinc fingers. Near the C terminus of STO is a highly basic region followed by acidic amino acid residues. CO also contains a relatively basic region near the C terminus, but there is no sequence similarity between the two proteins in these regions. Yeast calcineurin mutants producing STO had increased colony size on salt-containing medium relative to an isogenic wild-type strain. Expression of STO in calcineurin mutants produced phenotypes similar to those resulting from expression of active calcineurin, suggesting that STO might modulate a calcineurin-dependent pathway in yeast.

According to Southern Blot analysis a single *sto* gene is present in *Arabidopsis*. The results of the Northern Blot analysis with control and NaCl treated *Arabidopsis* plants showed that the steady state of *sto* mRNA was unchanged in plants treated with increasing NaCl concentrations (Lippuner *et al*., 1996).

Arabidopsis contains an additional *sto*-like gene and rice contains at least two *sto*-like genes. *Sto* was expressed at the highest levels in leaves but also expressed in roots and flowers. STO might represent a component of the salt tolerance mechanism that is conserved in yeast and plants (Takatsuji, 1998).

Holm's research group showed that STO interacts with the WD40 domain of COP1. COP1 can interact with STO, a putative transcription factor able to modulate Ca^{+2} signaling in yeast, since it suggests a direct link between a genetically identified repressor of light signals and a downstream target of Ca^{+2} signals. If the COP1-STO interaction results in STO degradation, it would provide a mechanism by which COP1 could modulate Ca^{+2}-dependent transcription (Holm *et al*., 2001).

STZ in *Arabidopsis* belong to TFIII-A type zinc-finger protein family. STZ in *Arabidopsis* complements the salt-sensitive phenotype of a yeast mutant, which is deficient in the phosphoprotein phosphatase calcineurin (Lippuner *et al*., 1996). The expression of the *stz* gene increases with salt treatment in plants. These observations suggest the role of STZ in the regulatory processes associated with salt tolerance in plants.

STZ consists of 228 amino acids with a calculated molecular mass of 24.6 kDa and an estimated pI of 8.3.STZ is 37-68% identical in amino acid sequence to a family of petunia DNA-binding Cys_2/His_2-type zinc finger proteins associated with flowers (termed EPF) being most similar to EPF2-7 (68%) (Takatsuji *et al*., 1994, Takatsuji *et al*., 1992). STZ also shows 47% amino acid identity with WZF1, a wheat zinc finger DNA-binding protein that is primarily expressed in the root apex (Sakamato *et al*., 1993).

STZ, like WZF1 and members of the EPF family, contains two Cys_2/His_2 zinc finger motifs. As is characteristic for zinc fingers in this family, the Cys-Xaa_2-Cys and His-Xaa_3-His regions in each zinc finger in STZ are separated by 12 residues including invariant hydrophobic residues, Phe and Leu (Berg, 1990). Both zinc fingers in STZ and in other plant members of this family contain 6 conserved consecutive residues (QALGGH), which appear to contact DNA

(Pavletich and Pabo, 1991, Jacobs, 1992). The region between the two zinc fingers ranges from 36 to 61 amino acid residues in these plant protein and is considerably larger than the 7-8 residues observed in many zinc finger proteins reported in yeast and animals (Sakamato *et al*., 1993, Evans and Hollenberg, 1988). The 35 residue separation between the two zinc fingers of STZ is similar to that of the petunia proteins. The basic B-Box, hypothesized to be a nuclear localization signal, is present in STZ and in all Cys_2/His_2 zinc finger proteins.

STZ belongs to a family of DNA-binding Cys_2/His_2 zinc finger proteins, which have thus far only been identified in plants, suggesting that STZ is also a DNA-binding protein. Thus, it is possible that STZ may act to directly regulate transcription and could represent the first eukaryotic transcription factor identified to play a role in salt tolerance. The high expression of *stz* and *stz* homolog in *Arabidopsis* roots, the organ through which all ions enter the plant, and the induction of these genes by NaCl support the hypothesis that STZ controls steps in Na^+ ion balance in plants.

According to Southern Blot analysis *stz* was observed to be having more than one gene consistent with the hypothesis that *stz* is a member of a multi-gene family. The *stz* gene expression responded differentially at any given salt concentration according to Northern Blot analysis (Lippuner *et al*., 1996).

The observation that STZ exhibits a phenotype only in response to cations suggests that STZ is involved in ion adaptation but does not eliminate the possibility that this gene product is regulating a subset of calcineurin-mediated pathways. STZ compensates for some, but not all, effects of calcineurin deficiency.

Expression of *sto* and *stz* not only increased salt tolerance of calcineurin mutants but also of wild-type yeast. Because steps in the salt stress response and salt-sensitive components in metabolic pathways appear to be conserved between yeast and plants, overexpression of *sto* and *stz* has the potential to be another method of increasing salt tolerance in plants (Lippuner *et al*., 1996)

1.5. Engineering of Plants Against Salt Stress

Classic genetic studies have demonstrated that the ability of plants to tolerate salt stress is a quantitative trait involving the action of many genes. As a result, it has been difficult to obtain salt tolerance in crop plants by traditional methods (Foolad and Lin, 1997). Improving salt tolerance in crop plants is limited by our poor understanding of the nature of the pathways that allow plants to adapt to salt stress. Identification of genes associated with an increase in salt tolerance would be a first step in engineering halotolerance.

Molecular genetic and plant transformation advances have made it feasible to asses biotechnological strategies based on activated signal cascades, engineered biosynthetic pathways, targeted gene or protein expression or alteration of the natural stress responsiveness of genes for development of salt tolerant crops (Hasegawa *et al*., 2000b, Zhu, 2001a). The strategy summarizing the development of stress resistant plants is shown in Figure1.7.

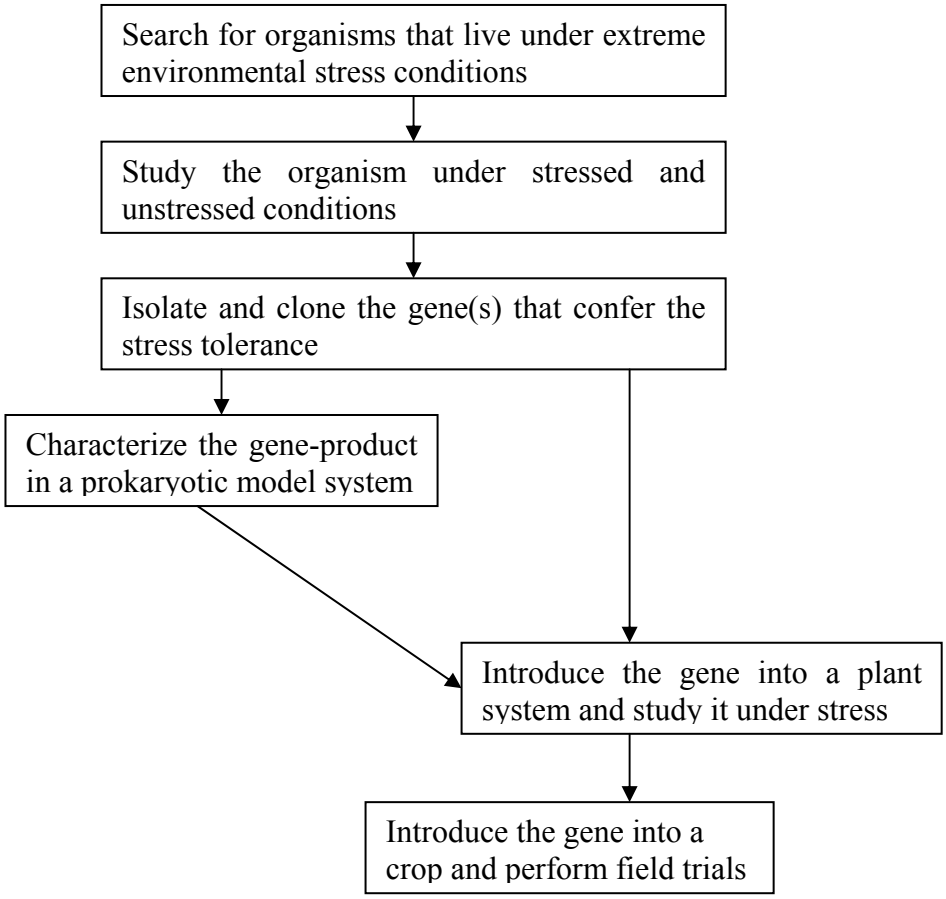


Figure 1.7 Strategy for creating a more stress-tolerant plant using genetic engineering (Holmberg and Bülow, 1998).

Phenotypic changes such as increases in salt tolerance caused by ectopic overexpression of regulatory genes in plants are also important for crop improvement, but in many cases may not be able to address the question of necessity, i.e. whether a regulatory protein is necessary for salt tolerance in plants. In one scenario, a gene of a multigene family may be sufficient to confer a stress-tolerance phenotype when overexpressed in plants but may not be adequate because other family members can substitute for its function owing to functional redundancies. In another scenario, a gene that normally does not function in salt stress responses may confer a stress-tolerance phenotype when ectopically expressed because signaling specificity can be lost during ectopic overexpression.

Progress might also be made by combining different strategies that are individually effective in enhancing stress tolerance. Targets for this approach include genes whose products are involved in the biosynthesis of compatible solutes; stress-induced genes, such as the Cold-Regulated (COR) and Late Embryogenesis-Abundant (LEA); and genes for regulatory proteins, such as stress-inducible transcription factors (Jaglo-Ottosen *et al*., 1998).

Numerous reports in the literature have shown improvement of salt tolerance via genetic engineering. The metabolic pathways and mechanisms modified to improve the salt tolerance can be grouped according to the genes employed in transgenic experiments. These genes can be classified into five groups according to their functions:

-Synthesis of osmolytes

-Protection of cell integrity

-Oxidative stress

-Ion homeostasis

-Transcription factors

The first phase of genetic engineering of stress hardiness has been to simply express one or several tolerance effector genes under constitutive or stress inducible promoters. Constitutive overexpression of transgenes in plants does not generally seem to compromise plant growth, suggesting that energy is not limiting for plant growth under normal conditions (Zhu, 2001). By contrast, the constitutive expression of several stress-related genes, including *CBF1, DREB1A, ATHB7* and yeast trehalose synthase, has been shown to cause slow growth of transgenic plants (Liu *et al*., 1998, Soderman, 2000, Holmström *et al*., 1996).

The second phase is to improve stress tolerance through engineering more effective signaling. The future promises to see a much clearer picture of salt and drought signal transduction pathways and more examples of genetic improvement of water stress tolerance by fine-tuning plant sensing and signaling systems (Zhu, 2002).

Genetic engineering of salt tolerance via different strategies has shown promising results. An important question that arises is the relative importance of the different strategies. Various transgenic plants should be compared in the same laboratories under identical test conditions to identify the most useful genes. This would be important not only for field applications but also for addressing a fundamental question: is detoxification or homeostasis more important for tolerance? Table 1.4 summarizes the transgenic plants developed to cope with salt stress and Table 1.5 lists the problems that can be faced during the development of transgenic plants.

Table 1.4 Salt tolerance of transgenic plants expressing several genes.

Source Species	**Gene Product/Name**	**Function**	**Target Species**	**Developmental Stage/ Location**	**Parameters studied**	**Reference**
E.coli	Choline dehydrogenase/*betA*	Betaine synthesis	Tobacco	Seedling	Dry weight	Lilius *et al*. (1996)
E.coli *S. oleracea*	Betaine dehydrogenase/ *BADH*	Betaine synthesis	Tobacco	Seedling	Increase of biomass protection of photosynthetic apparatus	Holmstrom *et al*. (2000) Liang *et al*. (1997)
A. globiformis	Choline oxidase/ *CodA*	Betaine synthesis	*Arabidopsis* Rice	Seedling Germination	Improve growth and photosynthetic activity	Hayashi *et al*. (1997) Sakamato *et al*. (1998)
A. pascens	Choline oxidase/ *COX*	Betaine synthesis	Arabidopsis Tobacco *Brassica napus*	Seedling	Improve shoot growth	Huang *et al*. (2000)
A. polyrriza	Inositol synthase/ *TUR1*	$InsP_3$ synthesis	*Arabidopsis*	Seedling	Slight allevation of NaCl stress	Smart and Flores (1997)
M. crystalinum	Myo-inositol*O*-metyltransferas/ *IMT1*	D-ononitol synthesis	Tobacco	Seedling	Improve photosynthetic activity	Sheveleva *et al*. (1997)
E.coli	Mannitol 1-phosphate dehydrogenase/ *MtlD*	Mannitol synthesis	*Arabidopsis* Tobacco	Seedling Germination	Germination and growth	Tarczynski *et al*. (1993) Thomas *et al*. (1995)
V. aconitifolia	Pyrroline-5-carboxylate/ *P5CS*	Proline synthesis	Tobacco Rice	Adult plant	Biomass and flower	Kavi Kishor *et al*. (1995)
A. thaliana	Proline dehydrogenase/ *ProDH*	Proline degradation	*Arabidopsis*	Adult plant	Turgency mantainement	Nanjo *et al*. (1999)
H. vulgare	LEA protein/ *HVA1*	Protein protection	Rice	Seedling	Growth	Xu *et al*. (1996)
N. tabacum	Glutathione-S-transferase/ glutathione peroxidase/ *Nt107*	GSSG synthesis	Tobacco	Seedling	Shoot length	Roxas *et al*. (1997)

Table 1.4 Salt tolerance of transgenic plants expressing several genes (continued).

Source Species	**Gene Product**	**Function**	**Harbourg Species**	**Stage development expressing tolerance**	**Parameters studied**	**Reference**
M. sativa	Transcription factor/ *Alfin1*	Improve gene expression	Alfalfa	Adult plant	Root growth	Winicov (2000)
O. sativa	Glutamine synthetase/ *GS*	Glutamine synthesis	Rice	Seedling	Photorespiration capacity	Hoshida *et al*. (2000)
S. cereviseae	K^+/Na^+ transport regulation/ *HAL1*	K^+/Na^+ homeostasis	Tomato Melon *Arabidopsis*	Seedling Shoot apex	Sustain K^+/Na^+ ratio Plant growth	Bordas *et al*. (1997) Gisbert *et al*. (2000) Yang *et al*. (2001)
S. cereviseae	FMN-binding protein/ *HAL3*	K^+/Na^+ homeostasis	*Arabidopsis*	Seedling		Albert *et al*. (2000)
A. thaliana	Vacuolar antiporter Na^+/H^+/ *AtNHX1*	Na^+ vacuolar sequestration	Tomato *Arabidopsis B. napus*	Adult plant	Biomass fruit and oil production	Apse *et al*. (1999) Zhang and Blumwald (2001) Zhang *et al*. (2001)
B. juncea	Glyoxylase/ *Gly1*	S-D-Lactoyl glutathione	Tobacco	Detached leaves	Chlorophyll content of detached leaves	Veena Reddy and Sopory (1999)
A. halophytica	Heat shock protein/ *DnaK*	Protein stabilization	Tobacco	Seedling	CO_2 fixation Na^+ content	Sugino *et al*. (1999)
S. cereviseae	Superoxide dismutase/ *MnSOD*	Reduction of O_2^-	Rice	Seedling	Oxidative stress	Tanaka *et al*. (1999)
S. cereviseae	Apoplastic yeast-derived invertase/ *Apo-Inv*	Sucrose synthesis	Tobacco	Seedling	Photosynthetic activity and osmotic pressure	Fukushima *et al*. (2001)
O. sativa	Protein kinase/ *OscCDPK7*	Improve gene expression	Rice	Seedling	Wilty phenotype	Saijo *et al*. (2000)
N. tabaccum	Peroxidase/ *TPX2*	Change cell wall properties	Tobacco	Seeds	Germination Water retention in seed walls	Amaya *et al*. (1999)
S. cereviseae	Trehalose synthase/ *TSP1*	Trehalose synthesis	Tobacco	Adult plant	Plant growth	Serrano *et al*. (1999)
A. thaliana	Transcription factor/ *DREB1A*	Improve gene expression	*Arabidopsis*	Adult plant	Plant growth and survival rate	Kasuga *et al*. (1999)
S. cereviseae	Calcineurin/ *CaN*	Improve Ca^{+2} signaling	Tobacco	Seedling	Plant growth	Pardo *et al*. (1998)

Table 1.5 Potential pitfalls when expressing foreign genes in higher plants.

- Transformation: Tecniques for transforming the host plant are available in most cases, but there are exceptions.
- Adequate expression: Adequate expression of the gene is essential, with high expression at the right time.
- Cellular localization: Expression in specific tissues and organelles is often essential for achieving the desired results.
- Post-translational modifications: Correct processing and folding are often prerequisites for function.
- Prosthetic-group acquisition: Limitations in prosthetic group acquisition may inactivate foreign enzymes.
- Precursor availability: Precursor shortage will place limitations on product formation.
- Inhibitory environments: Suboptimal enzyme activity may be because of abnormal pH, temperature or salt concentrations.
- Side-reactions of new compounds: Endogenous enzyme activities may deplete the product pool or form toxic compounds.

1.5.1. Osmolyte Synthesizing Genes

Metabolic engineering is generally defined as the redirection of one or more enzymatic reactions to produce new compounds in an organism, improve the production of existing compounds, or mediate the degradation of compounds (DellaPenna, 2001). Metabolic engineering of osmoprotectant pathways works for model plants subjected to more or less artificial laboratory tests of stress

resistance. But there is a long path ahead to raise accumulation levels, to overcome intracellular transport constraints, to restrict accumulation to when and where it is needed, to reduce side-effects, and to prove the value of engineered osmoprotectants in major crops under field stress conditions (Rontein *et al*., 2002).

Genetic transformation has allowed the introduction of new pathways for the biosynthesis of various compatible solutes into plants, resulting in the production of transgenic plants with improved tolerance to stress (McNeil *et al*., 1999). Plants engineered to synthesize and moderately accumulate a number of osmolytes showed marginally improved performance under abiotic stress conditions. The effects seen with modest increases in mannitol, fructans, trehalose, ononitol, glycine-betaine, or ectoine and with strong increases in proline amount indicate that the purely osmotic contribution of these metabolites to stress tolerance may not describe their function completely, i.e. that the pathway leading to a particular osmolyte may be more important than accumulation *per se* (Bohnert and Shen, 1999, Hare and Cress, 1997, Jain and Selvaraj, 1997, Nelson *et al*., 1998). Osmolytes are also believed to act in scavenging ROS since the levels of osmolytes in the transgenic plants are too low to be significant in osmotic adjustment (Shen *et al*., 1997).

The production of transgenic plants that can accumulate various compatible solutes, in particular plants of model species such as *Arabidopsis* and tobacco, has allowed this stress defense mechanism to be extended to crop plants, such as rice, potato and sugar beet, albeit with varying degrees of success (Chen and Murata, 2002).

Fructan producing tobacco transgenic plants performed significantly better than controls under drought conditions, having a 55% more rapid growth rate, 33% greater fresh weight and 59% greater dry weight than wild type plants (Pilon-Smits *et al*., 1995). Transgenic beet plants also accumulated fructan to about 0.5% of their dry weight in both roots and shoots. Moreover, these transgenic beets grew significantly better under drought conditions than did wild type plants (Pilon-Smits *et al*., 1999).

Tobacco and *Arabidopsis* plants do not usually contain mannitol. However, expression of *mtlD* gene for mannitol-1-phosphate dehydrogenase from *E.coli* in these two species resulted in the biosynthesis of mannitol. The mannitol-producing tobacco plants exhibited increased tolerance of high salinity (Tarczynski *et al*., 1993). In the seeds of mannitol-accumulating *Arabidopsis* plants, the concentration of mannitol reached 10 μmol g^{-1} dry weight. Mannitol expressing seeds were able to germinate in medium supplemented with up to 400 mM NaCl, whereas control seeds ceased to germinate at 100 mM NaCl (Thomas *et al*., 1995).

The increased level of proline significantly enhanced the ability of transgenic seedlings to grow in medium that contained up to 200 mM NaCl. The increased levels of free radicals, as determined by monitoring the production of malondialdehyde (MDA). Thus, it appears that, in addition to acting as an osmolyte, proline might play a role in reducing the oxidative stress that is brought on by osmotic stress (Hong *et al*., 2000).

Holmström K.O. *et al*. (1996) transformed tobacco with the gene for the trehalose-6-phosphate synthase (TPS1) subunit of yeast trehalose synthase, which was driven by the promoter of the *rbcS* gene from *Arabidopsis*. The accumulation of trehalose seemed to improve drought tolerance however they exhibited a 30-50% reduction in growth rate. The transgenic tobacco plants expressing the same gene exhibited significantly enhanced tolerance to drought. However, as in the case of the transgenic tobacco plants that expressed TPS1, the transgenics exhibited various morphological changes which ranged from severely retarded growth to yellowish, lancet-shaped leaves and the aberrant development of roots (Yeo *et al*., 2000).

1.5.2. Oxidative Stress Related Genes

Most of the transgenic improvements in plant salt tolerance reported to date have been achieved through detoxification of plants by scavenging ROS or prevent them from damaging cellular structures. This is obvious in the case of transgenic plants overexpressing enzymes involved in oxidative protection, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases and glutathione reductases (Roxas *et al*., 1997, Allen *et al*., 1997).

Transgenic plants have been generated to probe the effects of ROS scavenging on salinity stress tolerance, based on observations of gene expression changes in stressed plants. Overexpression of genes leading to increased amounts and activities of mitochondrial Mn-SOD, Fe-SOD, chloroplastic Cu/Zn-SOD, bacterial catalase and glutathione S-transferase/glutathione peroxidase can increase the performance of plants under stress (Bowler *et al*., 1991, Gupta *et al.*, 1993a-b, Roxas *et al*., 1997, Shikanai *et al*., 1998, Van Camp *et al*., 1996).

Preventing oxidative stress or reducing the level of the reactive molecules appears to be a promising approach to obtain plants with diverse tolerance to abiotic stress. Engineering crop plants that can cope with oxidative molecules could have a broad application in agriculture (Bartels, 2001).

1.5.3. Signal Transduction Genes

In nature, eukaryotic nuclear genes are tightly regulated at both the transcriptional and translational levels. Much of this control is achieved through DNA-binding transcription factors. The manipulation of plant traits in agricultural biotechnology would be greatly facilitated if preselected endogenous genes could be turned on or off in a controlled and selective manner. A conceptual approach to such manipulation is the engineered expression of specific native transcription factors that have evolved to control particular genes (Guan *et al*., 2002).

The transcription factor *DREB1A* (Dehydartion Response Element Binding) specifically interacts with the *DRE* (Dehydration Response Element) box promoter sequences and induces expression of stress tolerance genes with DRE elements in their promoters. The overexpression of *DREB1A* cDNA in *Arabidopsis* plants activated the expression of many of these stress tolerance genes under normal growing conditions. Transgenic plants with *DREB1A* ectopically expressed under the control of the CaMV 35S promoter showed morphological abnormalities under unstressed conditions (Liu *et al*., 1998). Kasuga's research group used the stress-inducible *rd29A* promoter to drive expression of *DREB1A*, with the aim of minimizing the negative effects on plant growth experienced with use of the 35S CaMV promoter. They observed improved stress tolerance of the transgenic plants and much improved growth under non-stressed conditions (Kasuga *et al*., 1999). These results demonstrate that stress-inducible promoters may be more desirable in order to generate plants that are tolerant to stress. However, this may not be always the case since growth was not adversely affected in transgenic alfalfa plants overexpressing the *Alfin1* transcription factor under the control of the 35S promoter (Winicov and Bastola, 1999). These plants showed both an enhanced expression of the salt-inducible *MsPRP2* gene in roots and an increase in root growth under salt stress.

Improvements provided by proteins such as barley HVA1 and CBF/DREBs in transgenic plants might also be credited to the detoxifying effect of the expressed protein or its downstream target proteins (Xu *et al*., 1996, Liu *et al*., 1998). One hallmark of the detoxification effect is its lack of specifity; that is, the transgenic plants have increased tolerance not only to high salts but also to drought, cold and in some cases even heat shock. The CBF/DREB transcription factors can bind to the DRE/CRT element that is found in the promoters of some stress-responsive genes (Stockinger *et al*., 1997).

Rational design of artificial transcription factors that target specific DNA sequences with non-native nucleotide binding domains fused to transcriptional activation or repression domains is another attractive option. An especially promising approach of this kind utilizes synthetic DNA binding domains of the

zinc finger protein (ZFP) class. Guan's research group showed that ZFP-based artificial transcription factors can be designed and synthesized to manipulate transgene and endogenous gene expression levels in transgenic plants (Guan *et al*., 2002).

The results obtained from transgenic approaches with many genes are encouraging, and recent results obtained in transgenic plants harboring genes encoding a Na^+/H^+ antiporter or a transcription factor show the possibility of increasing salt tolerance. Transformation of agronomic important crops and the identification of uncovered tolerance determinants and stress inducible promoters must be further explored to obtain plants with increased tolerance to salt stress (Borsani *et al*., 2003).

1.6. *Agrobacterium* Mediated Gene Transfer

The most widely used method for the introduction of new genes into plants is based on the natural DNA transfer capacity of *Agrobacterium tumefaciens*. In nature, this Gram-negative soil bacterium causes tumor formation (crown gall disease) on a large number of dicotyledonous as well as some monocotyledonous plant species and Gymnosperms (De Cleene and De Ley, 1976). The crown gall disease has been shown to be due to the transfer of a specific DNA fragment, the T-DNA (transferred DNA), from a large tumor-inducing (Ti) plasmid within the bacterium (Zaenen *et al*., 1974) to the plant cell. After transfer, the T-DNA becomes integrated into the plant nuclear genome (Chilton *et al*., 1977, Schell *et al*., 1979) and its subsequent expression leads to the crown gall phenotype.

Three bacterial genetic elements are required for T-DNA transfer to plants. The first of these elements are the T-DNA border sequences that consist of 24 or 25-bp direct repeats flanking and defining the T-DNA (Van Haeren *et al*., 1988). Usually, all DNA sequences between the borders are transferred to the plant. The second element consists of the virulence (*vir*) genes encoded by the Ti plasmid in a region outside of the T-DNA (Hooykaas and Beijersbergen, 1994). Some of

these *vir* genes are involved in the processing of the T-DNA from the Ti plasmid and T-DNA transfer from the bacterium to the plant cell. The third bacterial element necessary for T-DNA transfer consists of a number of chromosomal genes, of which some are important for attachment of the bacterium to the plant cell.

The development of plant transformation vectors using *A.tumefaciens* is based on the fact that besides the border repeats, none of the T-DNA sequences is required for transfer and integration. This means that the T-DNA genes can be replaced by any other DNA of interest, which will thus be transferred to the plant genome. As a removal of the plant hormone biosynthetic T-DNA genes, the transformed plant cells do not proliferate into tumorous tissues, but may regenerate into normal plants.

Two types of Ti plasmid-derived vectors can be distinguished

i. *cis* systems (cointegrate vectors) in which new genes are introduced via homologous recombination into a non-oncogenic Ti plasmid (Zambryski *et al*., 1983, Deblaere *et al*., 1985).

ii. *trans* systems (binary systems) in which new genes are cloned into a plasmid containing a non-oncogenic T-DNA, which is subsequently introduced into an *Agrobacterium* strain harboring a Ti plasmid with an intact *vir* region, but lacking the T-DNA region (De Framond *et al*., 1983, Hoekema *et al*., 1983).

In both *cis* and *trans* vectors, DNA of interest is first cloned into *E.coli* and then transferred to *Agrobacterium* by conjugation (Van Haute *et al*., 1983) or electroporation (Mersereau *et al*., 1990).

One of the main reasons why *Agrobacterium*-mediated transformation usually is the method of choice, is the delivery of a well-defined piece of DNA into the plant chromosomes. Integration of the T-DNA in the plant chromosomes is accomplished by the plants' illegitimate recombination system, but with the help of agrobacterial proteins, which enhance the precision and the efficiency of the integration process.

Some studies predominantly find single T-DNA copy inserts (Koncz and Schell, 1986, Spielmann and Simpson, 1986, Deroles and Gardner, 1988), in other transformation experiments these simple insertion patterns are less frequent than arrays of two or more T-DNA copies in tandem and/or inverted repetition (Jorgensen *et al*., 1987, Grevelding *et al*., 1993, De Neve *et al*., 1997). The factors that have been suggested as influencing copy number and conformation of integrated T-DNAs are the type of vector used (binary cointegrate), specific characteristics of the T-DNA (Mlynarova *et al*., 1994), the transformation method (Grevelding *et al*., 1993), number of infecting bacteria and the T-strand copies in the bacteria, transformation conditions and bacterial strain or vector used.

As in any technology for plant transformation, there are multiple factors involved in *Agrobacterium*-mediated transformation that influence the success or failure of the transfer of gene of interest into plants and their subsequent stable integration and expression. The different factors can affect transformation differently, depending in part on the plant species.

Aspects of transformation that affect success include:

i. Maturity of the plant - as a general rule young plants are easier to transform than old ones

ii. Selected tissue to be transformed

iii. *Agrobacterium* strain selected for transformation

iv. Extent of time and conditions for inoculation of the tissue with *Agrobacterium*

v. Growth of *Agrobacterium* with respect to the transformed plant cells. If there is overgrowth of *Agrobacterium*, the chances of regenerating complete plants from the transformed tissue dwindle.

vi. Plant tissue necrosis caused by *Agrobacterium*.

A major requirement for the long-term successful application of transformed plants is the stability and Mendelian segregation of the transgenic phenotype. Hybridization analysis of T-DNA structure in transgenic plants and their progeny revealed that the T-DNA remains stable during vegetative proliferation and during sexual transmission (Müller *et al*., 1987).

A majority of the plants with low or no transgene expression are probably the result of different silencing mechanisms that are not necessarily correlated with the chromosomal location of the T-DNA. These silencing mechanisms can be immediately active in the primary transformant resulting in low or no expression. In other transformants, silencing is induced later in the development, by environmental conditions, or during transmission of the transgene to the progeny, resulting in instability of the phenotype (Meyer, 1995).

Another related problem besides the stability is the level of transgene expression. The amount of protein produced commonly varies more than 100-fold among individual transformants within the same experiment and such substantial inter-transformant variability has also been seen at the transcript level (Peach and Velten, 1991, Breyne *et al*., 1992a-b).

Most importantly, high expression is more frequently obtained with single inserts, whereas clustered multiple inserts cause transgene silencing with a high frequency. Silencing of transgenes from single inserts may be due to the chromosomal position (Pröls and Meyer, 1992) or to initially too high expression levels (Elmayan and Vaucheret, 1996).

1.6.1. Regeneration Dependent Transformation Using *Agrobacterium*

Target cells for transformation and regeneration can be protoplasts, suspension cultured cells, callus cells, or cells forming part of an organ or tissue explant. The purpose of the regeneration procedure is twofold: it allows the recovery of uniformly transformed shoots and, importantly, the selection of such

shoots. Strict selection is essential for an efficient transformation system as the fraction of stably transformed cells is usually small. For example with a commonly used transformation method for *A.thaliana* (Valvekens *et al*., 1988), none of 172 plants regenerated on non-selective medium was transgenic (De Buck *et al*., 1998).

The transgenic plants are, in general, phenotypically indistinguishable from untransformed plants, new markers had to be developed to detect or select such transformed plant cells. Many of the commonly used markers (antibiotic resistance, colour-, or light-producing systems) functional in bacterial or animal systems are applicable to plants. Most selectable marker systems rely on detoxification or sequestration of a phytotoxic product or on expression of an insensitive version of the target of the toxic product. Selective agents include antibiotics, herbicides, and toxic levels of amino acids or amino acid analogues.

The choice of the selectable marker gene, the selective agent, and its concentration and timing of application are also very important as strict selection of transformed cells is required on the one hand, but, on the other hand, regeneration should not be impeded.

For many plant species, the lack of a suitable regeneration method is undoubtedly one of the main bottlenecks in developing a transformation procedure. Moreover, a particular regeneration method is usually only efficient with a limited number of genotypes within a species. Somaclonal variation may also be problematic with some regeneration procedures.

1.6.2. Regeneration Independent Meristem Transformation Using *Agrobacterium*

The shoot apex forms an attractive target for transformation as it contains the meristematic cells from which all aerial parts of a plant derive during the normal course of plant development. Targeting these cells for transformation has,

therefore, the advantage that transformed cell lineages, forming part of an organized shoot, can be obtained without the involvement of a *de novo* regeneration pathway. Transformation can be applied *in situ*, i.e., on meristems that are still part of an embryo or seedling, or on excised meristems or shoot tips. *In vitro* development of these explants into plantelets can be accomplished with minimal tissue culture manipulations and largely in a genotype-independent fashion. Moreover, plants originating from the culture of apical or axillary meristems are considered to show no somaclonal variability.

Meristems are multicellular so the primary transformants from a meristem transformation procedure are expected to be chimeric, consisting of transformed and untransformed sectors. Meristems are potentially very interesting targets for transformation. They are destined to form new shoots and can, therefore, develop into plants with minimal tissue culture manipulations and in a genotype-independent way. These characteristics are clearly the main advantage of a meristem transformation approach.

1.6.3. *In planta* Transformation Methods for *A.thaliana* Using *Agrobacterium*

Over the last decade, several methods for *Agrobacterium* mediated transformation of *A.thaliana* have been developed that do not involve any tissue culture steps. Bechtold *et al.* (1993) inoculated flowering *A.thaliana* plants by vacuum infiltration with an *Agrobacterium* suspension.

The method entails suspending *Agrobacterium* cells containing a vector with a gene of interest in vacuum infiltration medium, and the plant portions to be transformed are immersed in the suspension and subjected to vacuum infiltration. Physically, vacuum generates a negative atmospheric pressure that causes the air spaces between the cells in the plant tissue to decrease. The longer the duration and the lower the pressure of the vacuum, the less air space within the plant tissue.

The increase in the pressure allows the infiltration medium, including the infective transformation vector to relocate into the plant tissue. For plant transformation, vacuum is applied to a plant part in the presence of *Agrobacterium* for a certain time period. The length of time that a plant part or tissue is exposed to vacuum is critical as prolonged exposure causes hyperhydricity. Infiltrated plants subsequently produce transformed seeds from which transformed plants are obtained.

Transformants are found with much higher frequency in the offspring of the inoculated plants: several transgenic plants are obtained per infiltrated plant. With this method effective transformation takes place late in flower development and targets for transformation are the gametes or even the zygote (Feldmann, 1991, Bechtold *et al*., 1993). As these procedures are totally devoid of tissue culture steps, somaclonal variation does not occur.

1.7. Aim of the Study

As salinity stress is a continuing and increasingly deleterious obstacle to the growth and yield of crop plants, owing to irrigation practices and increasing demands on fresh water supply, the engineering of salt tolerant crop plants has been a long-held and intensively sought objective (Apse and Blumwald, 2002). Since salt tolerance is a multigenic trait, the acquisition of a salt tolerant crop may depend on the transfer of several genes (Bohnert *et al.,* 2001). Identification of genes associated with an increase in salt tolerance would be a first step in engineering halotolerance. The transfer of individual genes provides a valuable tool to achieve some improvement in salt tolerance, and to identify key genes actually involved on the process. In a different approach, the identification of genes in model organisms whose expression is functionally related to salt tolerance by genetic analysis could be of great value for transformation of crop plants.

In nature, eukaryotic nuclear genes are tightly regulated at both the transcriptional and translational levels. Much of this control is achieved through DNA-binding transcriptional factors. The manipulation of plant traits in agricultural biotechnology would be greatly facilitated if preselected endogenous genes could be turned on or off in a controlled manner. A conceptual approach to such manipulation is the engineered expression of specific native transcription factors that have evolved to control particular genes (Guan *et al*, 2002).

For this purpose in study we focused on:

i. Construction of plant expression vectors carrying *sto* under CaMV35S constitutive promoter in sense and anti-sense orientations.

ii. Transformation of tobacco and *Arabidopsis* plants with the newly constructed plant transformation vectors.

iii. Molecular analysis of transgenic plants to verify the integration of the gene and determination of the level of expression.

iv. Physiological and biochemical analysis of transgenic plants under salt stress conditions.

v. Expression analysis of *sto* gene in wild type *Arabidopsis* under stress and plant growth regulator treatments.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strains and Plasmids

Throughout the study *Eschericia coli* XL-1 BLUE cells were used as competent cells. GV3101/MP90RK strain of *Agrobacterium tumefaciens* (Koncz *et al.,* 1994) was used for plant transformation. pBSK-STO (Appendix A) is a pBluescript SK^+ vector, carrying a full-length cDNA encoding *Arabidopsis thaliana sto* gene (Appendix B). pPCVB1-STO-S is the binary vector carrying the AtSTO in sense orientation whereas pPCVB1-STO-AS is the binary vector carrying AtSTO in anti-sense orientation (Appendix A). Both of these vectors were used for the transformation of *Nicotiana tabacum* and *Arabidopsis thaliana*. The vector pPCVB812-STO (Appendix A) carrying the *sto* gene in sense orientation was only used for the transformation of *Arabidopsis thaliana*.

2.1.2. Bacterial Growth Media and Culture Conditions

The complex medium used for *E.coli* strains was Luria-Bertani Broth (Appendix C) supplemented with ampicillin (100 mg/l) with 1.5 % bacterial agar for solid media. The bacterial cultures were incubated at 37^{o}C overnight with vigorous shaking for liquid cultures. For *Agrobacterium tumefaciens* strains YEB (Appendix C) supplemented with kanamycin (100 mg/l), carbenicillin (100 mg/l) and rifampicillin (100 mg/l) with 1.5 % bacterial agar for solid media was used. The bacterial cultures were incubated at 28^{o}C for 2 days with vigorous shaking (150 rpm) for liquid cultures. A liquid aliquot of each bacterial clone carrying a specific plasmid was kept at –80^{o}C in 20% glycerol for longer storage.

2.1.3. Plant Material and Plant Tissue Culture Media

Nicotiana tabacum SR-1 cultivar Petit Havanna and *Arabidopsis thaliana* ecotype Columbia were provided by Dr.Laszlo Szabados and used in this study. MS (Murashige and Skoog, 1962) basal medium supplemented with sucrose and agar was used at tissue culture studies. For tobacco 3% sucrose and for *Arabidopsis* 2% sucrose was added to MS media (Appendix D). Before usage the medium was dissolved in distilled water, the pH was adjusted to 5.7-5.8 with NaOH and sterilized at 121^{o}C for 20 minutes. Plant growth regulators and antibiotics (ampicillin, hygromycin, kanamycin, rifampicin, carbenicillin) were sterilized by 0.2 μm pore sized filters and added freshly to sterile medium. The compositions and the purposes of the media are given in Table 2.1.

Table 2.1. The compositions and purposes of plant tissue culture media for tobacco.

Liquid MSA	M9274 + 3% sucrose + 1mg/l NAA + 1mg/l Benzylaminopurin BA	Diluting *Agrobacterium* culture before transformation
MSA	M9274 + 3% sucrose + 1.5 % plant agar + 1mg/l NAA + 1mg/l BA	Callus and shoot formation for control plants, co-cultivation for *Agrobacterium* transformed plants
MSB	M9274 + 3% sucrose + 1.5 % plant agar + 0.1 mg/l NAA + 1mg/l BA + 5mg/l PPT + 500 mg/l Cefotaxime	Selection of transformed plants and their regeneration
MSC	MSB without plant growth regulators and PPT for control shoots MSB without plant growth regulators for transgenic shoots	Induction of root formation on regenerated shoots

2.1.4. Chemicals

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

2.2. Methods

2.2.1. Transformation Studies for Bacterial Cells

All vectors were first transformed to *E.coli* cells, selected on ampicillin containing medium and tested for the presence of *sto* gene. These vectors were then transferred to *Agrobacterium* GV3101/MP90RK and used for gene transfer studies to tobacco and *A.thaliana*.

2.2.1.1. Competent Cell Preparation

E.coli and *A.tumefaciens* cells have to be competent to be transformed with the vectors. By using different protocols competent cells from these strains were prepared.

2.2.1.1.1. Competent *E.coli* Preparation

Competent cells of *E.coli* were prepared according to Inoue *et al*. (1990). *E.coli* XL-1 cells, taken from glycerol stock, were streaked on an LB agar plate and grown overnight at 37^{o}C. Next morning a single colony was transferred to 100 ml sterile SOB medium (Appendix C) in a 1 liter flask. The culture was grown at 18^{o}C with vigorous shaking (200-250 rpm) until an OD_{600} value of 0.6 was reached. The culture was transferred to centrifuge tubes and incubated on ice for 10 minutes. After the incubation, centrifugation at 3000 rpm for 10 minutes at 4^{o}C was carried out in Sigma 3K30 centrifuge. The pellet was suspended in 32 ml ice cold TB (Appendix C) and incubated on ice for another 10 minutes. Second centrifugation was done in the same manner. The pellet was suspended in 7.5 ml

TB and DMSO (dimethyl sulfoxide) were added with gentle swirling to a final concentration of 7%. After incubation on ice for 10 minutes, prepared competent cells were transferred into eppendorf tubes in 100 µl aliquots, and immediately chilled by immersing in liquid nitrogen. The frozen competent cells were stored at –80^{o}C for a few months without a detectable loss in their competencies.

2.2.1.1.2. Competent *A. tumefaciens* Preparation

A.tumefaciens GV3101/MP90RK cells were streaked on YEB agar plates and grown 2 days at 28^{o}C. A single colony was transferred to 50 ml YEB medium supplemented with 100 µl 1M $MgSO_4$ and incubated at 28^{o}C with vigorous shaking. Next day the cells were harvested at 4500 rpm for 15 minutes. The supernatant was removed and pellet was dissolved in 1 ml ice-cold 10mM $CaCl_2$. The suspension was kept for 5 minutes on ice. 100 µl aliquots were taken to eppendorf tubes and frozen by liquid nitrogen. The competent cells were stored at –80^{o}C for further use.

2.2.1.2. Transformation of Bacteria with Plasmids

The vectors used in this study were transformed to competent *E.coli* and *A.tumefaciens* cells. After transformation positive colonies were selected and analyzed for the presence of the desired gene. The transformation studies were carried out according to Maniatis *et al*., 1989.

2.2.1.2.1. Transformation of *E.coli*

Frozen competent *E.coli* cells were thawed in ice and 5-20 µl (at least 10 ng) of plasmids were added for each transformation. The cells were incubated in ice for 45 minutes. Following cold treatment a heat shock at 42°C for 90 seconds was applied to enhance the entrance of plasmids into the cells. The cells were transferred to ice for another 5 minutes period. Later cells were suspended in 900 µl sterile LB and incubated at 37°C for 45 minutes with vigorous shaking. At the end of this period the cells were centrifuged at 3000 rpm for 3 minutes, 900 µl supernatant was removed and cells were suspended in the remaining 100 µl of solution. Finally transformed bacteria were spread on ampicillin containing agar plates and incubated at 37°C overnight. Presence of colonies was checked the next day and the plates were stored at 4°C for further use.

2.2.1.2.2. Transformation of *A.tumefaciens*

Frozen competent *A.tumefaciens* cells were transformed with 5 µl of plasmid. The heat shock was applied at 37°C for 5 minutes. After heat shock cells were suspended in 900 µl sterile YEB and incubated at 28°C for 2-4 hours with vigorous shaking. At the end of this incubation, centrifugation was carried out at 3000 rpm for 3 minutes in microcentrifuge, 900µl of supernatant was removed and pellets were suspended in the remaining volume. Finally transformed bacteria were spread on kanamycin, carbenicillin and rifampicin containing YEB agar plates and incubated at 28°C for 2 days. Presence of colonies was checked and the plates were stored at 4°C for further use.

2.2.1.3. Plasmid Isolation and Manipulation

The colonies growing on selective media do not always carry the plasmid bearing the desired foreign gene. Plasmid isolations from the transformed *E.coli* and *A.tumefaciens* were done to verify the transformants carrying the gene of interest. The plasmid isolation and manipulation studies were carried out according to Maniatis *et al*., 1989.

2.2.1.3.1. Mini-Scale Plasmid Isolation from *E.coli*

A single colony of *E.coli* XL-1 transformed with a plasmid was taken from the selective agar plate by the use of a sterile toothpick. It was transferred into a sterile Falcon tube containing 3 ml LB supplemented with ampicillin and culture was grown overnight at 37^{o}C.

Overnight grown cells were harvested by centrifugation at 13000 rpm for 30 seconds in microcentrifuge. The supernatant was discarded and pellet was suspended in 100 µl Sol I (Appendix E) with vigorous vortexing. After the addition of 200 µl Sol II (Appendix E), the suspension was inverted several times to obtain a clear mixture and incubated in ice for 5 minutes. 150 µl of Sol III (Appendix E) was mixed gently to the suspension and incubated in ice for 10 minutes. With the addition of 450 µl phenol-chloroform-isoamyl alcohol (25:24:1) cell debris and chromosomal DNA were separated in different phases. Following the centrifugation at 13000 rpm for 10 minutes in micromax RF microcentrifuge upper phase was collected into a new eppendorf tube. Plasmid DNA was precipitated with equal volume of isopropanol at -20^{o}C at least for 30 minutes. The plasmid DNA was obtained in the pellet after centrifugation at 13000 rpm for 10 minutes. Pellet was washed with 70% ethanol, centrifuged at 13000 rpm for 5 minutes and supernatant was removed. The pellet was dried completely and dissolved in 50 µl TE (Appendix E) and RNAse (20 µg/ml). After the incubation

for 30 minutes at 37^{o}C for RNAse activity, plasmid DNA was stored at -20^{o}C until usage. Purity and integrity of the DNA were confirmed by spectrophotometry (OD_{260}, OD_{280}) and agarose gel electrophoresis, respectively. The plasmids isolated from selected colonies were checked by restriction enzyme digestion and agarose gel electrophoresis for the presence of the gene of interest.

2.2.1.3.2. Mini-Scale Plasmid Isolation from *A.tumefaciens*

A single colony was selected from *A.tumefaciens* transformed with plasmid and grown at 28^{o}C overnight in 3 ml of kanamycin, carbenicillin, and rifampicin containing YEB.

Overnight grown cells were harvested by centrifugation at 13000 rpm for 30 seconds in microcentrifuge. The supernatant was discarded and pellet was suspended in 1ml of STE (Appendix E). A centrifugation at 5000 rpm for 10 minutes was carried out. The supernatant was discarded and from this point onward the plasmid isolation was done as described in section 2.2.1.3.1.

2.2.1.3.3. Agarose Gel Electrophoresis

DNA molecules obtained throughout the study were visualized on agarose gel. Agarose gel electrophoresis was carried out according to Maniatis *et al*., 1989. According to the purpose, different concentrations (0.8-1%) of gel solutions were prepared in 0.5 - 1X TBE or TAE electrophoresis buffer solutions (Appendix F). For 0.8% agarose gel, 0.8 g of agarose was melted completely in 100 ml of 0.5XTBE buffer by heating. Then the solution was left to cool around 50°C and 50 µl of ethidium bromide (10 mg/ml) added to the gel solution. The gel was poured into the electrophoresis tray having a comb, which will form the wells for the sample loading. The gel was left at room temperature until it was solidified and electrophoresis tank was filled with 0.5XTBE buffer.

The samples to be loaded were prepared by mixing the samples with 6X loading buffer to the final concentration of 1X and applied into the wells. Together with the samples DNA size markers like DNA ladder and λ-phage DNA digested with PstI or HindIII, were loaded into separate wells. Then the tank was connected to a power supply and run under constant voltage of 50-60 volts using Bio-Rad DNA SubCell agarose gel electrophoresis apparatus. The gel was visualized under UV transilluminator and photographed.

2.2.1.3.4. Restriction Enzyme Digestions and Insert Isolation

The digestion mixture used in digestion process contained,

- DNA
- Restriction endonuclease(s) (10u/μg DNA)
- 10X restriction endonuclease cutting buffer and sterile distilled water

The mixture then incubated specified temperatures according to the enzyme used for a few hours or overnight. In order to determine the presence of gene of interest in the sample, the sample was loaded in a 0.8% agarose gel and run by using 0.5XTBE buffer as described in 2.2.1.3.3.

For the isolation, DNA molecule (10-20 μg) was first digested with correct restriction endonuclease(s) and run on 1% agarose gel and run by using 0.5XTAE buffer as described in sections 2.2.1.3.3. Following the visualization under UV light, the expected band corresponding to size marker was cut out from the gel. Gel slice was put into an eppendorf tube and the isolation of fragment was completed according to instructions given in the Sephaglass™ Band Prep. Kit.

2.2.1.3.5. Ligation of Insert to Plasmids

Previously isolated fragment was ligated into a digested plasmid as described below;

- Plasmid vector (50-100 ng)
- DNA fragment (insert; 100-500 ng)
- 10X ligase buffer
- DNA ligase (1-2 μl)
- Sterile distilled water

This mixture was incubated overnight at 16 ºC. Ligation mixture was transformed into the competent cells (section 2.2.1.2.1) and mini-scale plasmid isolation was performed (section 2.2.1.3.1). Then plasmids were checked by PCR using gene specific primers for the presence of the desired gene and further analysis was carried out by digestions with correct restriction endonuclease(s) as described in section 2.2.1.3.4 to release the expected fragment. Agarose-gel electrophoresis was performed (section 2.2.1.3.3) to check the fragment.

2.2.1.4. Amplification and Cloning of *sto* Gene

The *sto* gene was amplified using cDNA library of *A.thaliana*. In order to achieve the amplification of an error free *sto* gene, elongase enzyme with proof reading activity was used in PCR reaction and fragment was ligated to pBSK. The sequence analysis confirmed the presence of an error free full-length *sto* gene that can be used for further experiments.

2.2.1.4.1. PCR Amplification

PCR amplification with gene specific primers, STO1 and STO2 (Table 2.2), was carried out to obtain *sto* gene. Primers were designed with BamHI sites that enable easy cloning (by Dr. Laszlo Szabados), according to the sequence of *A.thaliana.* The template DNA used was cDNA library from cell suspension of *A.thaliana*. The PCR conditions and program used for PCR amplification are given below (Table 2.3 and Table 2.4).

Table 2.2 Gene specific primers for *sto.* Bold letters show BamHI sites

STO1 gene specific primer	5'-CGT**GGATCC**CTAGATATTTTGGTTTCTTGTATCCG-3'
STO2 gene specific primer	5'-CTC**GGATCC**ACACATGCAGATAGGTTTCTTG-3'

Table 2.3 Optimized conditions for PCR using elongase enzyme.

Template DNA (1μg)	1 μl
STO1 (10 pM)	2 μl
STO2 (10 pM)	2 μl
DNTP (5mM)	2 μl
5X PCR buffer for Elongase	10 μl
$MgCl_2$ (25 mM)	2 μl
Elongase (Fermentas)	1 μl
DH_2O	30 μl
Total volume	50 μl

Table 2.4 PCR cycling conditions for amplification of *sto* using elongase.

94°C 2 min	
94°C 30 sec	
55°C 30 sec	35 cycles
68°C 4 min	
72°C 7 min	

The PCR amplified fragment was isolated from the agarose gel using MBI Fermentas, DNA Extraction Kit (section 2.2.1.3.4), and digested with BamHI restriction enzyme (section 2.2.1.3.4) and ligated to pBSK (section 2.2.1.3.5). The ligation product was transferred to *E.coli* XL-1 BLUE competent cells. Colonies that were growing on ampicillin containing media were tested for the presence of *sto* gene by using PCR.

2.2.1.4.2. Cloning in pBSK

BamHI site of pBSK was used to clone *sto* amplified by PCR. pBSK and amplified *sto* gene were digested with BamHI for 4 hours at 37^{o}C and ligated (section 2.2.1.3.5) overnight at 16^{o}C**.** The new plasmid construct was used to transform bacterial cells as described in transformation of competent *E.coli* XL-1 BLUE cells (section 2.2.1.2.1).

After ampicillin selection positive colonies were analyzed by PCR and with restriction enzyme test digestions for the presence of *sto*. Sequence analysis of positive colonies was done to confirm the presence of error free DNA sequence of full-length *sto* cDNA.

2.2.1.4.3. Sequence Analysis

Sequence analysis of the promising colonies were done at laboratory of Dr. Laszlo Szabados, Biological Research Center, Szeged, Hungary using ABI PRISM 377 DNA sequencer (Applied Biosytems).

2.2.1.5 Plant Transformation Studies

Nicotiana tabacum SR-1 cultivar Petit Havanna and *Arabidopsis thaliana* ecotype Columbia plants were used for transformation studies. *Nicotiana tabacum* was transformed by leaf disk transformation method described by Horsch *et al.* (1985) and vacuum infiltration method was used for the transformation of *Arabidopsis thaliana* described by Bechtold *et al*., 1993 with some modifications.

2.2.1.5.1. Construction of Plant Transformation Vector pPCVB1

The plant transformation vector, pPCVB1 (Appendix A), was constructed by the excision of RFP fragment from pPCVB-RFP (provided by Dr. Ferenc Nagy, BRC, Szeged, Hungary) by restriction enzyme digestions (SacI and SmaI). The plasmid contains strong, nominally constitutive, 35 S promoter from cauliflower mosaic virus, ampicillin resistance gene for bacterial selection and PPT resistance for plant selection.

The error free full-length *sto* cDNA was excised from pBSK and cloned at BamHI site of pPCVB812 (Appendix A) in sense and pPCVB1 both in sense, pPCVB1-STO-S (Appendix A) and anti-sense orientations**.**

2.2.1.5.2. Transformation of Vectors To *Agrobacterium*

The vectors pPCVB1-STO-S and pPCVB1–STO-AS were transferred to *Agrobacterium* cells by direct transformation method described in section 2.2.1.2.2. From positive colonies plasmids were isolated (2.2.1.3.2), digested and tested on agarose gel for the presence of *sto* gene.

2.2.1.5.3. *Agrobacterium tumefaciens* Mediated Transformation of *Nicotiana tabacum*

N. tabacum plants were grown in sterile jars on Murashige-Skoog (MS) medium under 12 hours light cycle at 24°C. *A.tumefaciens* cells transformed with plasmids were grown in 200 ml of YEB medium supplemented with 2mM $MgSO_4$, 100 mg/l kanamycin, 100 mg/l carbenicillin and 100 mg/l rifampicin overnight with shaking at 28°C. The cells were harvested at 3000 rpm for 15 minutes, supernatant was removed and pellet was suspended in 25 ml of liquid MSA medium. Tobacco leaves were cut aseptically into leaf disks and put into petri plates. The *Agrobacterium* cell suspension was poured onto leaf disks. After several minutes excess *Agrobacterium* suspension was removed and the leaves were blotted on sterile Watmann filter papers. Then the leaf disks were transferred to petri plates containing MSA medium. After 2 days of co-cultivation at 25°C with 16 hours photoperiod, leaf disks were transferred to MSB medium for selection and regeneration. Elimination of *Agrobacterium* cells after co-cultivation was done by addition of 500 mg/l cefotaxime to the medium. Plates were refreshed every two weeks. After callus formation and shooting plants on MSB medium (1 month later), shoots were cut point attachment to callus and transferred to jars containing MSC medium to induce root formation and further development. Plantlets were transferred to soil and grown in the growth chamber at 24±1°C with 16 hours light and 8 hours dark cycles. When flower buds emerged, they were covered with nylon bags to prevent cross-pollination. Seeds of putative transgenic plants were collected when they dry completely.

2.2.1.5.4 *Arabidopsis thaliana* Transformation with Vacuum Infiltration

A.thaliana seeds were germinated on MS medium supplemented with 2% sucrose and later seedlings were transferred to pots and grown several weeks in short day conditions (9 hours light, 22^{o}C). In order to promote bolting further growth was achieved at long day conditions (16 hours, 22^{o}C). After 2-3 weeks, primary inflorescence shoots were removed to induce the development of rosette inflorescence shoots. About a week later these plants were infiltrated with *A.tumefaciens* transformed with plasmids by using vacuum infiltration method (Bechtold *et al*.). A 25 ml liquid culture of *A.tumefaciens* (YEB supplemented with kanamycin, carbenicillin and rifampicin) was inoculated with a single colony and grown at 28^{o}C for 2 days. The day before the infiltration, 10 ml of culture was used to inoculate 500 ml of YEB medium supplemented with antibiotics. The following day the cells were harvested by centrifugation at 5000 rpm for 10 minutes using Sigma 3K30. The supernatant was removed and cells were resuspended in infiltration medium (Appendix G) to an OD_{600} of 0.8-1.2. Glass jars were filled with the 200 ml *Agrobacterium* suspension and placed in the vacuum chamber. Pots of *A.thaliana* were inverted into the suspension, only with their inflorescence shoots submerged in the suspension. A pressure of about 16 mbars was applied for 5 minutes. After 5 minutes the vacuum was released quickly. Infiltrated plants were taken back to green house to long day conditions. T_1 seeds of infiltrated plants were collected when they dried completely and selected on MS containing PPT (15 mg/l) and cefotaxime 100 mg/l. After 2 weeks they were transferred to MS without selection. Finally they were transferred to pots to be grown in green house. Putative transgenic T_2 seeds were collected when they dried completely. Seeds of *Arabidopsis* can be stored at 4^{o}C for further usage.

2.2.1.6. Expression Analysis of *sto*

The expression of *sto* gene was analyzed by Northern Blot Hybridization. *A.thaliana sto* gene expression was determined under certain stress conditions and plant growth regulator treatments after 1, 6 and 24 hours. Three different abiotic stress conditions were applied; 8% sucrose, 300 mM NaCl and 4^{o}C cold. Plant growth regulators applied were 100 μM abscisic acid (ABA) and gibberellic acid (GA), 10 μM jasmonic acid (JA) and 2-4 D, 10 mM salicylic acid (SA) and 10 mg/l ethylene.

Northern Blot Analysis was also used for the determination of *sto* expression in putative transgenic plants using leaf samples of individual lines of tobacco and *Arabidopsis*.

2.2.1.6.1. Total RNA Isolation

All the solutions and distilled water used in RNA isolation were treated with DEPC and autoclaved before use. 0.1 gram of leaf sample was grind in liquid nitrogen and the powder obtained was transferred to sterile Falcon tube. For 0.1 g fresh weight of starting material 1 ml of TRI reagent (Appendix H) was added. The sample was incubated at room temperature for 3 minutes with occasional stirring until all plant material dissolved completely. 0.2 ml of chloroform was added for each ml of TRI reagent added. The content was mixed for 15 seconds with vigorous shaking. After centrifugation at 13000 rpm for 5 minutes upper phase was transferred into eppendorf tube and equal volume of isopropanol was added and incubated at room temperature for 10 minutes. The isolated RNA was precipitated by centrifugation at 13000 rpm for 15 minutes. The supernatant was discarded and pellet was washed with 70% ethanol. Pellet was dried at 65^{o}C for few minutes and dissolved in 300μl DEPC treated water with vortexing and

heating at 65°C. Following complete dissolution 30 µl DEPC treated 3M NaAcetate pH: 5.2 and 150 µl phenol (equilibrated with Tris, pH 8) were added and content was mixed with occasional shaking for 3 minutes. After the addition of 150 µl chloroform, centrifugation at 13000 rpm for 5 minutes was carried out. Upper phase was taken to a new eppendorf tube and equal volume of isopropanol was added and incubated at room temperature for 10 minutes. After the centrifugation at 13000 rpm for 15 minutes, supernatant was removed and the pellet containing RNA was washed with 70% ethanol. Another centrifugation at 13000 rpm for 5 minutes was carried out and supernatant was removed. The pellet was dried at 65°C and dissolved in 50 µl DEPC treated water.

2.2.1.6.2 Northern Blotting

The purity and concentration of RNA samples were checked by spectrophotometry, measuring the absorbances at 260 nm and agarose gel electrophoresis and the concentration of RNA samples were set to 20 µg. The RNA extracts containing 20µg of RNA were vacuum dried and dissolved in 20 µl of sample buffer (Appendix H), denatured at 65°C. The samples were loaded to 1 % agarose gel containing, 1X MOPS (Appendix H) and 6% formaldehyde and electrophoresis was run for 2 hours. During electrophoresis a Hybond-N nylon membrane, and 3 pieces of 3 MM paper having the same sizes with the gel was cut. A plastic tray of a suitable size was half filled with transfer buffer (Appendix H) and a platform was established (Figure 2.1) and covered with a wick made from three sheets of 3 MM paper. The paper was saturated with transfer buffer. The treated gel was placed on the wick platform. The surrounding of the gel was covered with parafilm to prevent the absorption of transfer buffer directly into the paper towels. A Hybond–N nylon membrane with the same size of gel was placed on top of the gel and wetted with transfer buffer. On top of the membrane three sheets of 3MM paper was placed one by one and wetted with transfer buffer.

Formation of air bubbles was avoided by rolling a glass pipette in each step. A stack of absorbent paper towels (at least 5 cm) was placed on top of 3 MM papers. Finally, a glass plate and a 1 kg weight were placed on top of the towels and the capillary transfer was allowed to proceed overnight. Next morning the transfer apparatus was dismantled and the nucleic acids were fixed to the membrane by UV cross-linking.

2.2.1.6.3. Probe Labeling, Hybridization and Detection

The membrane was placed in hybridization tube and pre-hybridized with the use of an excess amount of hybridization buffer (Appendix H) for 2 hours at 42^{o}C in a rolling tube hybridization oven. Meanwhile the probe that will be used for hybridization was prepared. Labeling of the probe was done by using Megaprime^{TM} DNA labeling system. The primers and the labeling buffer were thawed at room temperature. 5 µl of primers and 25 ng of *sto* gene were put into an eppendorf tube and the volume was completed to 50µl with sterile distilled water. The contents were mixed with a vortex, denatured in boiling water for 5 minutes and briefly centrifuged to collect the contents at the bottom of the tube. After the addition of 10 µ labeling buffer the tube was placed in ice. Following the addition of 2 µl enzyme the tube was incubated at room temperature for several minutes. A 5µl aliquot of radiolabeled dCTP (P^{32}) was mixed gently by pipetting, eppendorf was placed into a metal container and incubated 30 minutes at 37^{o}C. During the incubation period a Sephadex G-50 Pasteur pipette column was prepared and washed with TE buffer. The labeled probe was taken out from 37^{o}C and applied to the column. Small fractions of labeled probe (200 µl) were obtained with the application TE buffer. From each fraction 2µl aliquot was analyzed with Beckmann Scintillation counter (detectable counts) and the tubes with the highest cpm values were selected for labeling. The contents of the selected tubes were mixed, denatured in boiling water for 5 minutes and then

chilled on ice for 5 minutes. The labeled probe was then added to the pre-hybridized membrane and left for hybridization at 42^{o}C overnight in rolling tube hybridization oven. The following day membrane was washed twice for 30 minutes with pre-heated stringency wash solution (Appendix H). Finally the excess wash solution on the membrane was placing the membrane between two sheets of Watmann paper and membrane was incubated 2 days in phosphoimager cassette. After 2 days the film was scanned by using Phosphoimager.

2.2.1.7. Analysis of Putative T_0 Transgenic Tobacco Plants

In order to verify the integration and expression of the transferred DNA, transformed plants were analyzed by different methods including Basta® leaf paint assay, PCR analysis and regeneration test under salt stress.

2.2.1.7.1. Basta® Leaf Paint Assay

The putative transgenic plants in the pots were tested with leaf paint assay for their tolerance to commercially available form of PPT, Basta®. Equal amounts of 0,75% concentration of the herbicide were applied with cotton pieces on selected leaves of putative transgenic and control tobacco plants. The photographs of herbicide applied leaves were taken at the same day of application and after 7 and 10 days of application.

2.2.1.7.2. PCR Analysis

Screening of putative transgenic tobacco lines was done by PCR. Genomic DNA was isolated from each line by avoiding contamination of foreign DNA and attack by DNAses. Genomic DNA of independent lines of putative transgenic and control plants were isolated and amplified by gene specific primers.

2.2.1.7.2.1. Genomic DNA Isolation

The genomic DNA isolation from plants was based on CTAB DNA extraction method of Saghai-Maroof *et al*., 1984 with some modifications. 1.0-1.5 grams of fresh leaf sample was grinded in mortar by using liquid N_2. The powder was transferred to sterile centrifuge tubes and 5 ml of pre-heated (65^{o}C) 2X CTAB solution (Appendix I) was added. The tube was incubated at 65^{o}C for at least 30 minutes with occasional stirring. After the incubation, 15 ml of chloroform:isoamylalcohol (24:1) was added and the content was mixed vigorously. The centrifugation at 3000 rpm for 10 minutes was carried out and upper phase was taken into sterile centrifuge tube containing 3 ml of 5X CTAB solution (Appendix I). Equal amount of chloroform:isoamylalcohol was added to the tube and another centrifugation at 3000 rpm for 10 minutes was carried out. The upper phase was transferred to a sterile tube and incubated with equal amount of isopropanol at –20^{o}C at least for 1 hour. The DNA was obtained in the pellet after the centrifugation at 9000 rpm for 15 minutes. DNA was washed with 15 ml of 70% ice-cold ethanol, centrifuged at 9000 rpm for 10 minutes and the supernatant was discarded. The pellet was dried at 37^{o}C completely and dissolved in 500 µl of TE-RNAse (20 mg/ml). It was incubated for 30 minutes at 37^{o}C for RNAse activity. The purity and concentration of DNA was checked by spectrophotometry and agarose gel. The prepared DNA can be stored at –20^{o}C for further usage.

2.2.1.7.2.2. PCR Conditions

The isolated DNA from putative transgenic plants and control plant were analyzed with PCR for the presence of the *sto* gene. The PCR conditions and program used for PCR amplification are given below.

Table 2.5. Optimized conditions to amplify *sto* from plant genomic DNA by PCR

Template DNA (1μg)	X μl
STO1 (10 pM)	1 μl
STO2 (10 pM)	1 μl
dNTP (2.5mM)	2 μl
10X PCR buffer for Taq	2.5 μl
$MgCl_2$ (25 mM)	2 μl
Taq DNA Polymerase	0.5 μl
DH_2O	16-X μl
Total volume	25 μl

Table 2.6. PCR cycling conditions for amplification of *sto* using Taq.

94^{o}C 5 min	
94^{o}C 30 sec	
55^{o}C 30 sec	40 cycles
72^{o}C 1.5 min	
72^{o}C 7 min	

An aliquot of amplification products were separated on 1 % agarose gels in TBE buffer under constant voltage of 60 Volts as described in 2.2.1.3.3. Gels were visualized under UV transilluminator and photos were taken.

2.2.1.7.3. Regeneration Tests Under Salt Stress

Leaf samples from control and putative transgenic plants were tested for their ability of regenerate on simple MSB without PPT, MSB+5 mg/L PPT, MSB+150 mM NaCl and MSB +5 mg/L PPT+150 mM NaCl. After one month the photographs of the plates were taken and the regeneration was scored by weighing the samples and counting the number of shoots emerging from each sample.

2.2.1.8. Analysis of Tobacco T_1 Progeny

In order to verify the integration, expression and inheritance of the transferred DNA, putative T_1 transgenic plants of tobacco were analyzed by different methods like Mendelian inheritance analysis, PCR analysis, seed germination test under stress conditions and MDA assays.

2.2.1.8.1. Mendelian Inheritance Pattern

Seeds of tobacco plants coming from putative transgenic and control plants were surface sterilized in 30% hypochloride for 20 minutes in eppendorf tubes with shaking. Later hypochloride was removed and seeds were rinsed 3 times with sterile distilled water. Surface sterilized seeds were placed on MS medium containing 5 mg/L PPT. The plates were incubated 1 month at 25±2^{o}C with 16 hours light and 8 hours dark cycle. The number of surviving plantlets on PPT medium was calculated and scored for the Mendelian inheritance pattern. PPT resistant plantlets were later used for the isolation of DNA for PCR analysis.

2.2.1.8.2. PCR Analysis

PCR analysis of T_1 plants was performed as described in section 2.2.1.7.2.2 using PPT resistant T_1 plantlet DNAs as template

2.2.1.8.3. Seed Germination Test Under Stress Conditions

Seeds of tobacco plants coming from putative transgenic and control plants were surface sterilized as described in 2.2.1.8.1. Surface sterilized seeds were placed on MS without NaCl (control), MS+150 mM NaCl and MS+200 mM NaCl medium plates for salt stress treatments. The plates were incubated 6 weeks at 25±2^{o}C with 16 hours light and 8 hours dark-light cycle.

After salt stress treatments photographs of the plates were taken and surviving plants were counted. The fresh weights of the individual plantlets were weighed and necessary amounts of samples were set a side for MDA and assays.

2.2.1.8.4. MDA Assay

About 0.2 g of tissue was extracted for MDA assay. The extraction was carried out in glass-glass homogenizer in 1 ml 5% trichloroacetic acid. The homogenate was centrifuged in microcentrifuge at 12000 rpm for 15 minutes at room temperature. Equal volumes of supernatant and 2-thiobarbituric acid (0.5% in 20% (w/v) trichloroacetic acid) were mixed in a new eppendorf tube and the contents were mixed with shaking. The mixture was incubated at 96^{o}C for 25 minutes. After incubation they were put into ice until they reach the room temperature.

Following centrifugation at 10000 rpm for 5 minutes the absorbance value of the supernatant was recorded at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. MDA contents were calculated using an extinction coefficient 155 $M^{-1}cm^{-1}$.

2.2.1.9. Analysis of *Arabidopsis* T_1 Progeny

In order to verify the integration, expression and inheritance of the transferred DNA, T_1 seeds coming from PPT selected T1 *Arabidopsis* plants were analyzed by different methods like seed germination test under salt stress conditions and MDA assay. Measurements were taken in Shimadzu UV mini 1240 UV-VIS spectrophotometer.

2.2.1.9.1. Seed Germination Test Under Stress Conditions

Seeds of *Arabidopsis* plants coming from putative transgenic and control plants were surface sterilized as described in 2.2.1.9.1. Surface sterilized seeds were placed on MS without NaCl (control), MS+50 mM NaCl, MS+100 mM NaCl and MS+200 mM NaCl medium plates for salt stress treatments. The plates were incubated 1 month at 25±2^{o}C with 16 hours light and 8 hours dark-light cycle.

After salt stress treatments photographs of the plates were taken and surviving plants were counted. The fresh weights of these individual plantlets were weighed and necessary amounts of samples were set a side for MDA (2.2.1.8.4) assay.

CHAPTER 3

RESULTS

3.1. Amplification of *sto* Gene

The amplification of *sto* gene was done by using PCR. The template DNA used was a cDNA library of *Arabidopsis thaliana* and the primers used were gene specific primers (STO1 and STO2) designed by Dr.Laszlo Szabados. The enzyme elongase, that has the 3'-5' proof-reading activity was chosen to decrease the error frequency. In Figure 3.1 a band with a size of 868 bp belonging to the amplified fragment can be observed.

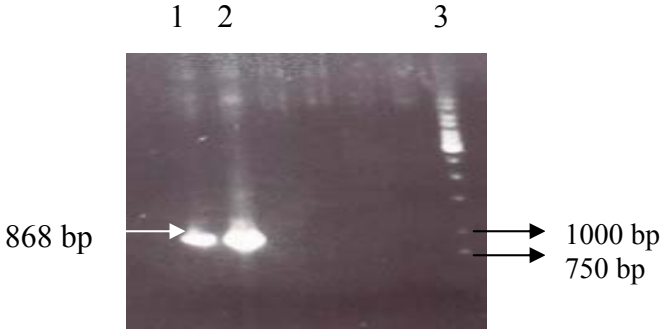


Figure 3.1. Agarose gel electrophoresis result of PCR amplified *sto* gene (Lane 1: PCR amplified *sto* gene using *Arabidopsis* cell suspension cDNA template, Lane 2: PCR amplified *sto* gene using *Arabidopsis* plant cDNA library template, Lane 3: DNA ruler).

The PCR amplified *sto* fragment was extracted from the agarose gel as described in section 2.2.1.4.1.

3.2. Cloning of *sto* in pBSK

Before cloning in plant transformation vector *sto* gene was cloned in pBSK. BamHI site of pBSK was used to clone *sto* amplified by PCR. For this purpose both pBSK and isolated fragment were digested with BamHI restriction enzyme and ligated. After the transformation of this plasmid to *E.coli*, positive colonies that grew on ampicillin plates were tested for the presence of the gene both by PCR (Figure 3.2.) with STO1 and STO2 primers and by test digestions with BamHI.

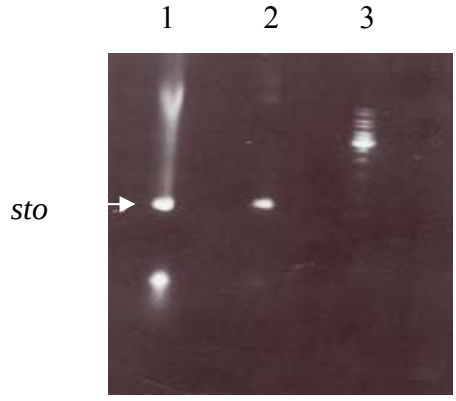


Figure 3.2. Analysis of pBSK-STO for the presence of *sto* gene (Lane 1: PCR amplification of *sto* gene using STO1 and STO2 primers using pBSK-STO template, Lane 2: BamHI digest of pBSK-STO, Lane3: DNA Ruler).

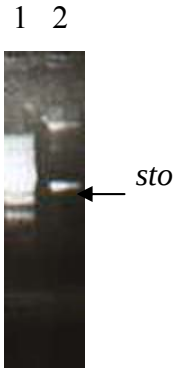


Figure 3.3. Agarose gel electrophoresis results of BamHI digestion of positive colonies (Lane 1: DNA Ruler, Lane 2: BamHI digest).

3.3. Sequence Analysis of *sto*

Sequence analysis of the promising colonies were done at laboratory of Dr. Laszlo Szabados, Biological Research Center, Szeged, Hungary using ABI PRISM 377 DNA sequencer (Applied Biosytems). Sequence analysis of positive colonies showed that an error free full-length *sto* was cloned in pBSK (Figure 3.4). The coding region of *sto* is located between 123 and 868 nucleotides. This region codes for a 249 amino acid protein.

```
    1 tctgaaccta cgcttctgct aagctattct aagagaagcc agactagcaa taaacccttc
   61 attttaagca ttctgtttcc ttcttgagaa acctagatat tttggtttct tgtatccggt
➔121 gATGaagata cagtgtgatg tgtgtgagaa agctccggcg acggtgattt gttgcgccga
  181 cgaagctgct ctctgtcctc aatgcgacat cgagattcac gccgctaaca aactcgctag
  241 caagcaccaa cgtcttcatc ttaattccct ctccaccaaa ttccctcgtt gcgatatctg
  301 ccaagagaag gcagctttca ttttctgtgt agaggataga gctctgcttt gcagggactg
  361 cgatgaatcc atccacgtgg ctaattctcg atctgctaat caccagaggt tcttagccac
  421 tgggatcaaa gtagctctga cctcaactat atgtagtaaa gaaattgaga agaatcaacc
  481 tgagccttcc aacaaccaac agaaggctaa tcagattcct gctaaatcca caagccagca
  541 gcaacaacaa ccttcttctg ctactccact tccctgggct gttgacgatt tctttcactt
  601 ctctgatatt gaatccaccg acaagaaagg acagcttgat cttggggcag gggagttgga
  661 ttggttttca gacatgggat tcttcggtga tcagattaat gacaaggctc ttcctgcagc
  721 tgaagttcct gagctttctg tttcgcattt aggtcatgtt cattcataca aacctatgaa
  781 gtcaaatgtt tcacacaaga agccgaggtt tgagaccaga tatgatgatg atgatgagga
➔841 acacttcatt gtccctgatc ttggcTAAaa agctatatgt aatctatgtg tagacattct
  901 tcaatgtaaa agaacaaaca agaaacctat ctgcatgtgt ggagttaatg tcatatacat
  961 tttagttttg tcttaagttg tgtaagatat gttgagagct tataacaaat gtctgtgttt
 1021 gagttaaaaa aaa
```

Figure 3.4. Sequence analysis of *sto*

3.4. Construction of Plant Transformation Vector

The plant transformation vector pPCVB1 was obtained by the removal of RFP (red fluorescence protein) gene from pPCVB-RFP. pPCVB-RFP contains Cauliflower mosaic virus 35S promoter that enables constitutive expression of the

cloned gene. The plasmid bears Basta® resistance for plant selection and ampicillin resistance for bacterial selection. The RFP gene present in the plasmid is for localization studies, which is not required for our transformation studies. For removal of RFP gene, pPCVB-RFP plasmid was digested with SacI and SmaI enzymes, treated with Klenow enzyme and religated. After the transformation to *E.coli* ampicillin resistant positive colonies were digested with several restriction enzymes to test the removal of RFP fragment.

First test digestion was done by SacI and SmaI enzymes and showed that out of 28 selected colonies only 4 of them were still containing the plasmid with RFP gene (Figure 3.5).

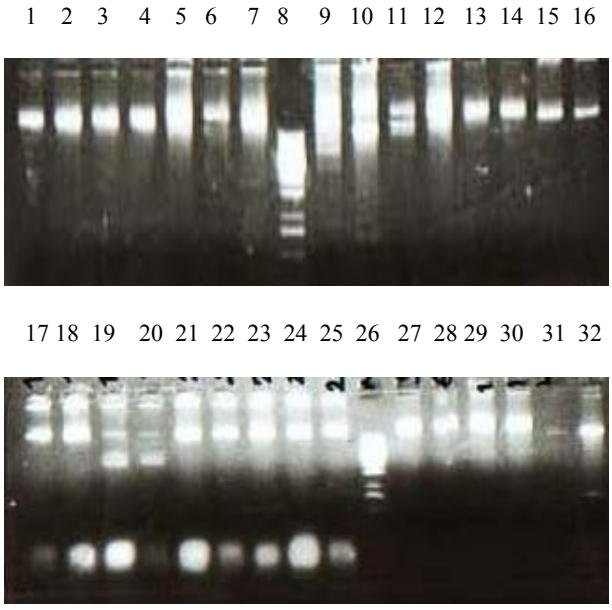


Figure 3.5. Agarose gel electrophoresis results of SacI and SmaI double digestion of positive colonies.

Lanes 1-7, 9, 12-18, 21-25, 27-32: plasmids lacking RFP gene, Lanes 10,11, 19, 20: plasmids containing RFP gene, Lanes 8, 26: DNA ruler.

Further restriction enzyme digestions of same colonies were done by using EcoRI and XbaI. The results of this digestions showed that there was 18 colonies containing the newly constructed plant transformation vector namely pPCVB1 that lacks the RFP gene (Figure 3.6).

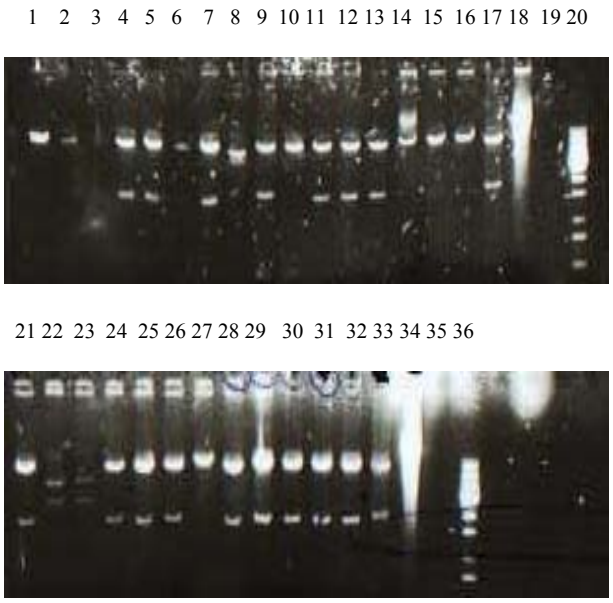


Figure 3.6. Agarose gel electrophoresis results of EcoRI and XbaI double digestion of positive colonies.

Lanes 4-5, 7,9, 11-13, 17, 21, 24-26, 28-33: plasmids lacking RFP gene, Lanes 18, 34: undigested plasmid, Lanes 20, 36: DNA ruler.

Out of these 18 colonies 6 of them were selected and tested again by a different restriction enzyme HindIII. Originally there are two cutting sites of this enzyme in pPCVB-RFP; one in promoter region and the other in RFP gene. As a result, if these selected colonies do not have the RFP gene the digestion will only result in linearization of the plasmid, whereas the presence of the RFP gene will give a band of size around 1000 bp. The results of this digestion (Figure 3.7) proved all of the selected colonies contain the newly constructed pPCVB1 plasmid lacking RFP gene. The plasmid pPCVB1 (Figure 3.8) was later used for the cloning of *sto* and transformation of *Arabidopsis* and tobacco.

Figure 3.7. Agarose gel results of HindIII digestion of positive colonies. Lanes 1-6,11-17: plasmids lacking RFP gene, Lane 7: pPCVB-RFP, Lane 8: undigested plasmid, Lane 9, 19: DNA ruler.

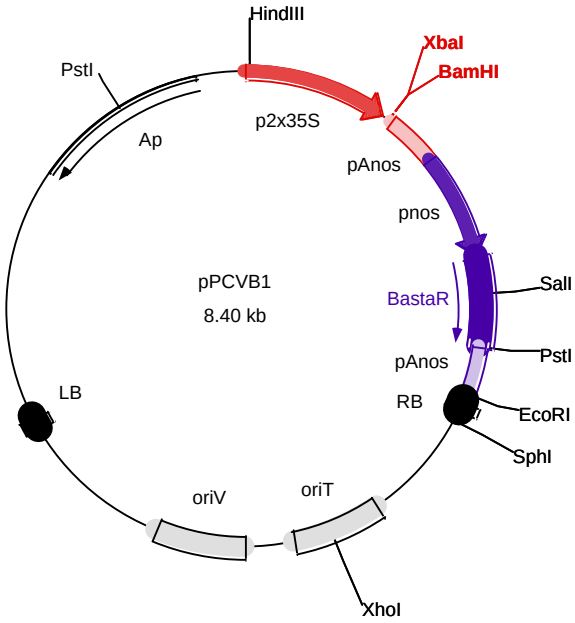


Figure 3.8. Map of pPCVB1.

p2X35S: CaMV promoter, pnos: nopaline synthease terminator, LB: left border RB: right border, Ap: ampicillin resistance gene, BastaR: Basta resistance gene, oriV, oriT: origin of replication.

3.5. Cloning of *sto* in Plant Transformation Vectors

The last step of the plasmid construction was to clone *sto* gene into the plant transformation vector. The *sto* gene (868 bp cDNA) was cloned into pPCVB1 vector under the control of a strong constitutive, CaMV 35S promoter both in sense and anti-sense orientations. The *sto* gene was also cloned in pPCVB812 in sense direction.

3.5.1. Cloning in pPCVB1

The gene *sto* was removed by digesting the pBSK-STO with BamHI and ligated into pPCVB1 that was also digested with the same enzyme. Digested samples were separated by agarose gel electrophoresis and extracted from the gel as described previously in section 2.2.1.3.3. and 2.2.1.3.4. Ligation of *sto* gene at BamHI site of pPCVB1 was carried out overnight and this plasmid was used to transform *E.coli* cells. From the ampicillin resistant colonies positive ones carrying the gene of interest was selected by restriction enzyme digestions.

First check digestion was done using BamHI restriction enzyme to cut out the *sto* gene if present. The result of digestion was shown by agarose gel electrophoresis in Figure 3.9. Out of 28 ampicillin resistant colonies 9 of them seemed to be containing the *sto* gene.

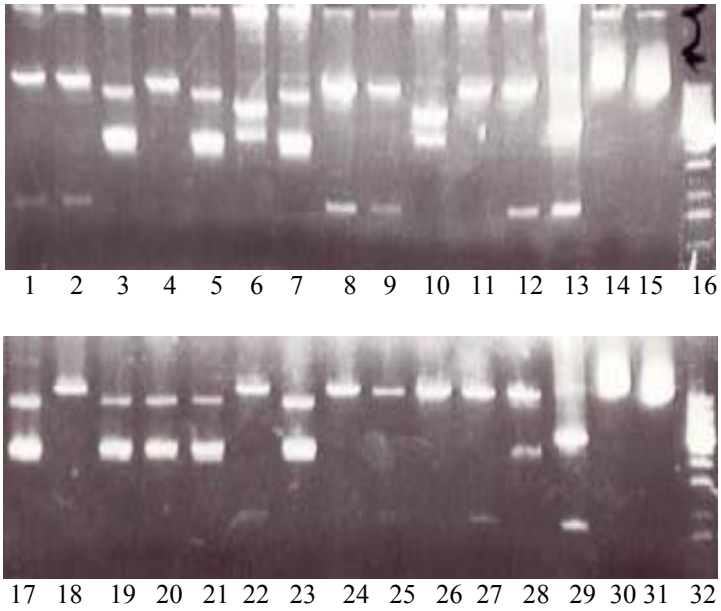


Figure 3.9. Agarose gel electrophoresis results of BamHI test digestion after ligation of *sto* to pPCVB1.

Lanes 1,2,8,9,12,18,22,25,27 contain pPCVB1-STO plasmids
Lanes 13, 29 pBSK-STO plasmids
Lanes 16, 32 DNA ruler

In order to check the orientation of *sto* gene in these 9 positive colonies having pPCVB1-STO, restriction enzyme digestions with XbaI-SacI and PstI was performed. The results of these digestions are shown in Figures 3.10 and 3.11.

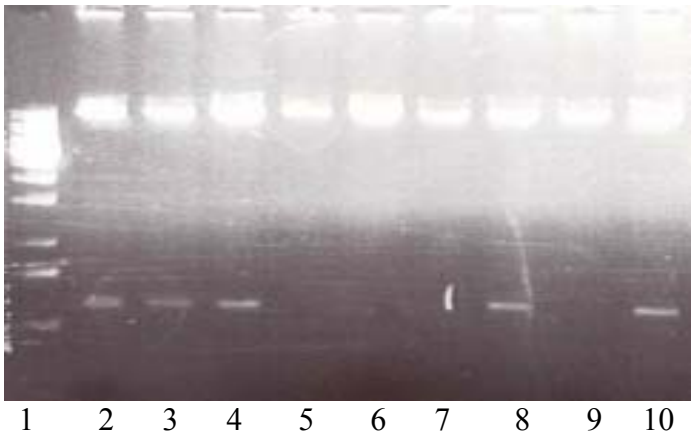


Figure 3.10. Agarose gel electrophoresis results of pPCVB1-STO digested with XbaI and SacI.
Lanes 2, 3,4,8,10 pPCVB1-STO-AS plasmids
Lanes 5,6,7,9 pPCVB1-STO-S plasmids

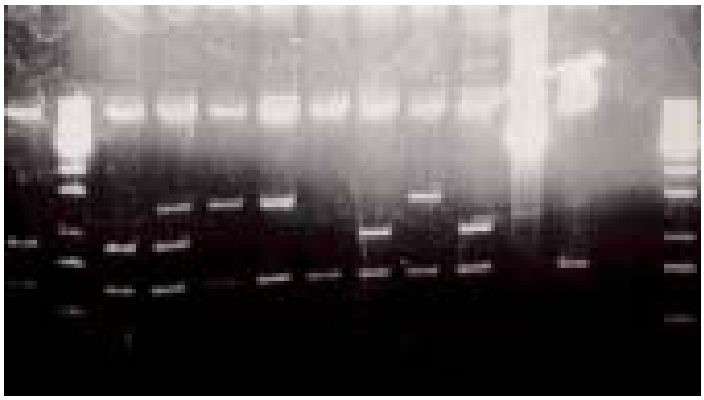


Figure 3.11. Agarose gel electrophoresis results of pPCVB1-STO digested with PstI.
Lanes 1,3,8,10 pPCVB1-STO-AS
Lanes 5,6,9 pPCVB1-STO-S
Lanes 2, 14 DNA ruler

The plant transformation vectors pPCVB1-STO-S (Figure 3.12) and pPCVB1-STO-AS were later used for the transformation of tobacco and *Arabidopsis*.

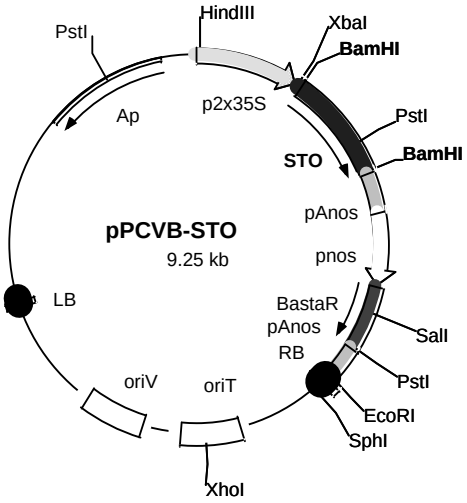


Figure 3.12. Map of pPCVB1-STO-S.

p2X35S: CaMV promoter, pnos: nopaline synthease terminator, LB: left border RB: right border, Ap: ampicillin resistance gene, BastaR: Basta resistance gene, oriV, oriT: origin of replication.

3.5.2. Cloning in pPCVB812

Another plant transformation vector was constructed for the transformation of *Arabidopsis*. The plasmid used was pPCVB812 (Figure 3.13) that has ampicillin resistance for bacterial selection and hygromycin resistance for plant selection. *Sto* gene was removed from pBSK-STO by BamHI digestion and ligated to BamHI site of pPCVB812 in sense direction. This plasmid was constructed in Dr.Laszlo Szabados' laboratory and named as pPCVB812-STO.

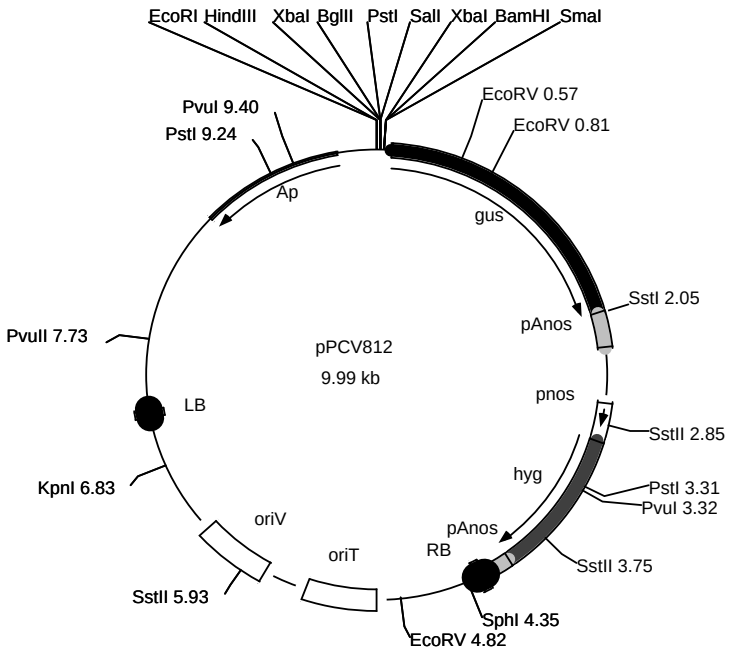


Figure 3.13. Map of pPCVB812.

pnos: nopaline synthease terminator, LB: left border RB:right border,
Ap: ampicillin resistance gene, oriV, oriT: origin of replication,
hyg: hygromycin resistance gene.

3.6. Transformation and Selection of Plants

Transformation studies to generate *sto* expressing plants were carried out for two different species; *Nicotiana tabacum* and *Arabidopsis thaliana.* For tobacco plants the aim was the expression of *sto* gene that is not originally present in its genome. However for *Arabidopsis* the goal was to produce lines that are either overexpressing *sto* that is naturally present in the plant or to produce lines that have *sto* in anti-sense orientation.

3.6.1. *Nicotiana tabacum*

Tobacco leaves were transformed with pPCVB1-STO-S and pPCVB1-STO-AS binary vectors using *Agrobacterium* mediated leaf disc gene transformation method. The first step was to transform these plants to *Agrobacterium tumefaciens* GV3101/MP90RK strain by direct transformation. After the selection on carbenicillin, kanamycin and rifampicin containing plates, plasmids from the positive colonies were isolated and digested with restriction enzymes to verify the presence of the gene of interest (Figure 3.14).

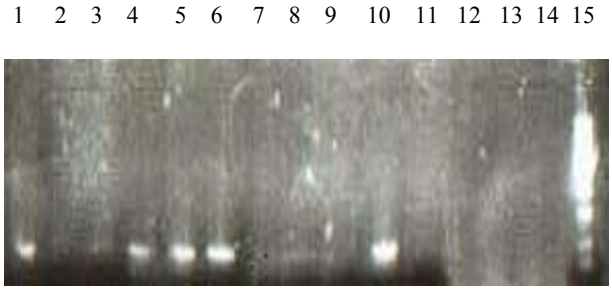
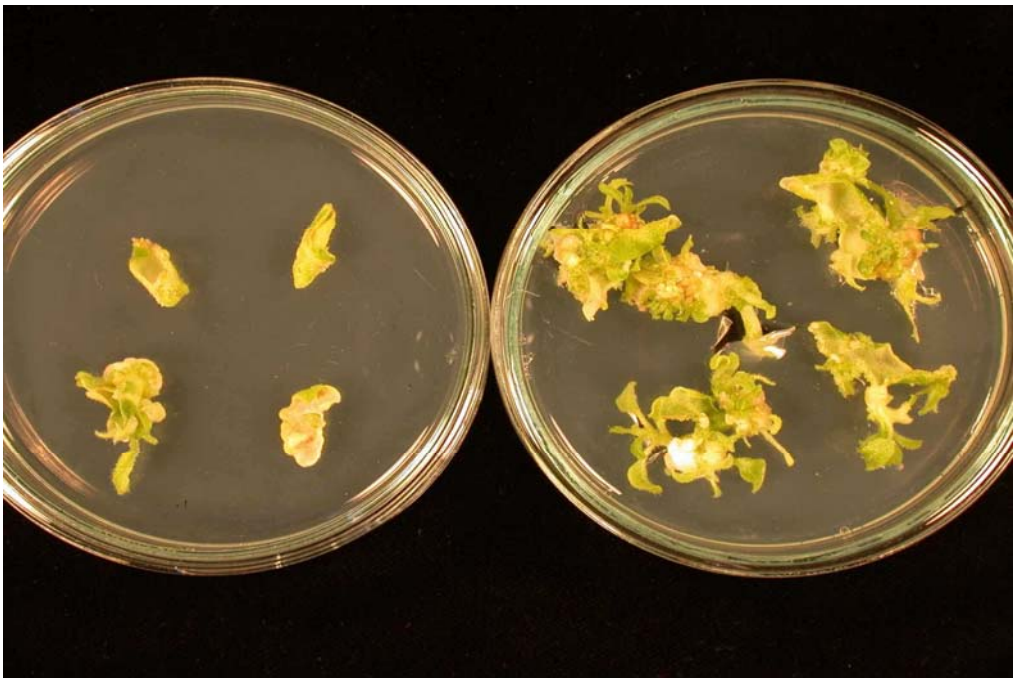


Figure 3.14. Agarose gel results of BamHI digestion of positive colonies isolated from *Agrobacterium.*

The leaves of tobacco of the same age were used for *Agrobacterium* mediated transformation. The putative transgenic plants were regenerated on MSB regeneration medium containing 5 mg/L PPT. 2-3 weeks after the transformation shoots started to emerge. Control plants did not initiate any roots and they died after 2 weeks (Figure 3.15). The regeneration efficiency of transgenic plants carrying *sto* in sense orientation was observed to be much more higher than that of anti-sense *sto* carrying plants (Figure 3.16 A,B, Figure 3.17). On the average each leaf disk transformed by pPCVB1-STO-S produced 3 shoots that can be transferred to root initiation media, whereas this value was only 1 for pPCVB1-STO-AS (Figure 3.18). These shoots were cut from their bases and transferred to root inducing media containing 5 mg/L PPT (Figure 3.19). Further selection and subculturing on PPT containing medium gave rise to 38 individual lines that were transformed by *sto* in sense direction. A representative photo of T_1 plant is given in Figure 3.20. On the other hand only 3 lines were obtained from the tobacco plants transformed by *sto* in anti-sense orientation. As a control wild type tobacco plants were also put to the same medium.

Figure 3.15. Control *Nicotiana tabacum* SR-1 leaf disks on PPT selection.



A **B**

Figure 3.16. Callus development of transformed tobacco leaf discs on PPT containing regeneration media.

A. Transformation with pPCVB1-STO-AS (5 mg/L PPT selection)

B. Transformation with pPCVB1-STO-S (5 mg/L PPT selection)

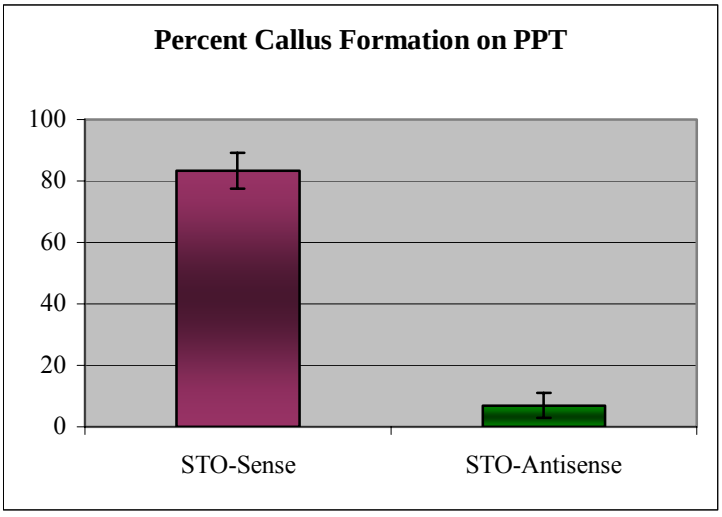


Figure 3.17. Regeneration efficiency of STO-Sense and STO-Antisense putative transgenics on selective media.

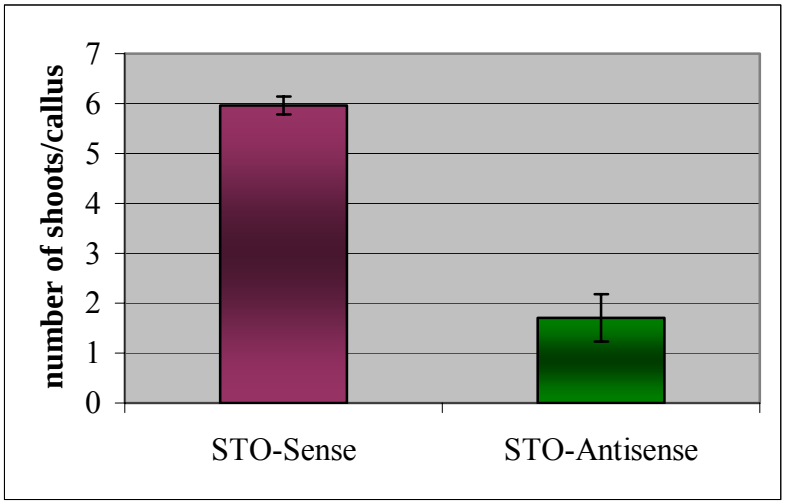
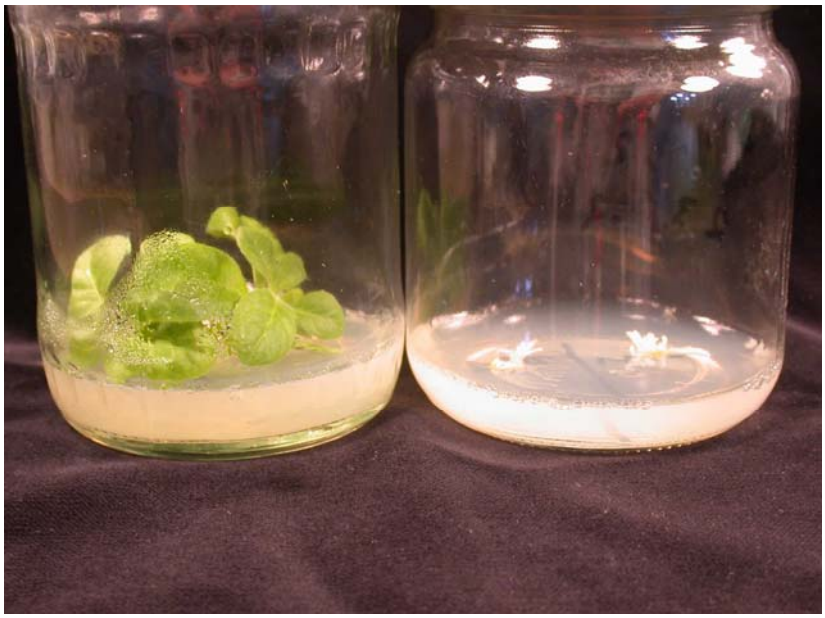


Figure 3.18. Number of shoots regenerated from STO-Sense and STO-Antisense putative transgenics on selective media.



A **B**

Figure 3.19. Development of plantlets on 5mg/L PPT containing media after transformation.

A. Putative transgenic plants carrying *sto*.

B. Control *Nicotiana tabacum* SR-1.

Later the plants were transferred into pots and further growth was achieved at growth chamber (Figure 3.21). Prior to flowering plants were covered with plastic bags to prevent cross-pollination. T_1 generation was obtained from all putative transgenic lines.

Figure 3.20. The growth of a putative transgenic tobacco plant containing pPCVB1-STO-S on 5 mg/L PPT containing media.

Figure 3.21. The growth of a putative transgenic tobacco plant containing pPCVB1-STO-S in soil.

3.6.2. *Arabidopsis thaliana*

The same binary vectors; pPCVB1-STO-S and pPCVB1-STO-AS and another binary vector pPCVB812-STO were used for the transformation of *Arabidopsis.* The procedure for the selection of positive colonies was the same, the only difference was the method used for plant transformation. *Arabidopsis* seedlings having rosette inflorescence shoots were transformed using vacuum infiltration method. Selection of transformed plants was carried out on MS containing 15 mg/L PPT (Figure 3.22). The efficiency of vacuum infiltration is not as high as leaf disc transformation method, as a result eight *Arabidopsis* plants carrying pPCVB1-STO-S and eight *Arabidopsis* plants carrying pPCVB1-STO-AS were observed to be resistant to PPT.

Plants carrying pPCVB812-STO were selected for their resistance to hygromycin (15 μg/ml) and 11 individual putative transgenics were obtained. After selection plants were transferred first to MS containing jars (Figure 3.23) and later to soil for further growth.

Figure 3.22. *A.thaliana* transformed with pPCVB1-STO-S selected on MS containing 15 mg/L PPT.

Figure 3.23. The growth of a putative transgenic *Arabidopsis* plant containing pPCVB1-STO-S on MS media.

3.7. Expression of *sto* Gene in Wild Type *Arabidopsis thaliana*

In order to examine whether expression of *sto* is induced by different treatments, *Arabidopsis* seedlings were subjected to various stress conditions and plant growth regulators. The results of the expression studies gave an idea at which conditions the expression of *sto* shows differences.

3.7.1. Analysis of *sto* Expression Under Abiotic Stress Conditions

Three weeks old *Arabidopsis* seedlings were transferred to liquid culture and certain stress treatments like NaCl (300 mM), sucrose (8%), cold (4^{o}C) were applied for 1, 6 and 24 hours. Total RNAs were isolated from treated and control plants, fractionated on 1% agarose gel and transferred onto nylon membrane and expressions were determined as described in section 2.2.1.6. The expression of *sto* under different conditions was compared with the expression of ubiqutine (ubq) that is constitutively expressed in the plant.

As shown in the Figure 3.24 the expression of *sto* was induced by sucrose and cold, however there was no change in expression at NaCl treatment.

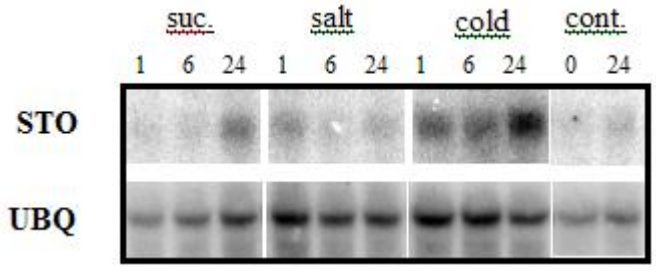


Figure 3.24. Expression of *sto* under abiotic stress conditions.

3.7.2. Analysis of *sto* Expression Under Plant Growth Regulator Treatments

The same kind of expression analysis under plant growth regulator treatments was also done for three weeks old *Arabidopsis* seedlings. As plant growth regulators treatments ABA (100 mM), 2,4-D (10 mM), Ethylene (10 mg/l), GA_3 (100 mM), JA (10 mM) and SA (10 mM) were applied for 1, 6 and 24 hours. Total RNAs were isolated from treated and control plants, fractionated on 1% agarose gel and transferred onto nylon membrane and expressions were determined as describe in section 2.2.1.6. The expression of *sto* under different conditions was compared with the expression of ubiqutine (ubq) that is constitutively expressed in the plant.

After ABA, ethylene and gibberellic acid treatments the expression of *sto* was observed to be slightly induced at 24 hours (Figure 3.25). Overall, growth regulators had apparently no or only mild effects on *sto* expression.

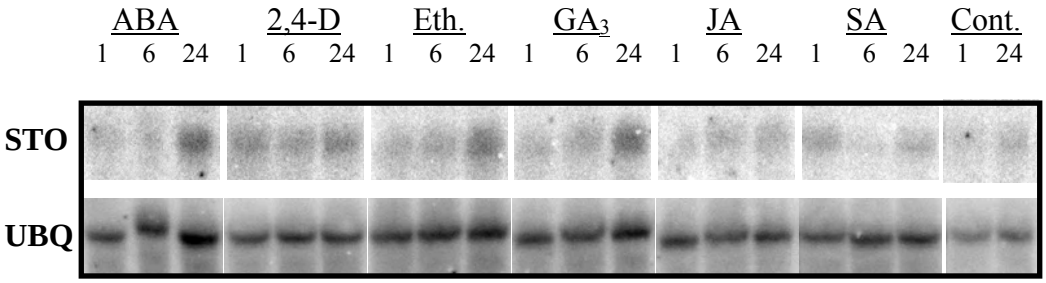


Figure 3.25. Expression of *sto* under plant growth regulator treatments.

3.8. Analysis of T_o Putative Tobacco Transgenic Plants

The putative transgenic tobacco T_0 lines were analyzed in order to prove the presence of *sto* by using certain tests. For this purpose Basta® leaf paint assay, PCR and Northern Blot Analysis were performed. Also these T_0 plants were analyzed for their ability to regenerate under salt stress.

3.8.1. Basta® Leaf Paint Assay

The putative transgenic plants were tested with leaf paint assay for their tolerance to commercially available form of PPT, Basta®. 0,75% concentration of herbicide was applied to the leaves of transgenic and control plants. The results (Figures 3.26, 3.27, 3.28) clearly indicated that the transgenic plants can tolerate the applied herbicide dosage without any significant observable lesion contrary to the lesions and necrosis taking place at the leaves of control plants.

A **B** **C**

Figure 3.26. Photographs of Basta® leaf paint assay of non-transformed tobacco plant.
A. The day of Basta® application,
B. 7 days after Basta® application,
C. 14 days after Basta® application.

A **B** **C**

Figure 3.27. Photographs of Basta® leaf paint assay of pPCVB-STO-S transformed tobacco plant (S/2-7)
A. The day of Basta® application
B. 7 days after Basta® application
C. 14 days after Basta® application

A B C

Figure 3.28. Photographs of Basta® leaf paint assay of pPCVB-STO-AS transformed tobacco plant (AS/3-1)
A. The day of Basta® application
B. 7 days after Basta® application
C. 14 days after Basta® application

3.8.2. PCR Analysis

Although selective media containing PPT was used at different stages of the regeneration and propagation experiments and Basta® leaf paint assay results also show the resistance of putative transgenic plants to the herbicide, further analysis was performed to verify the presence of T-DNA in T_0 putative transgenic plants. For this purpose CTAB DNA extraction was carried out for each putative T_o transgenic line. PCR analysis with STO specific primers was done to check the presence of *sto* gene.

From the results presented in Figure 3.29, it can be seen that out of 17 selected transgenic plants in 14 of them there was a band of expected size that is not present in control plants. This indicates the presence of the transferred *sto* gene in the transgenic plants.

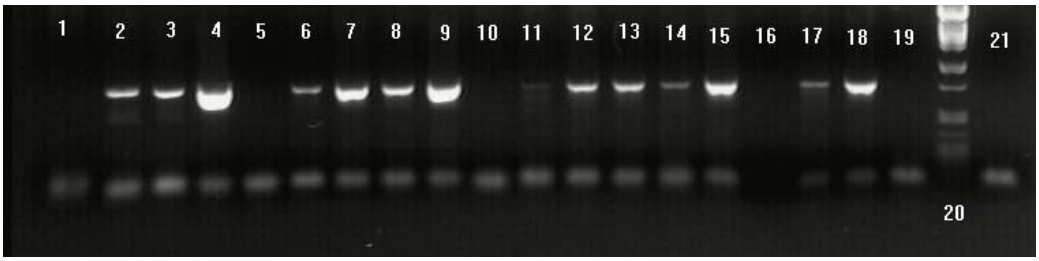


Figure 3.29. PCR analysis of T_o putative transgenic plants using STO gene specific primers. The samples used in PCR;

Lane 1. SR-1 Control **Lane 2.** STO-S/1-2 **Lane 3.** STO-S/1-3
Lane 4. STO-S/1-4 **Lane 5.** STO-S/1-10 **Lane 6.** STO-S/1-11
Lane 7. STO-S/1-12 **Lane 8.** STO-S/1-13 **Lane 9.** STO-S/1-25
Lane 10. STO-S/2-3 **Lane 11.** STO-S/2-4 **Lane 12.** STO-S/2-5
Lane 13. STO-S/2-7 **Lane 14.** STO-S/2-15 **Lane 15.** STO-S/3-6
Lane 16. Empty **Lane 17.** STO-S/3-1 **Lane 18.** STO-AS/3-1
Lane 19. STO-AS/3-2 **Lane 20.** λ PstI marker **Lane 21.** SR-1 Control

3.8.3. Northern Blot Analysis

The expression of *sto* in transgenic tobacco plants was determined by Northern Blot Analysis. The results of Northern hybridization showed the presence of a transcript that is lacking in the control plants. The intensities of the transcripts were observed to be variable at individual transgenic lines (Figure 3.30). According to the northern blot high level of expression was found in 7 transgenic lines S/1-25, S/2-3, S/2-7, S/2-10, S/3-6, S/3-7 and S/3-11. Medium level of expression was observed at lines S/1-3, S/1-9, S/1-12, S/2-5, S/2-14, S/2-15, S/3-2, S/3-9. The lines S/1-4, S/1-11, S/1-21, S/1-24, S/1-26, S/2-4, S/2-12, S/2-13, S/2-17, S/2-18, S/3-4, S/3-5, S/3-8, S/3-10, S/3-12 and AS/3-1, AS/3-2, AS/3-3 showed low or undetectable expression.

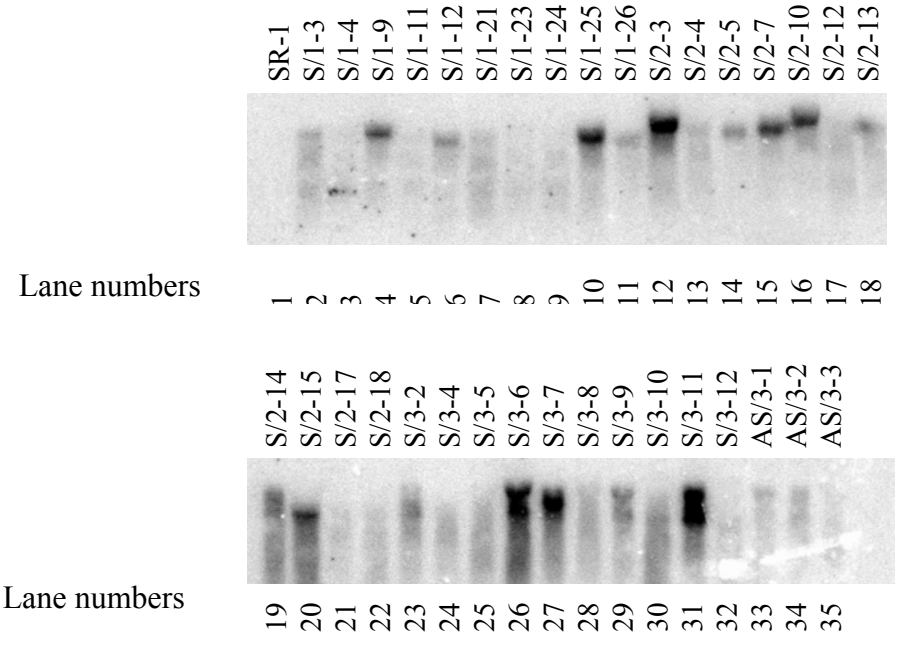


Figure 3.30. Northern blot analysis of T_0 tobacco plants.

Lane 1. Control SR-1 RNA

Lanes 2-32 STO-S putative transgenic plant RNAs

Lanes 33-35 STO-AS putative transgenic plant RNAs.

According to PCR results and Northern Blot Analysis certain lines that are expressing *sto* in low (S/1-4, S/2-4, AS/3-1), medium (S/1-3, S/1-12, S/2-4, S2-15), and high (S/1-25, S/2-3, S/2-7, S/3-11) levels were selected for further experiments.

3.8.4. Regeneration Test Under Salt Stress

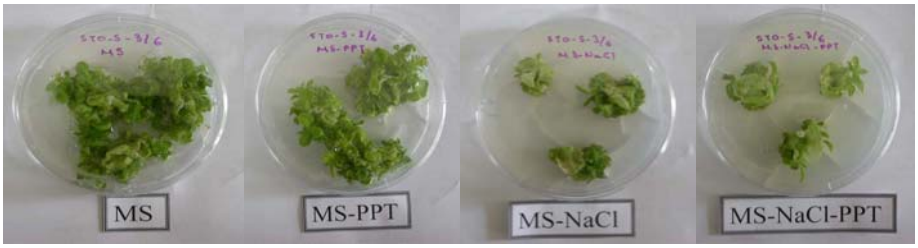
Leaf samples from control and putative transgenic plants were tested for their ability of callus formation and shoot initiation on different media.

i. Simple MSB without PPT: regeneration efficiency of PPT resistant and sensitive samples.
ii. MSB with 5 mg/L PPT: regeneration efficiency of PPT resistant samples.
iii. MSB with 150 mM NaCl without PPT: regeneration efficiency of PPT resistant and sensitive samples on NaCl containing media.
iv. MSB with 5 mg/L PPT and 150 mM NaCl: regeneration efficiency of PPT resistant samples on NaCl containing media.

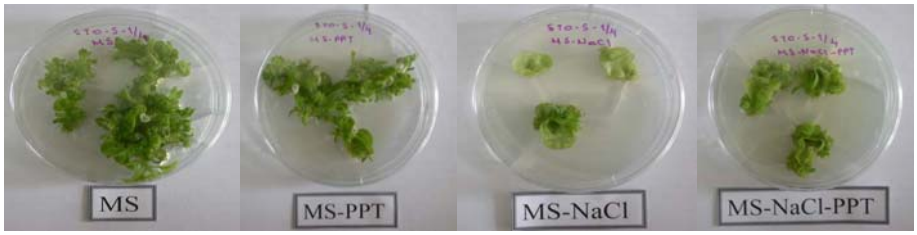
Representative photographs of the regeneration test are given in Figure 3.31. Figure 3.32 shows the regeneration efficiency on selective media with and without NaCl according to the callus weight that formed and Table 3.1 shows the number of shoots emerging from each sample on the media mentioned above.

All analyzed transgenic lines regenerated better on PPT containing and PPT+NaCl containing media.

A. STO-S/1-3



B. STO-S/3-6



C. STO-S/1-4

D. STO-AS/3-1

E. SR-1 Control

Figure 3.31. Regeneration photographs of putative STO transgenics and control plants on MS, MS+PPT, MS+NaCl and MS+PPT+NaCl containing plates

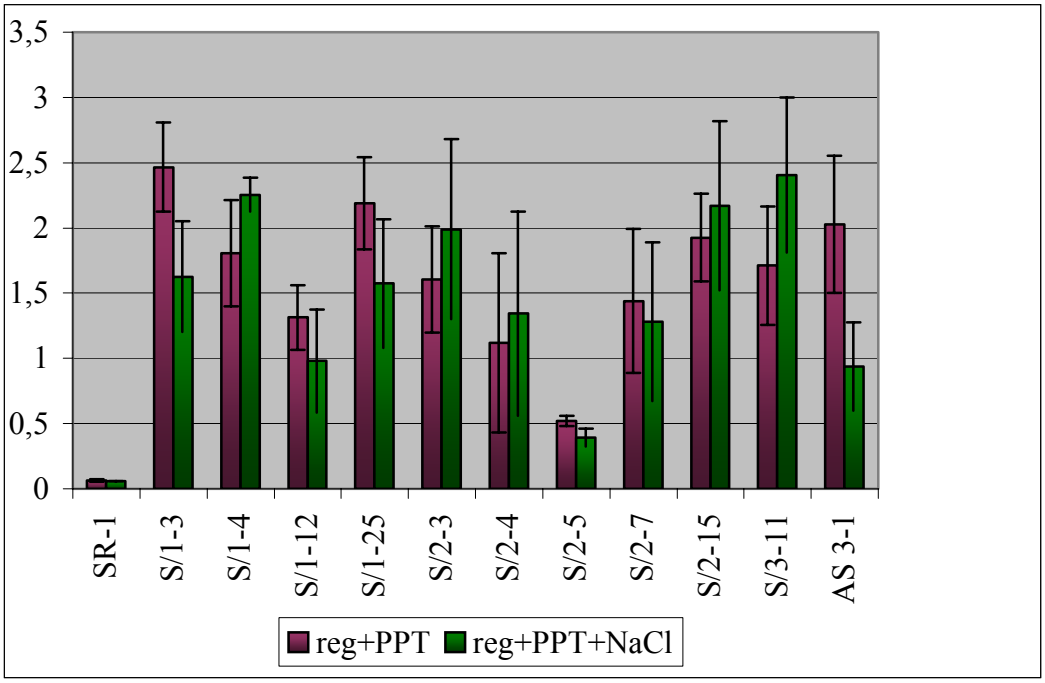


Figure 3.32. Regeneration efficiency of putative transgenic and control plants on selective media containing no NaCl and 150 mM NaCl with respect to the callus weight.

Table 3.1. Shoot formation of putative transgenic and control plants on MS, MS+PPT, MS+NaCl and MS+PPT+NaCl media.

	MS	MS+PPT	MS+150 mM NaCl	MS+PPT+ 150 mM NaCl
	# of shoots	# of shoots	# of shoots	# of shoots
SR-1	4,250±0,479	0	0	0
T_0 S/1-3	3,833±0,543	2,667±0,558	0,833±0,543	0,667±0,333
T_0 S/1-4	4,000±0,856	2,167±0,654	0	0,667±0,333
T_0 S/1-12	1,667±0,882	0,667±0,333	0,000±0	0
T_0 S/1-25	2,000±0,020	2,000±0,577	1,333±0,667	0
T_0 S/2-3	3,833±0,477	3,167±0,477	0	0,167±0,0567
T_0 S/2-4	2,667±0,882	0,000±0	0	0
T_0 S/2-5	4,000±0,577	1,333±0,333	0	0
T_0 S/2-7	5,667±1,450	2,000±0,577	1,667±0,333	0
T_0 S/2-15	2,833±0,654	1,833±0,307	0,167±0,0167	1,667±0,955
T_0 S/3-11	2,333±0,803	1,500±0,342	0,500±0,342	1,667±0,955
T_0 AS/3-1	5,333±0,843	3,000±0,856	0,167±0,016	0

3.9. Analysis of T_1 Tobacco Transgenic Plants

T_1 generation of transgenic plants was obtained by collecting the seeds from T_0 plants and germinating them in 5 mg/l PPT containing medium. The plants were later transferred to jars with selective rooting media. The rooted plants were transferred to soil and grown to maturity in green house (Figure 3.33). After self-pollination, flowering and capsule formation, T_2 seeds were collected.

Figure 3.33. Growth of T_1 transgenic plants in the green house.

T_1 generation seedlings were analyzed:

i. Molecular analysis; PCR and Northern Blot analysis.

ii. Physiological and biochemical analysis; Germination of seeds in PPT selective media, Germination test, fresh weight measurements and MDA assays under salt stress.

3.9.1. Molecular Analysis

Molecular analysis of T_1 transgenic plants was done by using Mendelian inheritance analysis, PCR and Northern Blot Analysis.

3.9.1.1 Mendelian Inheritance Analysis

In order to evaluate the segregation of the transformed gene, T_1 seeds were germinated in selective media containing 5 mg/l PPT. Phenotypic ratio for PPT resistant and sensitive seedlings were evaluated by counting the number of survivors on selective media. Figure 3.36 shows the appearance of PPT resistant and sensitive seedlings. For each line, the experiment was repeated three times by certain number of seeds and the cumulative results are given in Table 3.2. The expected 3:1 ratio of Mendelian inheritance is evaluated by using Chi-square Analysis with $p(\chi^2_1 \leq 3.841)=0.95$.

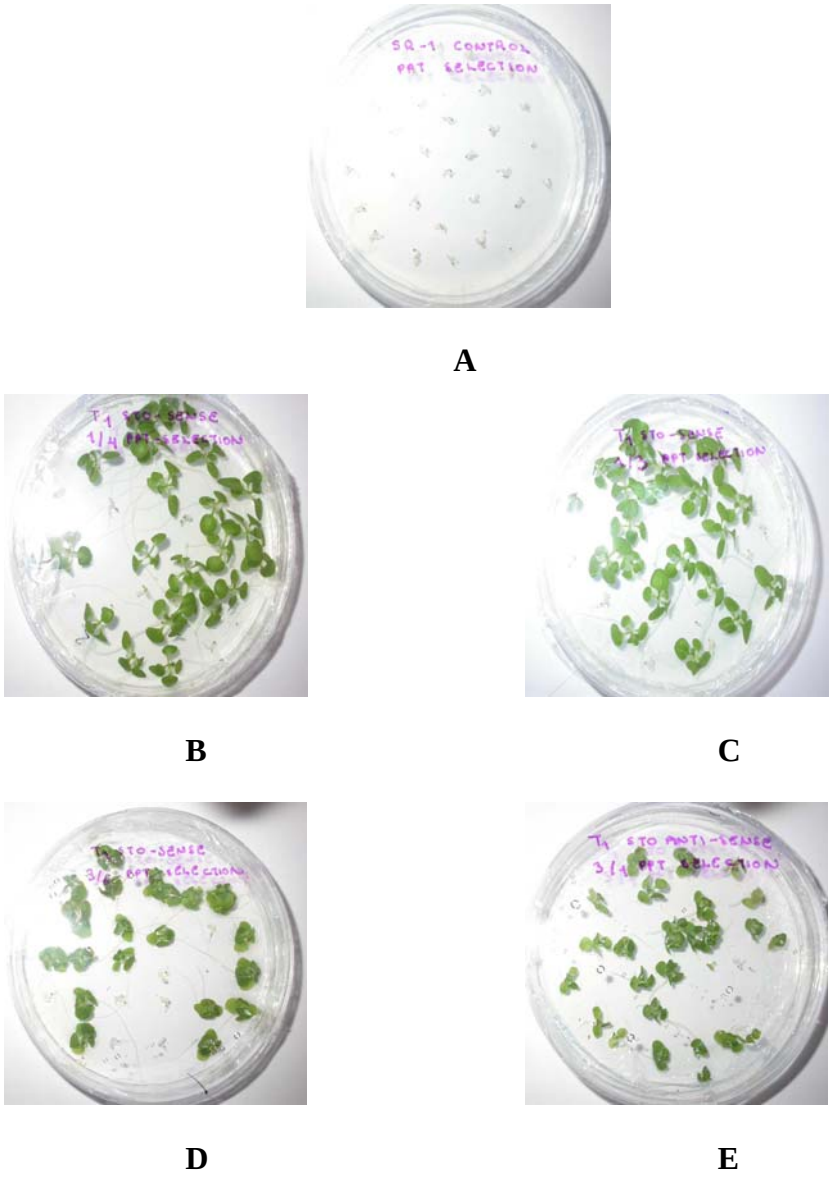


Figure 3.34. Representative photographs of T_1 progeny transgenic plants selected on PPT selective medium.

A. Seeds of untransformed control SR-1 plants
B. T_1 STO-S/1-4 transgenics
C. T_1 STO-S/1-3 transgenics
D. T_1 STO-S/3-6 transgenics
E. T_1 STO-AS/3-1 transgenics

Table 3.2. Mendelian inheritance analysis of T_1 progeny of tobacco.

NA represents the test cannot be applied to this line since there was no growth on selective media.

	Total seed number	**No of germinated seeds**	χ^2	$p(\chi^2_{0.05,1})$	**Decision**
SR-1	132	0	396,000	NA	NA
T_1 STO/S 1-2	123	95	0,328	<0,05	Fail to reject
T_1 STO/S 1-3	124	90	0,387	<0,05	Fail to reject
T_1 STO/S 1-4	152	116	0,140	<0,05	Fail to reject
T_1 STO/S 1-10	130	101	0,503	<0,05	Fail to reject
T_1 STO/S 1-11	116	113	31,080	>0,05	Reject
T_1 STO/S 1-12	84	60	0,571	<0,05	Fail to reject
T_1 STO/S 1-13	86	58	2,620	<0,05	Fail to reject
T_1 STO/S 1-15	130	121	22,656	>0,05	Reject
T_1 STO/S 1-25	127	97	0,129	<0,05	Fail to reject
T_1 STO/S 2-3	156	141	19,692	>0,05	Reject
T_1 STO/S 2-4	154	111	0,701	<0,05	Fail to reject
T_1 STO/S 2-5	87	1	253,061	>0,05	Reject
T_1 STO/S 2-7	176	121	3,667	<0,05	Fail to reject
T_1 STO/S 2-15	121	94	0,466	<0,05	Fail to reject
T_1 STO/S 3-6	155	108	2,342	<0,05	Fail to reject
T_1 STO/S 3-11	160	149	28,033	>0,05	Reject
T_1 STO/S 3-12	95	93	26,558	>0,05	Reject
T_1 STO/AS 3-1	173	158	24,603	>0,05	Reject
T_1 STO/AS 3-2	51	40	0,320	<0,05	Fail to reject
T_1 STO/S 3-3	139	5	377,959	>0,05	Reject

According to Mendelian Inheritance Analysis 8 lines do not obey the 3:1 rule while the other 12 lines obey the rule. Among the lines that do not obey the rule T_1 STO-S/2-5 and T_1 STO-AS/3-3 seem to be lines that do not contain *bar gene* and they can be categorized as non-transformants.

3.9.1.2. PCR Analysis

In order to verify the presence of *sto* to T_1 transgenic plants' chromosome, PCR analysis with STO specific primers were done. As negative control SR-1 DNA and as positive control pBSK-STO plasmid DNA were used.

As it can be seen in Figure 3.34, out of 13 selected transgenic plants in 12 of them there was a band belonging to *sto* of that is not present in control plants. Only in one line; STO-S/ 2-5 the amplification of *sto* gene was not observed and this line can be categorized as a non-transformant.

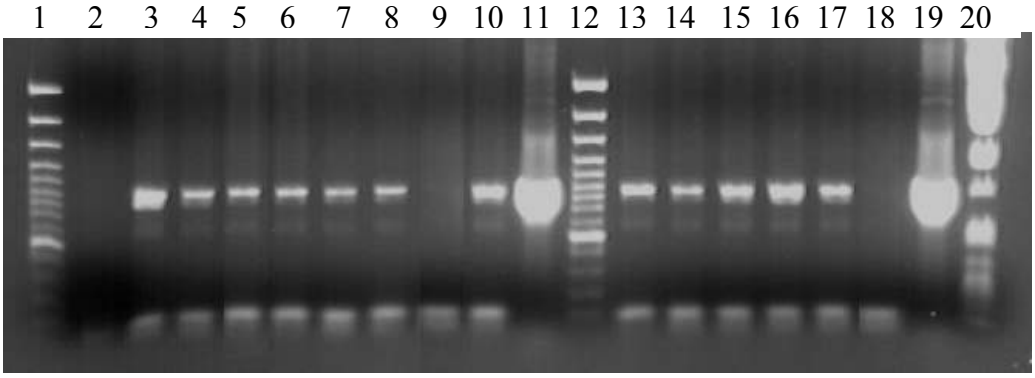


Figure 3.35. PCR analysis of T_1 transgenic plants using STO gene specific primers. The samples used in PCR;

Lane 1. DNA ruler **Lane 2.** SR-1 Control **Lane 3.** STO-S/1-3
Lane 4. STO-S/1-4 **Lane 5.** STO-S/1-12 **Lane 6.** STO-S/1-25
Lane 7. STO-S/2-3 **Lane 8.** STO-S/2-4 **Lane 9.** STO-S/2-5
Lane 10. STO-S/2-7 **Lane 11.** pBSK-STO **Lane 12.** DNA ruler
Lane 13. STO-S/2-15 **Lane 14.** STO-S/3-6 **Lane 15.** STO-S/3-11
Lane 16. STO-AS/3-1 **Lane 17.** STO-AS/3-2 **Lane 18.** SR-1 control
Lane 19. pBSK-STO **Lane 20.** λ PstI marker

3.9.1.3. Northern Blot Analysis

The expression of *sto* in T_1 transgenic tobacco plants was determined by Northern Blot Analysis. The results of Northern hybridization (Figure 3.35) showed the presence of a transcript that is lacking in the control plants. The intensities of the transcripts were observed to be variable at individual transgenic lines and it was lacking only in control and S/2-5 that does not contain *sto* according to PCR analysis.

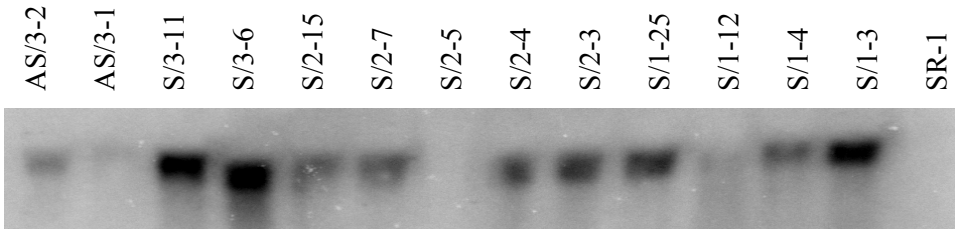


Figure 3.36. Northern blot analysis of T_1 transgenic tobacco plants.

3.9.2. Physiological and Biochemical Analysis

3.9.2.1. Analysis Under Salt Stress

It is known that *sto* takes a role in salt tolerance mechanism in yeast and plants. In order to evaluate the transgenic tobacco lines that express *sto*, a series of analysis were carried out for T_1 seeds and seedlings originated from these materials. These include some physiological tests like germination tests, fresh weight measurements and MDA content analysis of seedlings grown on media supplemented by salt.

3.9.2.1.1. Germination Test on 150 mM and 200 mM NaCl Containing Media

The seeds coming from individual transgenic lines that express *sto* gene at different levels were germinated on media containing 0 and 150 mM NaCl. The seedlings were grown in these media for 6 weeks and their photographs were taken (Figure 3.37.A-D). Uniform seed germination for all groups of plants was observed on medium containing no NaCl. On media containing 200 mM NaCl the transgenics germinated poorly with the exception of some lines like STO-S/1-12, 1-25 and 2-7. The photographs of the most salt resistant line STO-S/2-7 and control SR-1 plants were also taken under microscope, which clearly showed the increased salt tolerance of transgenic line compared to control (Figure 3.38).

The differences observed in 150 mM NaCl containing medium were much more significant according to germination percentages (Figure 3.39, Figure 3.40). All of the transgenic lines had higher germination rates and grow better on 150 mM NaCl containing medium when compared to control lines. The best responding line to salt stress was observed to be STO-S/2-7 with germination percentages of 98% on 150 mM NaCl and 88% on 200 mM NaCl containing medium. On the other hand the average germination percentages of control lines were 58% and 5% 150 mM and 200 mM NaCl concentrations.

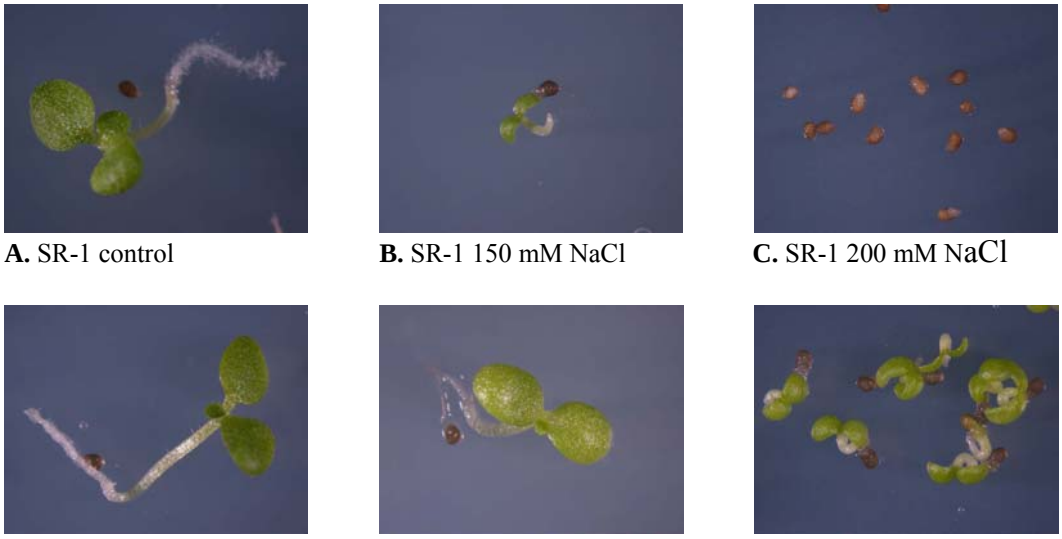
A. SR-1 seedlings

B. STO-S/1-12 seedlings

C. STO-S/2-15 seedlings

D. STO-S/3-11 seedlings

Figure 3.37. Photographs of control and transgenic tobacco plants under 150 mM NaCl stress after 6 weeks of treatment.



A. SR-1 control **B.** SR-1 150 mM NaCl **C.** SR-1 200 mM NaCl

C. STO-S/2-7 control **D.** STO-S/2-7 150 mM NaCl **E.**STO-S/2-7 200 mMNaCl

Figure 3.38. Photographs of SR-1 and STO/S 2-7 tobacco plants under control and salt stress conditions.

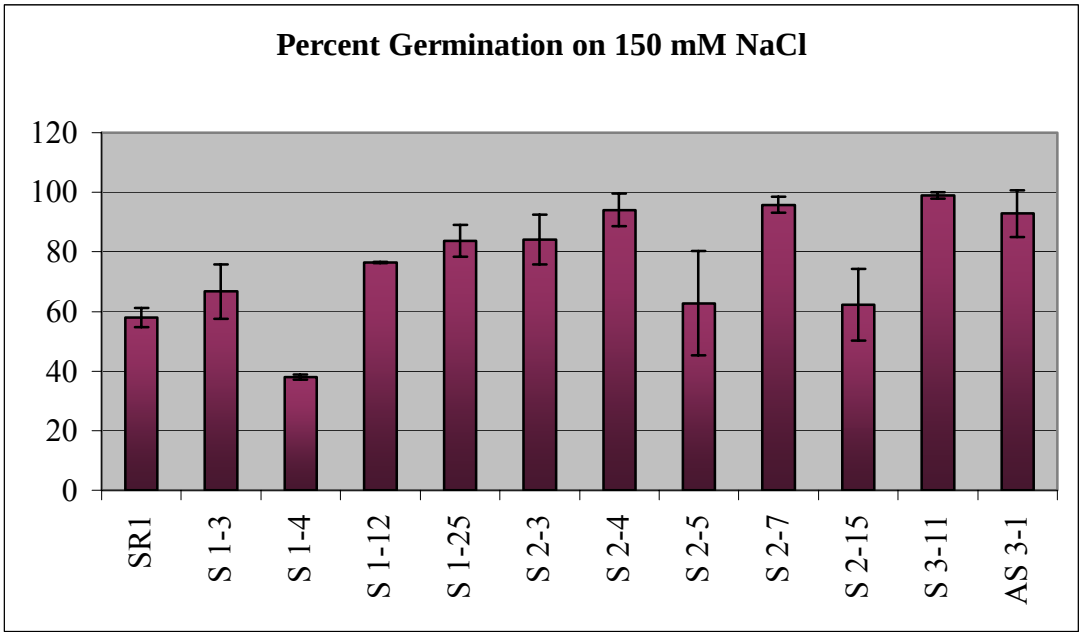


Figure 3.39. Percent germination of control and transgenic tobacco plants under 150 mM NaCl treatment.

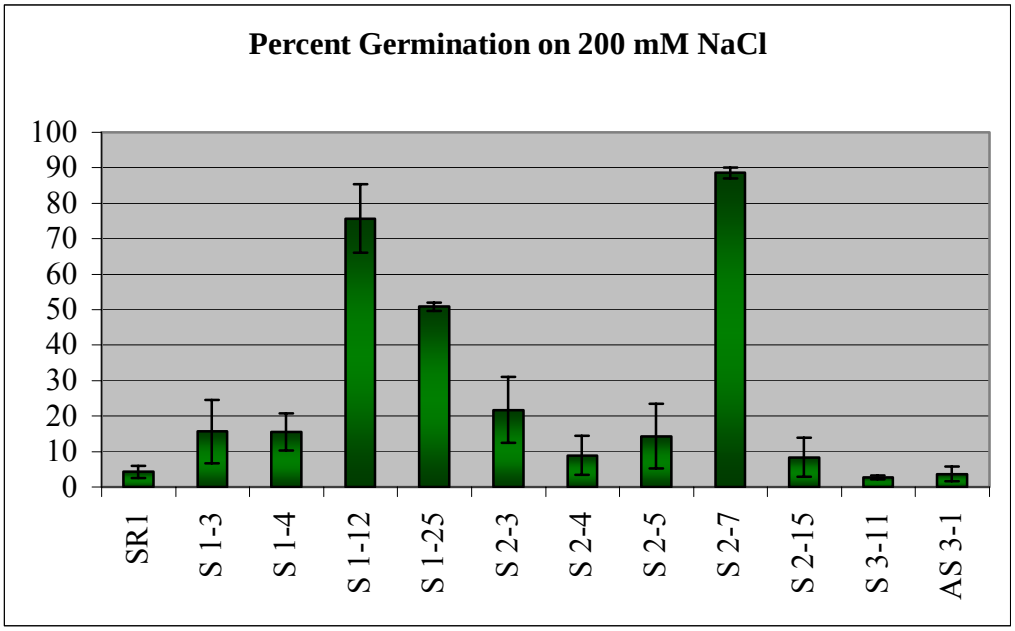


Figure 3.40. Percent germination of control and transgenic tobacco plants under 200 mM NaCl treatment.

3.9.2.1.2. Fresh Weight Measurements

The seedlings grown on 150 mM and 200 mM NaCl containing medium were evaluated in terms of fresh weight (Figure 3.41, 3.42). As it was the case in germination test again the line STO-S/2-7 was exhibited the highest fresh weight per seedling. With few exceptions all the transgenic lines had significantly higher fresh weights when compared to control.

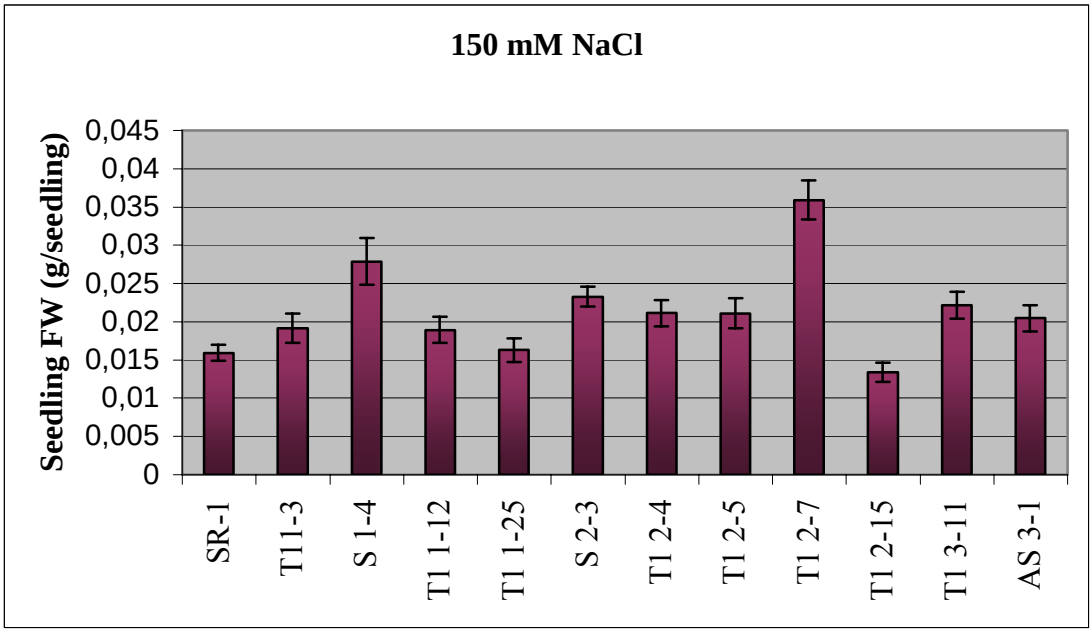


Figure 3.41. Seedling growth of transgenic tobacco lines after 6 weeks of growth on 150 mM NaCl. Values represented are the average of three independent experiments each containing 50 seeds and vertical bars represent SEM.

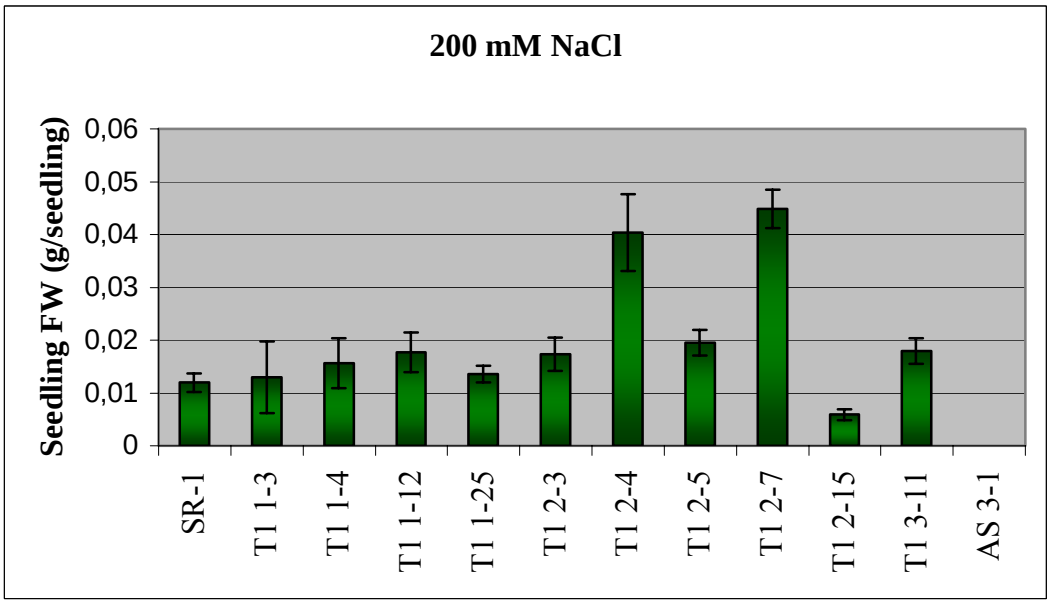


Figure 3.42. Seedling growth of transgenic tobacco lines after 6 weeks of growth on 200 mM NaCl. Values represented are the average of three independent experiments each containing 50 seeds and vertical bars represent SEM.

3.9.2.1.3. MDA Assay

Stress conditions lead to the accumulation of free radicals that have deleterious effects on plant growth and survival. One of the effects of these radicals is the peroxidation of lipids. MDA is a by product of lipid peroxidation and thus a representative of membrane damage occurring under stress conditions Its level indicates the amount of lipid peroxidation in a tissue and can give an idea about the free radical damage that occurred in the cell.

For this reason control and T_1 seeds were germinated on MS medium and 150 mM NaCl containing MS medium. After 6 weeks of growth the MDA contents were determined (Figure 3.43). According to this data significantly lower MDA amounts were found in all of the transgenic plants. Figure 3.44 shows the percent changes at MDA contents with respect to their controls.

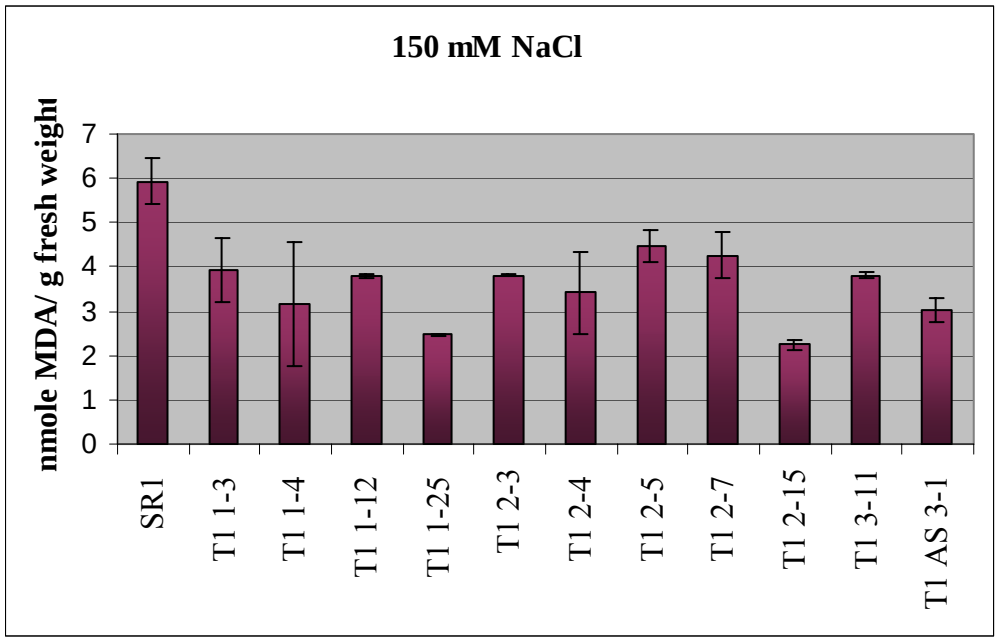


Figure 3.43. MDA contents of control and T_1 tobacco seedlings germinated on 150 mM NaCl containing MS medium.

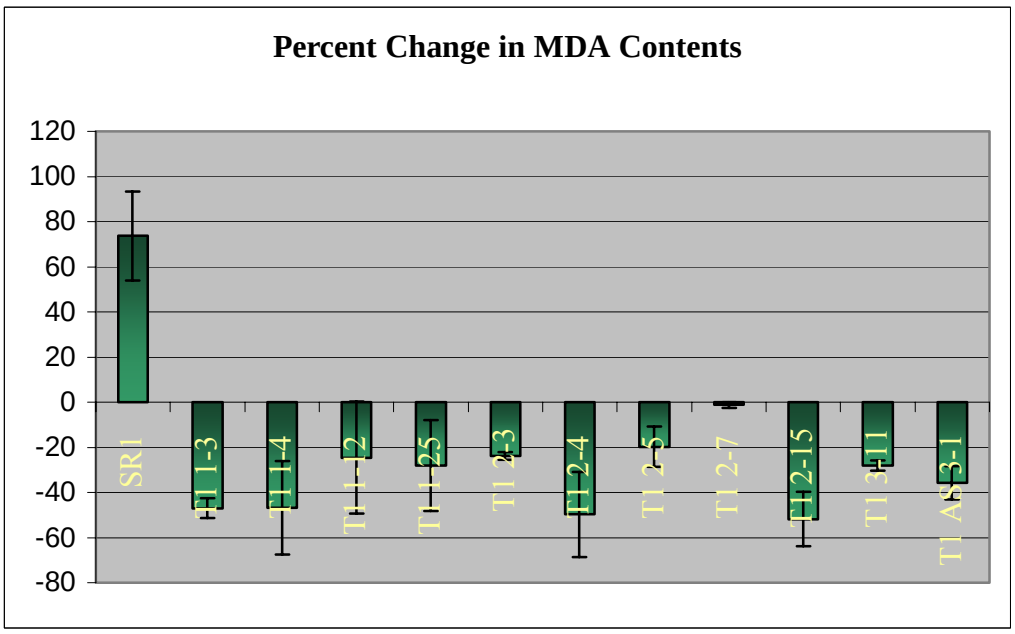


Figure 3.44. Percent changes at MDA contents of transgenic tobacco plants with respect to their controls.

3.10. Analysis of T_1 *Arabidopsis* Transgenic Plants

T_1 generation of transgenic *Arabidopsis* plants was obtained by collecting the seeds from T_0 plants and germinating them in 15 mg/l PPT or 15 μg/ml hygromycin containing medium according to the plasmid used during transformation. The lines named as S-2, S-3, S-4, S-5 were transformed with pPCVB1-STO-S, the others were transformed with pPCVB812-STO. The plants were later transferred to jars with selective rooting media. The rooted plants were transferred to soil and grown to maturity in growth chamber in order to obtain T_2 seeds.

By using the T_1 generation seeds certain molecular, physiological and biochemical analysis were carried out as in the case of tobacco plants. The results of these analyses gave an idea about the effect of overexpression and suppression of *sto* gene under non-stress and salt stress conditions.

3.10.1. Northern Blot Analysis

The expression of *sto* in T_1 transgenic *Arabidopsis* plants was determined by Northern Blot Analysis. According to the results of Northern hybridization (Figure 3.45) almost all transformed with *sto* in sense direction seemed to be overexpressing the gene when compared to control Columbia (Col). Only lines S-5, S-6 and S-11 were expressing the gene almost at the same level with Columbia.

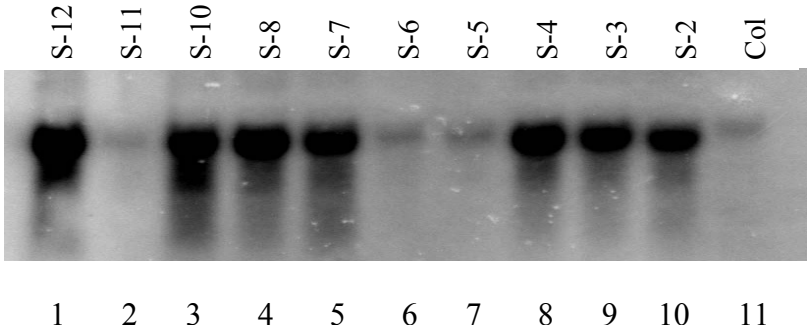


Figure 3.45. Northern blot analysis of T_1 transgenic *Arabidopsis* plants.

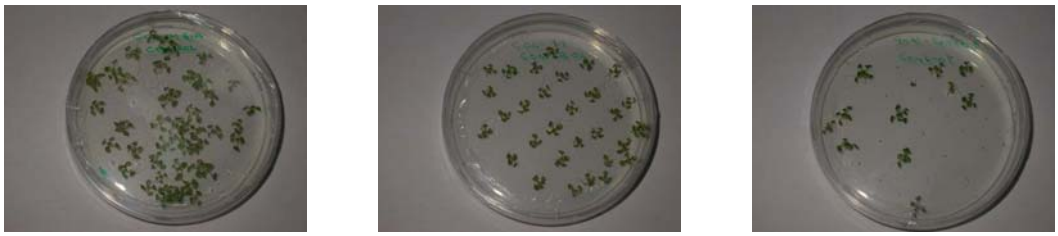
Lanes 1-10: *sto* transformants of *Arabidopsis*, Lane 11: Control *Arabidopsis*

3.10.2. Analysis Under Salt Stress

Physiological and biochemical analysis of T_1 transgenic *Arabidopsis* seedlings under salt stress consisted of the germination test, fresh weight measurements and MDA assays under control, 50 and 100 mM NaCl salt stress conditions.

3.10.2.1. Germination Test Under Salt Stress

Wild type and T_1 transgenic *Arabidopsis* seeds were germinated on 50 mM NaCl and 100 mM NaCl containing medium and seedlings were grown for 4 weeks in growth chamber. At the end of this period the seeds that germinated were counted. Figure 3.46 shows the representative photographs of wild type, STO-sense and STO-antisense *Arabidopsis* plants and Figure 3.47 shows the percent germination of transgenic and wild type seeds on these media. According to this data the transgenics overexpressing *sto* did not have any advantage under salt stress conditions with respect to germination. However, the STO-antisense *sto* transformants had significantly lower germination percentages (50-75%) when compared to control.



A. Columbia **B.** S-11 **C.** AS-5

Figure 3.46. Growth of wild type, STO-S and STO-AS transgenic *Arabidopsis* plants on MS media.

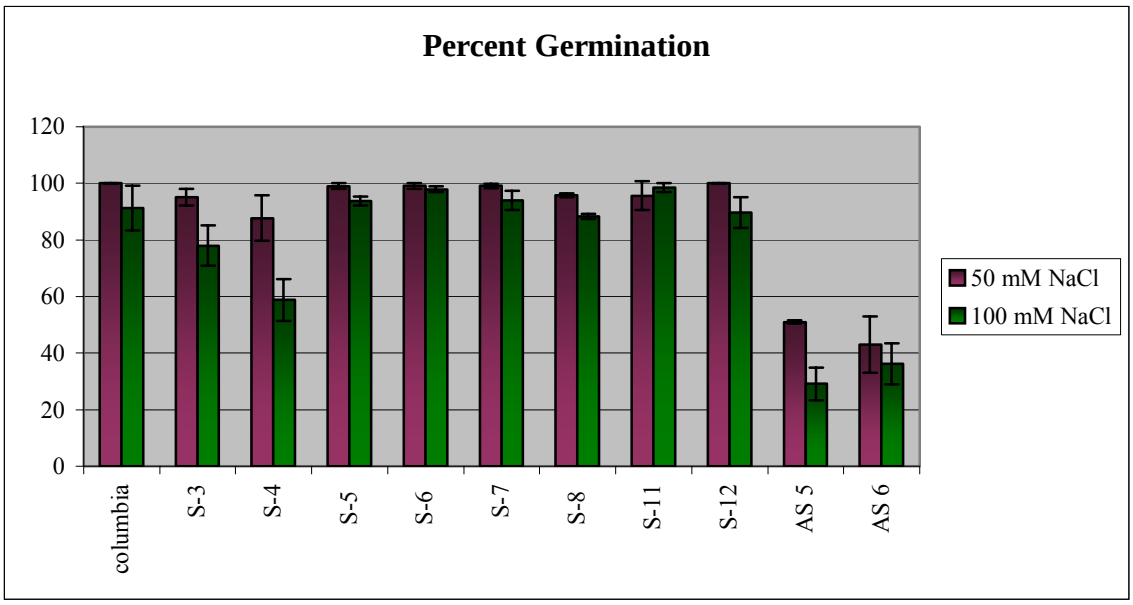


Figure 3.47. Percent germination of control and transgenic *Arabidopsis* plants under 50 and 100 mM NaCl treatments.

3.10.2.2. Fresh Weight Measurements

After 4 weeks of growth under control, 50 mM and 100 mM NaCl conditions transgenic and wild type seedling fresh weights were measured. Under 50 mM NaCl treatment almost all *sto* overexpressing transgenics had higher fresh

weights when compared to wild type (Figure 3.48). However under 100 mM NaCl treatment 5 lines (S-3, S-4, S-7, S-8, S-12) out of 8 had significantly higher fresh weights (Figure 3.49). The other 3 lines had seedling fresh weights close to wild type (Lines S-5, S-6, S-11).

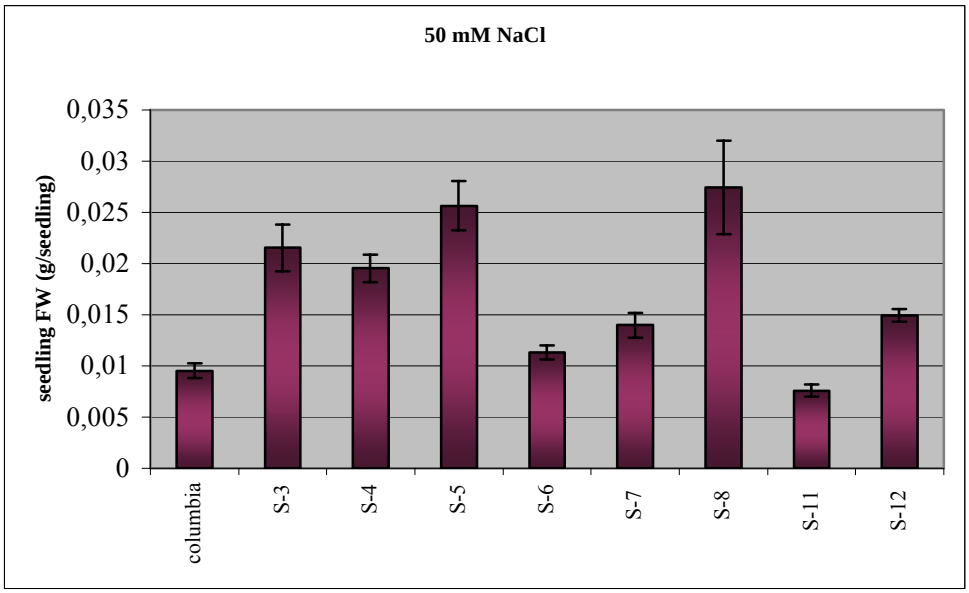


Figure 3.48. Seedling growth of transgenic *Arabidopsis* lines after 4 weeks of growth on 50 mM NaCl. Values represented are the average of three independent experiments each containing 50 seeds and vertical bars represent SEM.

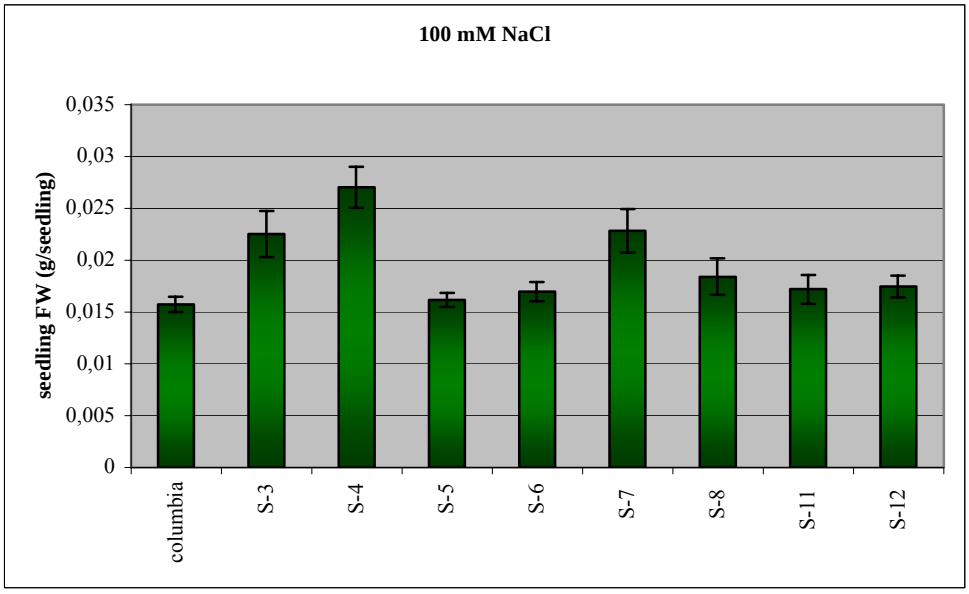


Figure 3.49. Seedling growth of transgenic *Arabidopsis* lines after 4 weeks of growth on 100 mM NaCl. Values represented are the average of three independent experiments each containing 50 seeds and vertical bars represent SEM.

3.10.2.3. MDA Assay

MDA analysis of control and transgenic *Arabidopsis* seedlings were carried out after the growth under 50 mM and 100 mM NaCl conditions. According to Figure 3.50 all of the transgenic lines overexpressing *sto* had lower MDA levels under 50 mM NaCl treatment. However under 100 mM NaCl treatment 5 lines S-3, S-6, S-8, S-11 and S-12 had significantly lower MDA levels when compared to wild type.

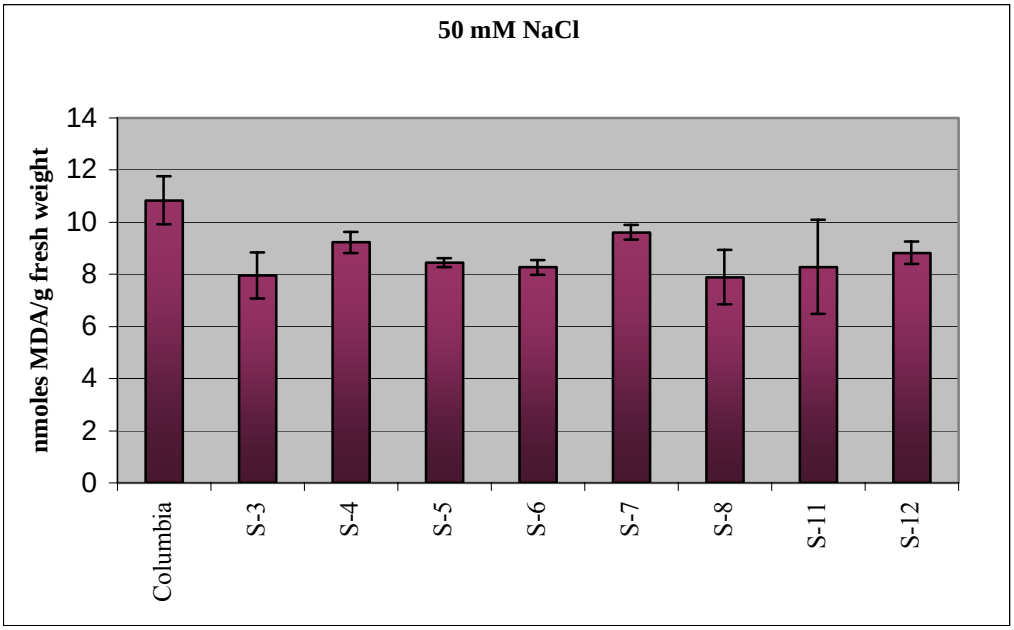


Figure 3.50. MDA contents of control and T_1 *Arabidopsis* seedlings germinated on 50 mM NaCl containing MS medium.

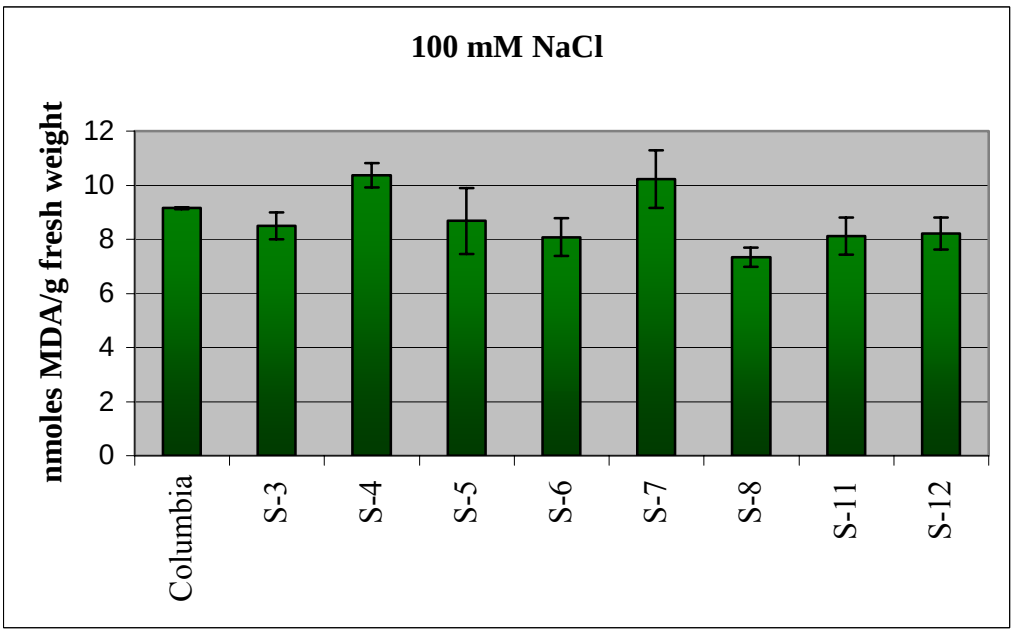


Figure 3.51. MDA contents of control and T_1 *Arabidopsis* seedlings germinated on 100 mM NaCl containing MS medium.

CHAPTER IV

DISCUSSION

Plant transformation technology has become a versatile platform to improve crop plants as well as studying gene function in plants. There is a great potential for genetic manipulation of crops to enhance productivity through increasing resistance to diseases, pests and environmental stresses. Beyond crop improvement, the ability to engineer transgenic plants is also a powerful and informative means for studying gene function and the regulation of physiological and developmental processes. Many genes that respond to osmotic stress have been described. These genes are thought to function not only to protect cells from water deficit by the production of important metabolic proteins but also to regulate the expression of genes required to mediate signal transduction in response to the stress. This fact makes the genes taking part in signal transduction pathways, especially transcription factor genes, good candidates for crop improvement against stress conditions.

Although there is significant effort in understanding the transcriptional level of signal transduction, the information obtained so far is still very limited. In this study we aimed to characterize genetically and molecularly STO, which is one of the putative zinc-finger transcription factors, that takes role in salt stress signal transduction pathway. The expression of *sto* under stress and plant growth regulator treatments was analyzed in wild type *Arabidopsis* to see in which conditions there is a change in the level of *sto* expression. Another part of the study was to transform tobacco and *Arabidopsis* plants with *sto* in order to test the transgenic plants' performance under salt stress.

4.1. Cloning of *sto* Gene

The first part of the study involved the isolation of an error free full length *sto* gene from *Arabidopsis*, its cloning in plant transformation vectors and later use of these vectors for the transformation of plants with different *Agrobacterium* mediated gene transfer techniques.

The full-length s*to* was amplified using a cDNA library of *Arabidopsis* by gene specific primers STO1 and STO2. These primers were designed with BamHI sites to provide easy cloning of the gene into vectors. After the amplification *sto* gene was cloned into BamHI site of pBSK and the sequence analysis of the positive colonies showed the integration of an error free full length *sto*.

The next part of the study was to clone amplified *sto* gene in plant transformation vectors and transform the selected model plants. The success of plant transformation depends on certain criteria. One of the most important among them is the selection of transformed tissues with a low frequency of false positives. In that respect the vector must be chosen to provide easy selection in bacterial and plant cells. Plant cloning vectors (PCVs) consist of two functional units; a conditional mini-RK2 replicon and the T-DNA. Between the left and right 25 bp borders the T-DNA carries plant selectable markers, reporter genes, cloning sites and a segment of plasmid pBR322 with a ColE1 replication origin, as well as a β-lactamase gene providing ampicillin and carbenicillin resistance for selection in *E.coli* and *Agrobacterium* (Koncz *et al*., 1994).

We used two different plant cloning vectors pPCVB1 and pPCVB812 containing CaMV35S promoter and ampicillin resistance gene for bacterial selection. PPCVB1 was modified and RFP gene was excised from the pPCVB-RFP vector. RFP protein is an important tool in localization studies that is not desired in our study. PPCVB1 has PPT resistance and pPCVB812 has hygromycin resistance genes for plant selection. In both of the vectors BamHI site was used to clone *sto*. The gene was integrated both in sense and anti-sense orientations in pPCVB1 and only in sense orientation in pPCVB812.

4.2. Transformation of Plants

As mentioned before successful transformation of plants demands that certain criteria be met (Hansen and Wright, 1999). Among the requirements for transformation are:

i. Target tissues competent for propagation and regeneration

ii. An efficient DNA delivery method

iii. Agents to select for transgenic tissues

iv. The ability to recover fertile transgenic plants at a reasonable frequency

v. A simple, efficient, reproducible, genotype-independent and cost-effective process

vi. A tight timeframe in culture to avoid somoclonal variation and possible sterility.

One of the methods that fulfill these criteria is *Agrobacterium* mediated transformation. The natural ability of the soil microorganism Agrobacterium to transform plants is exploited in the *Agrobacterium* mediated transformation method. During the process of transformation, a specific segment of the vector, T-DNA, which can be engineered to contain a selectable marker and/or genes of interest, is transferred from the bacterium to the host plant cells and inserted into the nuclear genome.

Agrobacterium strain GV3101 that we used in our study is a standard host for PCVs. This strain harbors a C58C1 chromosomal background marker by a rifampicin resistance mutation, and carries pMP90RK, a helper Ti plasmid encoding virulence functions for T-DNA transfer from *Agrobacterium* to plant cells.

In the literature there are several studies concerning the transformation of plants with transcription factors however there is only one study that involves the transformation of the model plant *Arabidopsis thaliana* with *sto* gene (Nagaoka and Takano, 2003). In our study we transformed *Nicotiana tabacum* to express *sto* that is not originally present in its genome and *Arabidopsis thaliana* to overexpress its own gene or inhibit the gene by antisense strategy. Therefore, in this respect, to our best knowledge, our experimental data demonstrates the first example of transgenic tobacco plant engineered with *A.thaliana* originated gene. Moreover, this is the first study for the production of *Arabidopsis* expressing *sto* in anti-sense orientation. The physiological and biochemical analysis of transgenic reveled that the tobacco transgenics expressing *sto* responded to salt stress better than wild type plants. However such an observation was not so significant for *Arabidopsis* transgenics overexpressing *sto*.

4.2.1. *Nicotiana tabacum* Transformation

Typically, *Agrobacterium* mediated transformation of dicots is performed using sterile leaf pieces, cotyledons, stem segments, callus suspension cultures and germinating seeds.

In this study tobacco leaf disk transformation with *Agrobacterium* was carried out. The gene transformed was under the control of the constitutive CaMV35S promoter both in sense and antisense orientations. At the end of transformation studies it was observed that the tobacco plants transformed with STO-S construct regenerated better on selective media when compared to STO-AS transformants. These plants also initiated more shoots than STO-AS transformants. As a result 38 independent transgenic lines transformed by *sto* in sense direction and 3 independent lines transformed by *sto* in anti-sense orientation were obtained. With PCR analysis the presence of *sto* gene in most of the transgenics was shown and differential expression of the gene in individual transgenic lines was analyzed by Northern Blot Analysis.

4.2.2. *Arabidopsis thaliana* Transformation

There is considerable interest in developing plant transformation methods that exclude the tissue culture steps and rely on simple protocols. These methods are called *in planta* transformation because transgenes are generally delivered into intact plants in the form of naked DNA or from *Agrobacterium*. The best example for *in planta* transformation is the successful transformation method for *Arabidopsis*. In this method, *Arabidopsis* flowers are infiltrated or dipped into an *Agrobacterium* suspension and subsequently some of the harvested seeds are transgenic (Bechtold *et al*., 1993, Clough and Bent, 1998). Although the overall efficiency is low, the sheer number of seeds recovered for screening and the ease of the method makes it an extremely attractive alternative.

In this study we used *in planta* transformation of *Arabidopsis thaliana* by vacuum infiltration. The number of transgenic *Arabidopsis* obtained by this method was less than the transgenic tobacco obtained by *Agrobacterium* mediated leaf disc transformation method. This is due to the efficiency of the transformation system. With the vacuum infiltration of *Arabidopsis* 8 lines having *sto* in sense and 8 lines in antisense orientations were obtained with pPCVB1-STO-S and pPCVB1-STO-AS transformations. Vacuum infiltration using pPCVB812-STO lead to the production of 11 independent *sto* overexpressing lines.

4.3. Expression of *sto* Gene in Wild Type *Arabidopsis thaliana*

Following the results of Lippuner's research group in 1996, there has not been many studies concerning the characterization of *sto*. In this respect the conditions that can effect the expression of *sto* stayed as an important question. Regarding this question we tried to analyze the changes of *sto* expression under different stress and plant growth regulator treatments.

When stress conditions were concerned expression of *sto* was only shown to be higher under cold and sucrose treatments. On the other hand NaCl treatment had no significant effect on *sto* expression. It is important to note that in the previous study (Lippuner *et al*., 1996) a similar result was obtained. Also in a recent research (Nagaoka and Takano, 2003) it was shown that although the transgenic plants had higher levels of *sto* transcript than wild-type plants, the expression of *sto* in these transgenic lines were not induced by salt stress. Although neither STO nor SOS3 is induced by NaCl stress, both genes are thought to have important functions in the response to NaCl stress (Nagaoka and Takano, 2003).

The effect of plant growth regulators on expression was not significant with only mild increase in expression with ABA, ethylene and giberellic acid. The effects of these hormones were only detectable in 24 hours of application.

4.4. Analysis of T_o Tobacco Transgenic Plants

In order to test the success of transformation and the presence of the transferred gene in putative transgenics, the T_o plants were tested with certain molecular and physiological analyses. The transmission of *sto* gene was proved by PPT selection, Basta® leaf paint assay, PCR and Northern Blot analysis.

4.4.1. Molecular Analysis of Transgenic Plants

Before obtaining T_1 progeny of transformants, it is possible to show the presence of *sto* gene in T_o putative transgenics. In our study we used two different strategies to prove the presence of the gene. The Northern Blot Analysis was carried out in order to test the level of *sto* expression in independent transgenic lines. According to expression analysis the gene was differentially expressed in transgenics ranging from high to low levels of expression.

The observed variability has often been referred to as “position effect”, based on the assumption that expression levels of the introduced genes redirectly influenced by the structural and functional properties of the host DNA at or near the site of integration.

The number of lines that will be used in further analysis was decreased by selecting certain number of lines from high, medium and low *sto* expressing lines. These lines were checked with PCR analysis. The results of PCR showed the presence of an amplified fragment belonging to *sto* in 17 selected independent transgenic lines. Such a fragment was not observed in wild type plants and in certain lines that can be categorized as non-transformants.

4.4.2. Physiological Analysis of Transgenic Plants

The simplest test that can be applied to putative transgenics is to determine their tolerance to the plant transformation selective agent. In our case this selective agent was PPT and its characteristics makes it a good selectable and screenable marker. The screenable characteristic of PPT was used in the leaf paint assay of T_o putative transgenics. The strategy was to test the resistance of putative transgenics for their tolerance to commercially available form of PPT, Basta®. The advantages of the leaf-paint assay are that the effect of the herbicide is limited within the application area causing very characteristic necrosis that cannot be confused and the treated plant survives after the leaf paint assay. The results of the leaf paint assay clearly indicated that the transgenic plants tolerated the applied herbicide dosage without any observable problem. However in control plants the herbicide applied portions of leaves were completely necrosed.

T_o putative transgenic plants were also analyzed for their regeneration efficiencies on different media. According to our results all transgenics had significantly higher callus weights on PPT containing media with or without NaCl. The number of shoots initiated from most of the transgenic lines on these media was also higher than control.

4.5. Analysis of T_1 Tobacco Transgenic Plants

T_1 seeds coming from T_o putative transgenic tobacco plants, were selected on PPT containing media and they were analyzed by PCR for the presence of the gene and by Northern Blot Analysis for the expression of the gene. The seeds of individual T_1 lines were germinated on NaCl containing media and the seedlings were later analyzed for their tolerance to salt stress.

4.5.1. Molecular Analysis of Transgenic Plants

Dominant characteristic of the PPT resistance trait enables the genetic selection of the herbicide mostly in a manner analogous to Mendelian inheritance of a single nuclear gene. In our study we used this characteristic in order to determine the Mendelian inheritance pattern of the transferred gene. It was observed that 3:1 ratio can not be observed in the lines S/2-5 and AS/3-3 that were categorized as non-transformants, which strengthens the idea that these lines do not carry the gene of interest. The 3:1 ratio was observed in 12 lines and it was not observed in 6 lines. The reason for a reduced ratio can be related to the expression level of the transgene. The high ratios that are observed in some lines can be due to the possibility of insertion and integration of more than one copy of the gene.

The PCR with T_1 transgenics amplified the *sto* gene present in these lines. The gene was not amplified only in one line (S/2-5). With the Northern Blot Analysis the differential expression of the gene was again shown as in the case of T_o expression analysis. Only in one line (S/2-5) the expression of *sto* could not be observed. Taking together the results of PCR and Northern Blot Analysis line S/2-5 can be categorized as non-transformant.

4.5.2. Physiological and Biochemical Analysis Under Salt Stress

In order to measure the effect of salt stress on germination at seedling level, T_1 seeds were germinated on media containing 150 and 200 mM NaCl. Seedling germination capacity, weight and MDA analysis were also investigated. According to germination capacity 7 lines out of 11 lines germinated better on 150 mM NaCl containing medium when compared to control. The number of better germinating lines on 200 mM NaCl was 6. When the tolerance to salt stress was scored according to seedling fresh weight 9 lines were having higher values than control plants. The line number more tolerant to 200 mM NaCl than control was 5. The best responding line to salt stress according to these criteria was S/2-7 that was obeying Mendelian inheritance law and expressing *sto* in high levels according to Northern blot analysis.

It was shown previously that the free radicals forming during osmotic stress and this can be measured as an increased level of MDA, which is a product of lipid peroxidation (Hong *et al*., 2000). The MDA contents can be used to measure the levels of free radicals in cells (Kunert and Ederer, 1985). MDA analysis under 150 mM NaCl treatment reveled that all transgenic lines were having lower MDA contents when compared to control plants and this can be concluded as a low level of lipid peroxidation and free radical formation in transgenic plants.

4.6. Analysis of T_1 *Arabidopsis* Transgenic Plants

The *Arabidopsis* originated *sto* gene was also used for the overexpression of the same gene in wild type plants. The other strategy was to transform the wild type plants with the construct having *sto* in antisense direction and see the effects on growth.

4.6.1.Northern Blot Analysis

In order to determine the level of expression in transgenics and compare this with control plants Northern Blot Analysis was carried out. The results showed that with the exception of 3 lines all other transgenics were expressing the gene in higher levels than control plants. The expression analysis could not be carried out for antisense transformants since these plants germinated poorly and the necessary amount of material for Northern Blot Analysis couldn't be obtained.

4.6.2. Analysis Under Salt Stress

The lines that were analyzed with Northern Blot Analysis were also tested for their tolerance to salt stress. Nagaoka and Takano (2003) used in their study two different salt concentrations (50 and 100 mM NaCl) to test the tolerance of *sto* overexpressing *Arabidopsis* transgenics with respect to root growth and they reported that all transgenics were having longer roots under these salt concentrations. In this respect these salt concentrations were also used in our study.

When the germination rates of STO-S and STO-AS were considered the significant change observed was only in STO-AS transgenics. These lines germinated poorly even under normal conditions. This result is consistent with the seedling lethal phenotype observed by Dr. Laszlo Szabados during the analysis of STO T-DNA mutants. Almost all STO-S transgenics on the other hand didn't have a significant tolerance to these salt concentrations and germinated almost at the same rate with control plants.

The fresh weight measurements of the seedlings under these salt concentrations have shown that 6 *sto* overexpressing lines out of 8, having higher fresh weights under 50 mM NaCl treatment. This result was valid in 5 *sto* overexpressing lines under 100 mM NaCl treatments. It should be noted that the lower fresh weights were observed in lines expressing *sto* in low levels.

The MDA amounts under 50 mM NaCl salt concentration were lower in all transgenics with variations between the lines. On the other hand MDA contents were only significantly lower in 5 lines under 100 mM NaCl treatment. The MDA analysis could not be performed with antisense *Arabidopsis* transformants since the material obtained after germination even in normal growth medium was not enough to carry out the analysis.

CHAPTER V

CONCLUSION

In this study we aimed to characterize STO, the putative transcription factor taking role in salt stress signaling pathway. For this purpose we conducted experiments towards the engineering of tobacco and *Arabidopsis* plants using different strategies. The original gene was isolated from *Arabidopsis*, amplified and used in the transformation of model plants. Moreover, the conditions that may effect the level of *sto* expression was determined under stress and plant growth regulator treatments.

Transformation studies were conducted both with sense and anti-sense *sto* constructs for tobacco and *Arabidopsis* plants. In both of the cases the gene was under the control of the 35S Cauliflower Mosaic virus (CaMV35S) constitutive promoter. As a result of tobacco transformations 38 independent lines transformed with *sto* in sense orientation, and 3 lines in antisense orientations were obtained. After the PCR and expression analysis of these lines some lines that are expressing *sto* in high, medium and low levels were selected and these lines were used for further analysis.

Certain tests for T_0 and T_1 *sto* transformants were carried out to evaluate the performance of these plants under salt stress. These analyses include regeneration tests on different media, germination percentages, fresh weight determinations and MDA content determinations under salt stress. According to the cumulative results of the above mentioned analysis almost all transgenics respond better to salt stress with STO/S-7 being the most tolerant one.

When the results obtained from the analysis of *Arabidopsis* transgenics were considered it was observed that the overexpression of *sto* gene in transgenic lines did not change the germination efficiencies under salt stress. However most transgenic lines had higher fresh weights and lower MDA contents. The most significant result obtained as a result of *Arabidopsis* transformations is that the decreased germination rates of antisense *sto* transformants even under normal conditions. With our results and the seedling lethal phenotype observed at T-DNA mutants, it can be concluded that STO may be having important roles in the germination and growth of plants under normal conditions.

The studies to be conducted in the future would include:

i. Further analysis of T_1 plants under different osmotic stress conditions (like cold) with respect to their osmotolerance,

ii. The analysis of T_2 progenies under osmotic stress conditions,

iii. The detection of the STO protein in the transgenic lines with Western Blot Analysis,

iv. Analysis of transgenic lines by Southern Blot Analysis.

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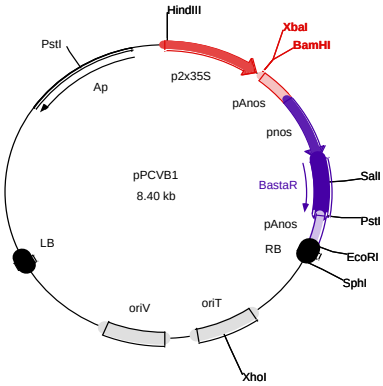
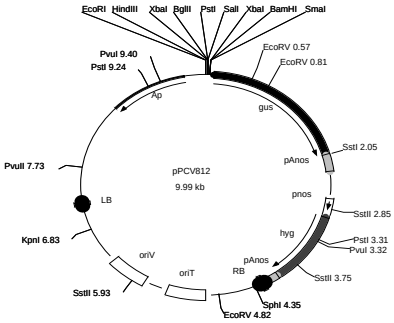
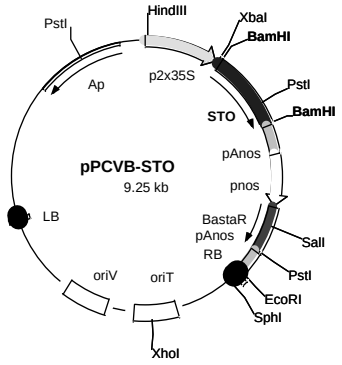
APPENDIX A

PLASMID MAPS





Plasmid name: pPCV812
Plasmid size: 9.99 kb
Constructed by: C. Koncz
Construction date: ?
Comments: promoter testing vector with promoterless gus gene and polycloning site in front of it.



APPENDIX B

AtSTO COMPLETE SEQUENCE

Sto nucleotide sequence

```
   1tctgaaccta cgcttctgct aagctattct aagagaagcc agactagcaa taaacccttc
  61 attttaagca ttctgtttcc ttcttgagaa acctagatat tttggtttct tgtatccggt
 121 gATGaagata cagtgtgatg tgtgtgagaa agctccggcg acggtgattt gttgcgccga
 181 cgaagctgct ctctgtcctc aatgcgacat cgagattcac gccgctaaca aactcgctag
 241 caagcaccaa cgtcttcatc ttaattccct ctccaccaaa ttccctcgtt gcgatatctg
 301 ccaagagaag gcagctttca ttttctgtgt agaggataga gctctgcttt gcagggactg
 361 cgatgaatcc atccacgtgg ctaattctcg atctgctaat caccagaggt tcttagccac
 421 tgggatcaaa gtagctctga cctcaactat atgtagtaaa gaaattgaga agaatcaacc
 481 tgagccttcc aacaaccaac agaaggctaa tcagattcct gctaaatcca caagccagca
 541 gcaacaacaa ccttcttctg ctactccact tccctgggct gttgacgatt tctttcactt
 601 ctctgatatt gaatccaccg acaagaaagg acagcttgat cttggggcag gggagttgga
 661 ttggttttca gacatgggat tcttcggtga tcagattaat gacaaggctc ttcctgcagc
 721 tgaagttcct gagctttctg tttcgcattt aggtcatgtt cattcataca aacctatgaa
 781 gtcaaatgtt tcacacaaga agccgaggtt tgagaccaga tatgatgatg atgatgagga
 841 acacttcatt gtccctgatc ttggcTAAaa agctatatgt aatctatgtg tagacattct
 901 tcaatgtaaa agaacaaaca agaaacctat ctgcatgtgt ggagttaatg tcatatacat
 961 tttagttttg tcttaagttg tgtaagatat gttgagagct tataacaaat gtctgtgttt
1021 gagttaaaaa aaa
```

Amino acid sequence of STO

MKIQCDVCEKAPATVICCADEAALCPQCDIEIHAANKLASKHQRLHLNSLSTKFP
RCDICQEKAAFIFCVEDRALLCRDCDESIHVANSRSANHQRFLATGIKVALTSTIC
SKEIEKNQPEPSNNQQKANQIPAKSTSQQQQQPSSATPLPWAVDDFFHFSDIESTD
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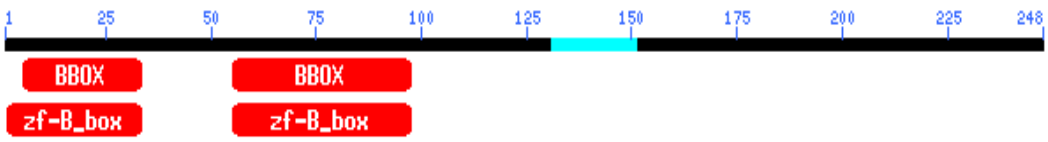

APPENDIX C

BACTERIAL CULTURE MEDIA

Luria-Bertani Broth (1 lt)

Yeast extract	5 gr
Tryptone	10 gr
NaCl	10 gr
1 N NaOH	1 ml
Bacterial agar	15 g

The pH of the medium is adjusted to 7.4 and autoclaved at 121^{o}C for 20 minutes.

Yeast Extract Broth Medium (1 lt)

Yeast extract	1 gr
Beef extract	1 gr
Peptone	5 gr
Sucrose	5 gr
Bacterial agar	15 g

The pH of the medium is adjusted to 7.2. After autoclaving the medium at 121^{o}C for 20 minutes, 2 ml of 1M sterile $MgSO_4$ is added.

SOB Medium (1 lt)

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

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

TB Buffer (100 ml)

HEPES	0.24 gr
$CaCl_2.2H_2O$	0.221 gr
KCl	1.864 gr
$MnCl_2.4H_2O$	1.09 gr

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

APPENDIX D

COMPOSITIONS OF PLANT TISSUE CULTURE MEDIA

From Sigma Plant Cell Cultures	**M9274**
Component	**mg/l**
Ammonium Nitrate	1650
Boric Acid	6,2
Calcium chloride Anhydrous	332,2
Cobalt Chloride. $6H_2O$	0,025
Cubric Sulfate. $5H_2O$	0,025
Na_2EDTA	37,26
Ferrous Sulfate./H_2O	27,8
Magnesium Sulfate	180,7
Magnesium Sulfate.H_2O	16,9
MolybdicAcid(Sodium salt).$2H_2O$	0,25
Potassium Iodide	0,83
Potassium Nitrate	1900
Potassium Phosphate Monobasic	170
Zinc Sulfate.$7H_2O$	8,6
Agar	8000
Glycine	2
Myoinositol	100
Nicotinic Acid	0,5
Pyrodoxine.HCl	0,5
Sucrose	30000
Thiamine.HCl	0,1
Grams of powder to prepare 1liter	42,4

APPENDIX E

PLASMID ISOLATION SOLUTION

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

STE

NaCl	0.1 M
Tris.Cl (pH:8)	10 mM
EDTA (pH:8)	1mM

APPENDIX F

TBE AND TAE SOLUTIONS

10X TBE (Tris-borate) Buffer (1L)

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

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

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

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

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

APPENDIX G

INFILTRATION MEDIUM

Macro nutrients	826 mg/L
Micro nutrients	500 mg/L
Sucrose	5%

pH: 5.7

Medium was autoclaved at 121^{o}C for 20 minutes and BAP and SILWET were added.

BAP	0.044μM
SILWET L-77 (surfactant)	0.005 %

APPENDIX H

NORTHERN BLOT ANALYSIS SOLUTIONS

TRI Reagent (110 ml)

Guanidine thiocyanate	23.7 g
Na-citrate (pH: 7)	12.5 mL 100 mM

The contents were dissolved in 50 mL H_2O.

Sarcosyl	715 µl 35%
β-mercaptoethanol	340 µl
Water-saturated phenol	50 ml
KOAc (pH: 4.8)	10 ml 2M

Pre-hybridization and Hybridization Buffer

		100 ml
Deionized formamide	50 %	50 ml 100%
EDTA (pH:7)	5 mM	1 ml 0.5M
Na(PO_4)	50 mM	5 mL 1M
Salmon sperm DNA	200 µg	2 ml 100µg/ml
Denhardt solution	10X	20 ml 50X
SDS	0.1%	0.5 ml 20%
		21.5 mlH_2O

Transfer Buffer (20X SSC)

Tri-sodium citrate	88.23 g
NaCl	175.32 g

All are dissolved in 800 ml dH_2O, the pH is adjusted around 7-8 and the volume is completed to 1l with dH_2O.

Stringency Wash Solution

SSC	0.2X
SDS	0.1%

APPENDIX I

CTAB DNA ISOLATION SOLUTIONS

2X CTAB Extraction Solution (100 ml)

CTAB	2 g
Tris.Cl	10 ml 1M (pH:8.0)
EDTA	4ml 0.5 M (pH:8.0)
NaCl	28 ml 5 M

All are dissolved in dH_20 and volume is completed to 100 ml.

5X CTAB Extraction Solution (100 ml)

CTAB	5 g
Tris.Cl	10 ml 1M (pH:8.0)
EDTA	4ml 0.5 M (pH:8.0)
NaCl	28 ml 5 M

All are dissolved in dH_20 and volume is completed to 100 ml.

VITA

Miss.Feyza Selçuk

January , 21th, 1976, Ankara Turkey

Turkish

Single

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Education

1999- Sciences, M.E.T.U	Graduate School of Natural & Applied Ph.D in Biology, CGPA 3.29/4
1997-1999	Graduate School of Natural & Applied Sciences, M.E.T.U Master of Science in Biology (MSc., High Honours in Biology, CGPA 3.64/4)

1993-1997	Middle East Technical University M.E.T.U, Ankara, Turkey Bachelor of Science in Biology (Cumulative Grade Point Average: 3.09/4)
1982-1993	T.E.D. Ankara College (primary, secondary and high school) graduation with Diploma

Professional Experiences and Previous Employment

1999-	Teaching Assistant in Biochemistry and Experimental Plant Physiology Laboratories, Department of Biology, M.E.T.U
1998-	Research Assistantship at METU, Institute of Natural and Applied Sciences
1997-1999	MSc. Thesis on "Physiological and Biochemical Characterization of Turkish Wheat Varieties Exposed to Boron Stress"
1996-1997	Special project study on "Effects of Boron Stress on the Activities of Superoxide Dismutase Isoenzymes in Turkish Wheat Varieties"
1996	Summer practice at Hacettepe University Hospital, Microbiology Laboratory (6 weeks)

Languages

- English: fluent (English Proficiency Exam score: 79/100, Foreign Language Proficiency Examination For State Employees score: 80/100)
- Turkish: native

Professional Skills

- Recombinant DNA techniques including cloning, vector construction, analysis of gene expression
- Plant transformation systems (direct DNA uptake, vacuum and *Agrobacterium* mediated transformation, microprojectile bombardment) and plant tissue culture
- Preparation and Analysis of DNA and RNA (PCR, Southern and Northern Blot)
- Qualitative and quantitative analysis of proteins by colorimetric methods, 1-D and 2-D gel electrophoresis, activity staining for determination of SOD isoenzymes
- Physiological and biochemical analysis of plants under stress conditions; determination of chlorophyll contents and PS-II activities, quantification of osmoprotectants under stress conditions (proline, trehalose)

Other Skills

- Competent with all common Microsoft-Office based software and internet applications

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Fellowships, Awards and Technical Courses Attended

- Intensive Course on Plant Biotechnology and Applications, Mediterranean Agronomic Institute of Chania, Greece, May 15th- June 2nd , 2000.
- TUBITAK fellowship, Agricultural Biotechnology Center, Godollo, Hungary, September 1st-October1st, 2001.
- UNESCO-ITC course, Biological Research Center, Szeged, Hungary, October 1st – September 1st, 2001-2002.

Fields of Interest

Physiological and biochemical changes of crop plants at various stress conditions (osmotic stress, boron stress)

- Determination of changes at chlorophyll contents and PS-II activities under such stress conditions.
- Characterization of SOD isoezymes and determination of changes at SOD isoenzyme patterns at stress conditions.
- Roles of osmoprotectants (proline, trehalose) during stress conditions.

Crop improvement by transgenic technologies; genetic modifications against stress factors

- Characterization of stress related genes of model plants

Seminars Attended

- Speaker at Seminar in Biology "Physiological and Biochemical Characterization of Turkish Wheat Varieties Exposed to Boron Stress", Department of Biology, METU, April,1999
- Speaker at XI. KÜKEM Biotechnology Congress, Isparta, Turkey, September, 1999
- Second Balkan Botanical Congress, Istanbul, Turkey, May,2000
- Speaker at Seminar in Biology "Genetic Manipulation of Crop Plants Against
  Osmotic Stress", Department of Biology, METU, May,2001

Related Publications

- **Selçuk, Feyza**. "Physiological and Biochemical Characterization of Turkish Wheat Varieties Exposed to Boron Stress" Master of Science Thesis, September, 1999, Middle East Technical University, Ankara, TURKEY

- **Selçuk, Feyza** and Szabados, Laszlo (2002) "Genetical and Molecular Characterization of *Arabidopsis thaliana sto* Gene" International Training Course, Szeged, Hungary, Proceeding Book.

Selected Presentations and Meeting Abstracts

- **Selcuk F.**., Öktem H.A., Yücel M. " Physiological and Biochemical Characterization of Turkish Wheat Varieties Exposed to Boron Stress" Second Balkan Botanical Congress, 14-18 May 2000, İstanbul, Turkey.
- **Selcuk F**. and Szabados L. "Genetical and Molecular Characterization of *Arabidopsis thaliana sto* Gene" ITC Closing Seminar, Szeged, Hungary.

Projects

- **DPT2001K122060**

Development of Transgenic Crop Plants Against Drought and Salt Stress. Yucel M., Oktem H.A., Ellialtioglu S.,Hamamci H., Inci F, Ertugrul F., **Selcuk F**., Karamollaoglu İ. (2001-2003)

- **BAP-2001-07-02-00-61**

Genetic manipulation of crop plants against osmotic stress. Yucel M., Oktem H.A., Selcuk F. (2001-2003)

- **TARP-2366**

Yucel M., Oktem H.A., Hamboobi H., Selcuk F. Physiological and Biochemical Analysis of Wheat and Barley Exposed to Boron Stress.

Accreditations and Licenses

- Class B driving license.
- Classical ballet certificate of MEB.
- Royal Academy of Dancing Grade 1-8 Certificates with a highly recommended grade.

References

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