EFFECT OF SALT STRESS ON CHLOROPLAST PROTEIN IMPORT

THESIS SUBMITTED TO
JAWAHARLAL NEHRU UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF
MASTER OF PHILOSOPHY
IN LIFE SCIENCES

BY

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CERTIFICATE

The research work embodied in this thesis entitled "Effect of Salt Stress on Chloroplast Protein Import" has been carried out in the school of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or full for any other degree or diploma of any other university.

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ACKNOWLEDGEMENT

I feel privileged to express my gratitude to my supervisor, Prof. B.C.Tripathy for his excellent guidance and thoughtful suggestions during the course of my work. I shall remain indebted for his valuable guidance, encouragement, patience and help at every stage.

I am extremely thankful to the Dean of School of Life Sciences, Prof. R.N.K.Bamezai for extending the facility to carry out present work.

I express my profound appreciation and thanks to my seniors, Dr. Suchi, Dr. Ajay, Dr. Rupesh, Gopal, Ansuman, Siddhartha, Satpal and my colleagues, Naveen, Manas, Vijay, Namrata. I express my thanks to Mr. Alexander, Mr. B.A. Khan, Mr. S. K. Mishra, Mr. Sharma and all members of CIF for their technical assistance. I am thankful to Sumer Singh and Ramesh for lab assistance.

The financial assistance of CSIR is dully acknowledged.

I owe very much to my parents, brother Ashu, sister Deepika and friends Ratnesh, Anupma, Santosh, Naumi, Pradumn, Nikhil, Ramesh for their constant encouragement, affection and support, without which it would not have been possible to complete the work.

Last but not the least; I bow my head before the God for the courage and blessings.

Rajneesh Singhal.
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ABBREVIATIONS

APS	Ammonium Per Sulphate
Chl a	Chlorophyll a fluorescence
CPE	Chloroplast Processing Enzyme
ETR	Electron Transport Rate
Fo	Minimal fluorescence level from dark adapted leaves
Fm	Maximal fluorescence level from dark adapted leaves
Fv/Fm	Ratio of maximum variable to maximum total fluorescence
GTP	Guanosine Triphosphate
h	Hours
HEPES	N-[2- hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HKT	high efficiency potassium transporter
HSP	Heat shock protein
kDa	Kilo Dalton
NaCl	Sodium Chloride
NHX	Na^+/H^+ exchanger
PAM	Pulse Amplitude Modulator
pSSU	Precursor of small subunit of Rubisco
qN	Non-Photochemical Quenching
qP	Photochemical Quenching
SDS	Sodium Dodecyl Sulphate
SOS	Salt Overly Sensitive
SPP	Stromal Processing Peptidase
TEMED	N-N-N-N'-tetramethyl-ethylenediamine
TIC	Translocon at the inner envelope membrane of chloroplast

TOC	Translocon at the outer envelope membrane of chloroplast
Tris	Tris (hydroxymethyl) amino methane
Yield	Quantum yield of photsystem II (φPSII)

CONTENTS

INTRODUCTION

The productivity of plants is greatly affected by various environmental stresses. Environmental stresses encompass a wide range of physical conditions, which can significantly alter plant metabolism, growth and development, leading at their extremes to plant death. These abiotic stresses include drought, high salinity, extremes of temperature (both high and low), heavy metal toxicity, ultraviolet radiation, nutrient deprivation, high light stress and hypoxia. Salt stress is the most typical abiotic stress affecting plants. Soil salinity limits agricultural production throughout the world. The most common cause of soil salinity is irrigation water, because salt dissolved in the irrigation water is left in the soil following evaporation (Zhu, 2001). High salinity causes ion imbalance, toxic levels of cytoplasmic sodium and drought stress. A high level of Na^+ is toxic to plants because it interferes with K^+ nutrition and thus affect K^+ stimulated enzyme activities, metabolism and photosynthesis.

Plastids-such as chloroplasts, which carry out photosynthesis in the green parts of plants, are present in most cells. Chlorophyll fluorescence has been applied as an effective and non-invasive tool for elucidating various aspects of the physiological state of the photosynthetic apparatus in intact leaves of higher plants (Krause and Weis 1991).

Chloroplasts are organelles of endosymbiotic origin, and they transferred most of their genetic information to the host nucleus during this process. Therefore, the vast majority of chloroplast proteins are synthesized as precursor proteins (preproteins) in the cytosol and are imported post translationally into the organelle. The preproteins are recognized in a GTP regulated manner by receptors of the outer-envelope translocon, which is called the TOC complex. The preproteins cross the outer envelope through an aqueous pore and are then transferred to the translocon in the inner envelope, which is called the TIC complex. The TOC and TIC translocon functions together during the translocation process (Soll and Schleiff, 2004).

Subcellular organelles such as mitochondria and plastids are potential targets for damage during stress. Photosynthesis is profoundly affected by salinity stress. Chloroplast biogenesis is dependent on the import of proteins synthesized in the cytosol and hence the proper functioning of photosynthesis. Therefore it is essential to study the impact of salinity stress on chloroplast protein import machinery. The effect

of salinity stress on the import of proteins into chloroplast is studied by taking pea as a model plant. The efficiency of protein import into chloroplast is studied by using in-vitro translated precursor of small subunit of rubisco. The expression level of different protein import components under salinity stress is also studied by RT-PCR.

REVIEW OF LITERATURE

Salinity Stress and Plant Response

Salt Stress

The productivity of plants is greatly affected by various environmental stresses. Salinity stress negatively impacts agricultural yield throughout the world affecting production whether it is for subsistence or economic gain. The plant response to salinity consists of numerous processes that function in coordination to alleviate both cellular hyperosmolarity and ion disequilibrium. High salt concentrations decrease the osmotic potential of soil solution creating a water stress in plants. Secondly, they cause severe ion toxicity, since Na^+ is not sequestered into vacuoles as in halophytes. Finally, the interaction of salts with mineral nutrition may result in nutrient imbalances and deficiencies. The consequence of all these can ultimately lead to plant death as a result of growth arrest and molecular damage (McCue and Hanson, 1990).

Mechanism of salt tolerance

Plants develop a plethora of biochemical and molecular mechanisms to cope with salt stress. Biochemical strategies lead to products and processes that improve salt tolerance. These include (i) selective accumulation or exclusion of ions, (ii) control of ion uptake by roots and transport into leaves, (iii) compartmentalization of ions, (iv) synthesis of compatible solutes, (v) change is photosynthetic pathway, (vi) alteration in membrane structure, and (vii) induction of antioxidative enzymes. Under the molecular mechanism there is induction of large number of genes and proteins that confer salt tolerance to the plants. The genes under this category include: genes for photosynthetic enzymes, genes for synthesis of compatible solutes, genes for vacuolar sequestering enzymes etc.

Ion Homeostasis

The homeostasis of intracellular ion concentrations is fundamental to the physiology of living cells. Plant cell employ primary active transport, mediated by H^+ ATPases, and secondary transport, mediated by channels and co-transporters, to maintain characteristically high concentrations of K^+ and low concentrations of Na^+ in the cytosol. A high level of Na^+ is toxic to plants because it interferes with K+

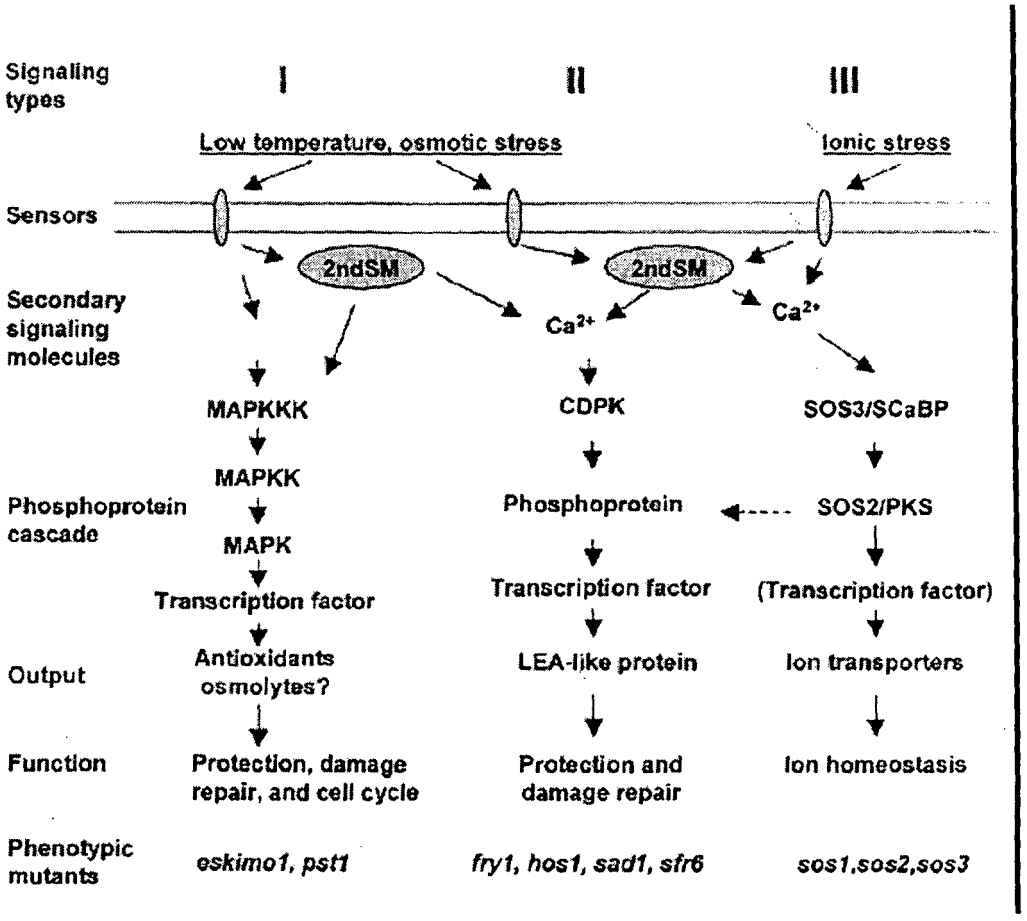


Figure 1. **Major types of signaling for plants during cold, drought and salt stress (Adapted from Xiong et al., 2002).**

nutrition and thus affects K^+ stimulated enzyme activities, metabolism, and photosynthesis. To prevent Na^+ accumulation in the cytoplasm, three likely mechanisms include (i) reducing Na^+ entry into the cell, (ii) active Na^+ efflux from the cell and (iii) active sequestration of Na^+ in the vacuole. Although Na^+ is not an essential nutrient, the ion is taken up into cells down the electrochemical gradient via several pathways, such as the non- selective cation channels (Demidchik et. al., 2002) and the sodium transporter HKT1 (Uozumi et. al., 2000).

Sensing Salt Stress

Na^+ can be sensed either before or after entering the cell, or both, though little is known about this phenomenon. Extracellular Na^+ may be sensed by a membrane receptor, whereas intracellular Na^+ may be sensed either by membrane proteins or by any of the many Na^+ sensitive enzymes in the cytoplasm. The plasma membrane Na^+/H^+ antiporter SOS1 (SALT OVERLY SENSITIVE1) is a possible Na^+ sensor. The SOS1 protein has 10-12 tansmembrane domains, and a long tail (of more than 700 amino acids) that is predicted to reside in the cytoplasm (Shi et.al., 2000). SOS1 has Na^+/H^+ exchanger activity, and this transport activity is essential for Na^+ efflux from *Arabidopsis* cells (Qiu et. al., 2002 and Quintero et. al., 2002). Several transporters with long cytoplasmic tail or loops have been demonstrated to be sensors, like the sugar permease BglF in Escherichia coli has a dual role in sensing and transporting β-glucosides (Chen et. al., 1997).

Na^+ Influx

The enormous negative membrane potential across the plasma membrane of plant cells favors the passive transport of Na^+ into cells. Na^+ enters plant cells through the high-affinity K^+ transporter HKT1 (Rus et al., 2001; Maser et al., 2002) and through non-selective cation channels (Amtmann and Sanders 1999). Additionally, in some plant species such as rice, Na^+ leakage into the transpiration stream via the apoplast can account for a major part of Na^+ entry into plants (Yeo et al., 1999). Na^+ currents that are mediated by non-selective cation channels are partially sensitive to calcium, and this correlates with the inhibition of Na^+ entry into roots by calcium (Tester and Davenport 2003).

Na^+ Efflux

In *Arabidopsis*, N^+ efflux is catalyzed by the plasma membrane Na^+/H^+ antiporter encoded by the *SOS1* gene (Shi et al., 2000; Qiu et al., 2002; Quintero 2002; Shi et al., 2002). SOS1 activity is detected in salt stressed but not in unstressed plants (Qiu et al., 2002). It is electroneutral Na^+/H^+ exchanger that is specific for Na^+ (Qiu et al., 2002; Qiu et al., 2003). The greatest activity of the SOS1 promoter is found in root epidermal cells and in cells bordering the vascular tissue throughout the plant (Shi et al., 2002). The SOS1 has several roles, first, Na^+ efflux into the root medium, second buying time for Na^+ storage in the vacuole by slowing down Na^+ accumulation in the cytoplasm; and third controlling long distance Na^+ transport between roots and leaves by loading Na^+ into and unloading Na^+ from the xylem and phloem.

The transcript level of SOS1 is upregulated by salt stress at the posttranscriptional level. The salt stress upregulation of SOS1 is partly under the control of SOS2 and SOS3 (Shi et al., 2000). Plasma membrane H^+-ATPases generate the driving force for Na^+ transport by SOS1. Disruption of the root-endodermis-specific plasma membrane H^+-ATPase, AHA4, in mutant Arabidopsis plants causes increased salt sensitivity (Vitart et al 2001). Activation of the Na^+/H^+ antiport activity of SOS1 by salt stress is controlled by SOS3 and SOS2 (Qiu et al., 2002; Quintero 2002). SOS3 is a myristoylated calcium-binding protein that is capable of sensing the cytosolic calcium signal elicited by salt stress (Liu and Zhu 1998; Ishitani et al., 2000). SOS2 is a serine/threonine protein kinase that has a unique carboxy-terminal regulatory domain and an amino terminal catalytic domain. The amino-terminal catalytic domain of SOS2 interacts with the carboxy terminal regulatory domain. The carboxy terminal regulatory domain of SOS2 also interacts with SOS3, and this interaction is mediated by 21-amino acid sequence, the FISL motif (Guo et al., 2001). In the presence of calcium, SOS3 activates the substrate phosphorylation activity of SOS2 (Halfter et al., 2000). The FISL motif is autoinhibitory and its deletion results in constitutive activation of SOS2 (Qiu et al., 2002; Guo et al., 2001). Constitutive activation of SOS2 can also be achieved by introducing mutations into the kinase activation loop (Guo et al., 2001; Gong et al., 2002).

Na^+ compartmentation

Vacuolar sequestration of Na^+ not only lowers Na^+ concentration in the cytoplasm but also contributes to osmotic adjustment to maintain water uptake from saline solutions. In Arabidopsis, the AtNHX family of Na^+/H^+ antiporters functions in Na^+ compartmentation (Blumwald 2000). AtNHX1 and AtNHX2 are localized in the tonoplast membrane and their transcript levels are upregulated by ABA or osmotic stress (Yokoi et al., 2002). The transcript levels of vacuolar H^+-ATPase components also increase in response to salt stress (Dietz et al., 2001).

K^+ Homeostasis

A high cytosolic K^+/Na^+ ratio is important for maintaining cellular metabolism. Under salt stress, Na^+ competes with K^+ for uptake into roots. The transcript levels of several K^+ transporter genes are either down- or upregulated by salt stress. Salt stress increases the transcript level of the Arabidopsis root K^+ transporter gene *AtKC1* (Pilot et al.,2003). In the common ice plant, salt stress upregulates the expression of KMT1 (a AKT/KAT family member) and various HAK/KUP (high affinity K^+ transporter/K^+ uptake transporter) type genes (Su et al., 2001; Su et al., 2002).

Compatible solutes

Under osmotic stress, there is accumulation of osmotically active compounds called osmolytes in order to lower the osmotic potential. These osmolytes do not inhibit normal metabolic reactions. Frequently observed metabolites with an osmolyte function are sugars (mainly sucrose and fructose), sugar alcohols (glycerol, methylated inositols), and complex sugars (trehalose, raffinose, fructans). In addition, ions (K+) or charged metabolites [glycine betaine, dimethyl sulfonium propionate (DMSP), proline and ectoine (1,4,5,6-tetrahydro-2-methyl-4-carboxy pyrimidine] are encountered. 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. Compatible solutes at high concentrations can reduce inhibitory effects of ions on enzyme activity to increase thermal stability of enzymes, and to prevent dissociation of enzyme complexes, for example, the oxygen-evolving complex of photosystemII. Trehalose- producing, transgenic rice (*Oryza sativa*) plants showed increased

tolerance to drought, salt, cold, as shown by chlorophyll fluorescence and growth inhibition analyses (Jang et al., 2003). Glycine betaine preserves thylakoid and plasma membrane integrity after exposure to saline solutions or to freezing or high temperatures (Rhodes and Hanson, 1993).

Induction of antioxidative enzymes

The formation of reactive oxygen species such as superoxide, hydrogen peroxide, hydroxyl radical (Halliwell and gutteridge, 1985), and single oxygen (Elstner, 1987) can seriously disrupt normal metabolism through oxidative damage to lipids (Frisovich, 1986; Wise and Naylor, 1987) and to proteins and nucleic acids (Fridovich, 1986; Imlay and Linn, 1988). Since internal oxygen concentrations are high during photosynthesis (Steiger et al., 1977) chloroplasts are especially prone to generate activated oxygen species (Asada and Takahashi, 1987). The plant possesses a number of antioxidant enzymes such as superoxide dismutase, catalase, peroxidases, and glutathione reductase to cope up with activated oxygen species. A putative phospholipids hydroperoxide glutathione peroxidase transcript was reported to increase during salt stress in Arabidopsis and citrus (Gueta-Dahan et al., 1997; Sugimoto and sakamoto, 1997); also in citrus transcripts and enzyme activities of Cu/Zn-SOD, glutathione peroxidase, and cytosolic ascorbate peroxidase increases (Gueta-Dahan et al., 1997).

Meloni et al. (2003) have shown that two cultivars of cotton, Guazuncho and Pora showed different response to salt stress. The superoxide dismutase (SOD: EC 1.15.1.1) activity in Pora increases with increase in the intensity of NaCl stress, but salt treatment has no significant effect on this enzyme activity in Guazuncho. The peroxidase (POD) and gutathione reductase (GR) activities showed similar trends under salt stress, in both cultivars. Net photosynthesis and stomatal conductance decreased in response to salt stress, but Pora showed a smaller reduction in photosynthesis than Guazuncho. Stomatal aperture limited leaf photosynthetic activity in the NaCl treated plants of both cultivars. However significant reduction in the leaf chlorophyll contents due to NaCl stress was observed only in Guazuncho. This suggests that salt tolerant cotton varieties may have a better protection against reactive oxygen species (ROS) by increasing the activity of antioxidant enzymes under salt stress. Wang et al. (2004) have shown that in *Suaeda salsa* L. Photosynthetic capacity was not decreased by NaCl treatment but seven SOD activity bands were detected in

S. salsa leaf extracts, including a Mn-SOD and several isoforms of Fe-SOD and CuZn-SOD indicating that *S. salsa* possesses an effective antioxidative response system for avoiding oxidative damage. According to Badawi et al. (2004) over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit.

Effect of salinity on photosynthetic pigments and proteins

The chlorophyll and total carotenoid contents of leaves decrease in general under salt stress. The oldest leaves start to develop chlorosis and fall with prolonged period of salt stress (Hernandez et al., 1995; Agastian et al., 2000). However, Wang and Nil (2000) have reported that chlorophyll content increases under conditions of salinity in *Amaranthus*. Kennedy and De Fillippis, 1999 have reported in *Grevilea* that protochlorophyll, chlorophylls and carotenoids are significantly reduced under NaCl stress, but the rate of decline of protochlorophyll and chlorophyll is greater than tht of Chl-a and carotenoids. Tezara et al.(2003) have shown that salt stress has no significant effect on chlorophyll content in *Lycium nodosum*.

Soluble protein contents of leaves decrease in response to salinity (Alamgir and Ali, 1999; Wang and Nil, 2000; Parida et al., 2002). Soluble protein increases at low salinity and decreases at high salinity in mulberry (Agastian et al.2000). In *Rhizobium* certain outer membrane proteins of molecular weight 22, 38, 40, 42, 62 and 68kDa markedly decrease in the presence of salt (Unni and Rao, 2001).

Effect of salinity on chloroplast ultrastructure

Hernandez et al., (1995) have shown in *Pisum sativum* that thylakoidal structure of the chloroplasts becomes disorganized, the number and size of plastoglobuli increases, and their starch content decreases in plants treated with NaCl. In mesophyll of sweet potato leaves thylakoid membranes of chloroplasts are swollen and most are lost under severe salt stress (Mitsuya et al., 2000). In leaves of NaCl-treated plants of tomato the transmission electron microscopy shows that the chloroplasts are aggregated, the cell membranes are distorted and wrinkled, and there are no signs of grana or thylakoid structures in chloroplasts (Khavarinejad and Mostofi, 1998).

Salinity effects on photosynthesis

Plant growth as biomass production is a measure of net photosynthesis and, therefore, environmental stresses affecting growth also affect photosynthesis. Salt stress causes either short or long term effects on photosynthesis. The short term effect occurs after a few hours or within 1or 2 days of the onset of exposure and this response is important, as there is complete cessation of carbon assimilation within hours. The long term effect occurs after several days of exposure to salt and reduction in carbon assimilation is due to the salt accumulation in developing leaves (Munns and Termatt, 1986). Although suppression of photosynthesis upon salt stress have been reported by many (Chaudhuri and choudhuri, 1997; Kao et al., 2001) there are also reports that photosynthesis is stimulated by low salt concentration (Rajesh et al., 1998; Kurban et al., 1999). Kurban et al., (1999) have reported that in A.*pseudoalhagi* (a leguminous plant), the leaf Co_2 assimilation rate increases at low salinity(50mM NaCl) but is not affected significantly by 100mM NaCl, while it is reduced to about 60% of the control in 200mM NaCl. In *Bruguiera parviflora* net Co_2 assimilation increases at low salinity and decreases at high salinity whereas stomatal conductance remains the same as in control at low salinity and decreases at high salinity (Parida et al., 2004).

Polyamines have been suggested to play an important role in stress protection. However, attempts to determine the function of polyamines have been complicated by the fact that polyamine contents could increase or decrease during stress. Kasinathan & Wingler (2004) have shown the accumulation of polyamines in control and mutated plants of *Arabidopsis thaliana* with reduced activity of arginine decarboxylase (EC 4.1.1.19). Polyamines accumulated in wild-type that were pre-treated with 100mM NaCl before transfer to 125mM NaCl, but not in plants that were directly transferred to 125mM NaCl without prior treatment with 100mM NaCl. This shows that polyamine accumulation depends on acclimation to salinity. The salt treatment that induced polyamine accumulation in wild-type plants did not lead to polyamine accumulation in mutants. Decreased fresh weight, chlorophyll content and photosynthetic efficiency indicated that mutants were more severely affected by salt stress than the wild type.

Photochemical Parameters

PhotosystemII (PSII) plays a key role in the response of photosynthesis to environmental perturbations (Baker, 1991). The results of salinity stress on PSII photochemistry are conflicting. Some studies have shown that salt stress inhibits PSII activity (Bongi and Loreto, 1989; Baelkhodja., 1994; Everard et al.., 1994), whereas other studies have indicated that salt stress has no effects on PSII (Robinson et al.., 1983; Brugnoli and Bjorkman, 1992; Abadia et al. ., 1999).

Chen et al. (2004) have shown that NaCl treatment in *Rumex* leaves alone had no effect on the maximal photochemistry of PSH or the polyphasic rise of chlorophyll fluorescence but prompted heat resistance of the O_2-evolving complex (OEC) and also causes lesser heat-induced decrease in photochemical quenching (qP), efficiency of excitation energy capture by open PSII reaction centers (Fv'/Fm'), and quantum yield of PSII electron transport (Φ PSII). Salt stress reduced the heat stress on PSII photochemistry by causing lesser heat-induced decrease in photochemical quenching (qP), efficiency of excitation energy capture by open PSII reaction centers (Fv'/Fm'), and quantum yield of PSII electron transport (ΦPSII) (Allakhvediv et al. 2000).

Effects of light and salt stress on photosystem II (PSII) in the cyanobacterium *Synechocystis* sp. PCC 6803 are completely different. Strong light induced photodamage to PSII, whereas salt stress inhibited the repair of the photodamaged PSII and did not accelerate damage to PSII directly. The combination of light and salt stress appeared to inactivate PSII very rapidly as a consequence of their synergistic effects. Radioactive labeling of cells revealed that salt stress inhibited the synthesis of proteins de novo and, in particular, the synthesis of the D1 protein (Allakhverdiev et al., 2002).

PROTEIN IMPORT INTO CHLOROPLAST

Plastids are a heterogeneous family of organelles found ubiquitously in plant and algal cells (Whatley 1978). Most prominent among these are the chloroplasts, which contain the green pigment, chlorophyll and are responsible for the light harvesting and carbon fixation reactions of photosynthesis, as well as synthesis of many essential metabolites such as fatty acids and amino acids. Chloroplasts are complex organelles comprising six distinct compartments: they have three different membranes (the two envelope membranes and the internal thylakoid membrane), and

three distinct aqueous compartments (the intermembrane space of the envelope, the stroma and the thylakoid lumen). As it is known that chloroplasts are organelles of endosymbiotic origin, and they have transferred most of their genetic information to the host nucleus during this process. Therefore they have to import several nuclear encoded proteins from cytosol to their proper suborganellar compartment. Targeting is mediated by a set of targeting signals that are intrinsic to the nuclear encoded proteins. These signals act independently to initiate translocation of the protein across one or more of the chloroplast membranes en route to their proper destination (Cline and Henry, 1996).

TRANSIT PEPTIDE

The majority of chloroplast preproteins contain a cleavable N-terminal transit peptide leading to post-translational import (Soll and Schleiff, 2004). The transit peptide is essential and sufficient for targeting to the surface and transfer across the translocation machinery of the two envelope membranes of chloroplasts. However, recent studies have identified two classes of proteins that are localized in chloroplasts and which do not contain such a targeting signal. While most of the outer envelope proteins do not contain such a signal proteome analysis of chloroplastic proteins has revealed stromal and inner envelope proteins that are not containing such a targeting signal (Kleffmann et al., 2004). In case of the outer envelope proteins the information for directing the protein to its proper location is contained within the mature protein. They are deficient in acidic amino acids and abundant in serine and threonine. The targeting information contained within the mature proteins generally is located in membrane spanning domains; it also serve as a membrane insertion or a stop transfer signal (Keegestra and Cline, 1999).

Although the transit peptide of different precursors proteins share only slight similarity at the amino acid level and in length (25 to 150 amino acids), some of the following common features do exist (i) Transit peptides are enriched for hydroxylated amino acids such as serine or threonine. (ii)They possess small hydrophobic amino acids and only a few acidic residues, resulting in an overall positive charge (Cline, 2000). (iii)In aqueous solutions the transit peptides are largely unstructured with a small content of β sheet structure (Pilon et al., 1992; Theg and Geske, 1992).

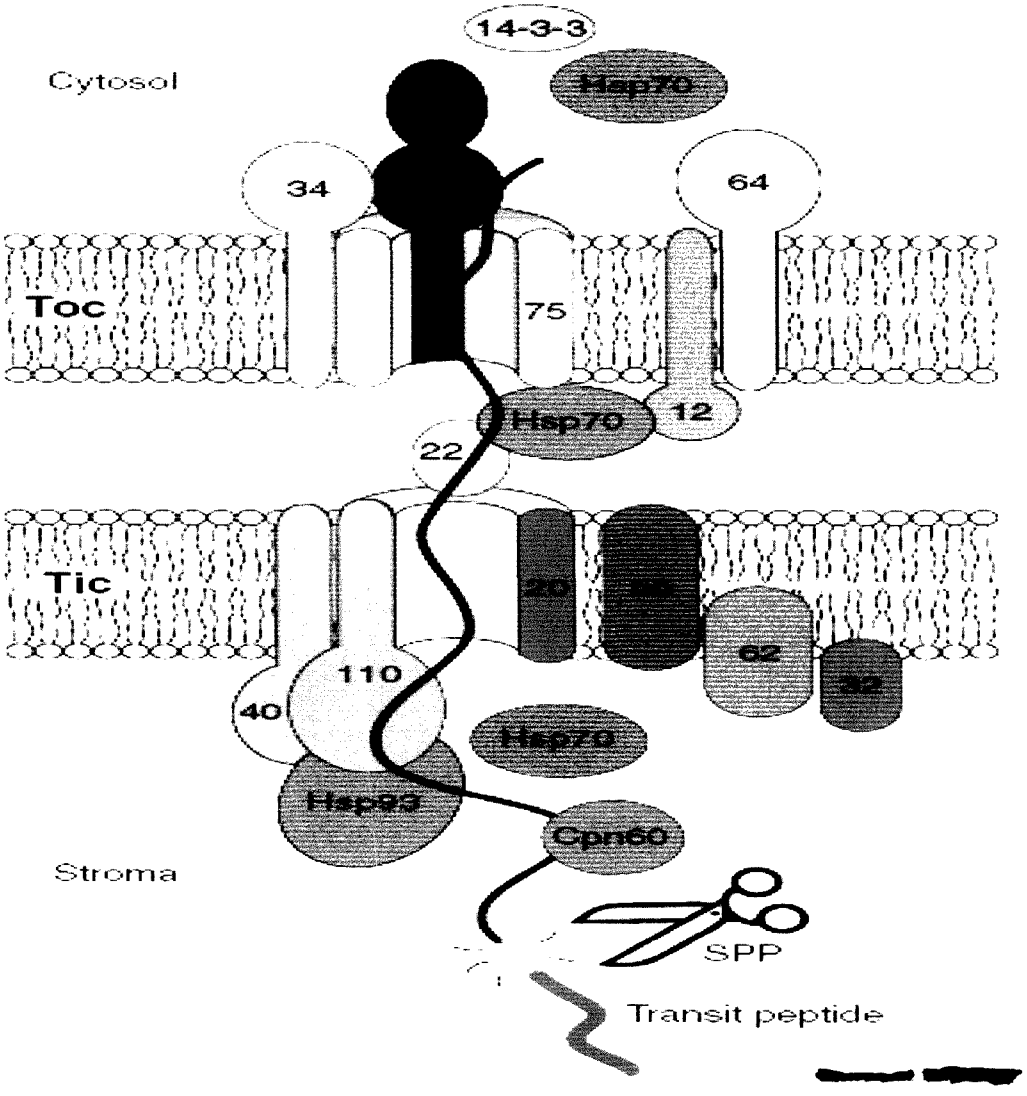


Figure 2. The chloroplast protein import apparatus. (Adapted from, Jarvis, [redacted] and Robinson, [redacted] 2004)

ENVELOPE TRANSLOCATION

Currently, four different pathways are proposed for translocation of chloroplast precursor proteins to their final destination: (i)Direct membrane insertion of the outer membrane proteins, (ii)an interaction of non-phosphorylated precursors proteins with as yet unidentified components of the cytosolic transport machinery, (iii) guiding of phosphorylated precursors to the chloroplast surface by a hetero oligomeric protein complex called the guidance complex (consisting of 14-3-3, Hsp70 and perhaps other so far unknown components), and (iv)Shuttling by the outer envelope receptor Toc 159.

All precursors bind to cytosolic Hsp 70 after translation. In addition, many of the precursor proteins contain a phosphorylation motif within the N-terminal transit peptide which shares similarities to 14-3-3 binding sites. This motif within the transit peptide of pSSU or pOE33 can be phosphorylated on serine or threonine in vitro by a cytosolic kinase (Waegemann and Soll, 1996).

The import of precursors into chloroplasts can be divided into 3 distinct stages:

1. The precursor proteins interact with chloroplasts in an energy independent and readily reversible manner.

2. The precursor proteins interact with the translocation apparatus in an irreversible manner. Olsen et al. (1989) showed that a low level of ATP (>100um) is required for this stage. The hydrolysis of ATP during this stage promotes the insertion of precursor proteins into the protein conducting complex of both outer and inner membranes, which has been defined as the formation of 'early import intermediate' (Ma et al., 1996;` Nielson et al., 1997).

3. The third stage of import requires higher levels of stromal ATP (1-3mM) for complete translocation of precursors across the envelope membranes (Theg et al., 1989). During or after translocation, the TP is removed by the stromal processing peptidase (Ritcher and Lamppa, 1998). A membrane potential across the envelope membrane is not required for protein transport into chloroplast distinguishing this process from mitochondrial protein import (Theg et. al., 1989).

The translocation apparatus comprises protein complexes in the outer and inner membranes called TOC (translocon at the outer envelope membranes of chloroplasts) and TIC (translocon at the inner envelope membranes of chloroplasts) respectively. During import, the TOC and TIC complexes come together at contact

sites and the precursor protein passes through both membranes simultaneously (Kouranov et al., 1998; Kuchler and Soll, 2001).

TOC APPARATUS

The TOC core complex is more than 500kDa in size and comprises four different proteins, each named for their molecular weights: Toc159, Toc34, Toc75 and Toc64. Structural studies of Toc complex reveals a solid ring with a less dense interior which is divided into four parts by a central, finger like domain. It was suggested that Toc 159 represents the central finger like domain, whereas the four Toc75 polypeptides form the surrounding channels (Schleiff et. al., 2003b)

Toc 159

Toc 159 is the GTP binding subunit of translocon. The full length protein can be divided into three regions: an amino-terminal A-domain, which is rich in acidic amino acids; a central G-domain, which contains the GTP binding site and finally the carboxy-terminal membrane or M-domain (Bauer et al., 2000 and Hiltbrunner et al., 2001). Toc 159 appears to be a major point of contact for precursor proteins arriving at the translocon complex, and has therefore been regarded as a chloroplast protein import receptor (Perry et al., 1994; Kessler et. al., 1994; Hirsch et. al., 1994). It has been proposed to function as a soluble receptor that shuttles between the cytosol, where it binds with preproteins, and the chloroplast surface it delivers the preproteins (Hiltbrunner et. al., 2001). Cross linking experiments under conditions favouring energy independent binding identified Toc 159 as the translocon component most closely associated with bound precursor proteins. Toc 159 is a highly labile protein and was originally identified as an 86kDa proteolytic fragment called Toc86 (Waegemann et. al., 1991; Chen et. al., 2000).

Toc 159 also possesses characteristic GTP binding site motifs within its central domain (Kessler et al., 1994). GTP is required for progression to the early import intermediate stage only and not for precursor translocation (Young et. al., 1999; Chen et. al., 2000). Thus, Toc159 undergoes one or more rounds of GTP hydrolysis after energy- independent binding in order to transfer the preprotein to

other subunits of Toc complex or to initiate insertion across the outer envelope membrane (Young et al., 1999).

In Arabidopsis thaliana, four isoforms of Toc 159 are described namely, atToc159, atToc132, atToc120 and atToc90 (Soll and Schleiff, 2004).

TOC 34

The second receptor of the Toc complex is the GTPase Toc34. It is anchored by its carboxy terminal tail and its N-terminal 266 amino acids long GTPase domain projects into the cytosol (Kessler et al., 1994; Seedorf et al., 1995). Toc 34 was co-purified with trapped precursor protein by a linear sucrose gradient and associated with Toc159 and Toc75 (Seedorf et al., 1995; Nielsen et al., 1997; Kouranov and Schnell, 1997). The cytosolic region of Toc34 reveals 34% α-helical and 26% β-strand structure and is stabilized by intramolecular electostatic interaction. The crystal structure of the Toc34 GTP-binding domain suggests that GTP-regulated dimerisation of the Toc GTPase domains controls the targeting and translocation of preproteins at the choroplast envelope (Kessler and Schnell, 2002).

Jarvis *et al* (1998) have isolated an *Arabidopsis* mutant lacking a new component of import machinery (Toc33, a 33 kDa protein). The functional similarity of Toc33 to Toc34 implies that multiple different translocon complexes are present in plants (Chen *et al.,* 2000). Processes that are mediated by Toc33 operate during early stages of plastid and leaf development. Toc 34 and 33 seem to have distinct but overlapping functions (Gutensohn *et al.*, 2000).). Two Arabidopsis mutants, defective in atToc33 and atToc159 have been isolated from T-DNA-tagged mutants (Jarvis *et al.,* 1998; Bauer *et al.,* 2000).

Toc159 and Toc34 reveal considerable sequence homology also outside the nucleotide-binding motifs. Furthermore, both proteins are phosphorylated by protein kinases; Nucleotide binding and phosphorylation might represent further regulatory checkpoints during translocation (May and Soll, 1999).

TOC 75

Toc75 is one of the most abundant protein of the outer envelope of chloroplasts. It was discovered as a Toc component by the stable interaction with precursor proteins and by association with Toc159 and Toc34 (Perry and Keegstra,

1994; Schnell et al., 1994). The protein is deeply embedded into the membrane by a β barrel structure that consists of 18 predicted sheets (Hinnah et al., 1997; Schleiff et al., 2003a). Heterologously expressed TOC75 forms a cation-selective, high conductance ion channel when it is reconstituted into planar lipid bilayers (Hinnah et al., 1997). Electrophysiological measurements indicated that the channel is 25 Å wide at the entrance, constricts to 15-17 Å wide inside the channel. A width of 15 Å could accommodate a polypeptide stretch that still retains some secondary structure (Hinnah et al., 2002). The electrophysiological data also showed that TOC75 has a cytosolic preprotein binding site, which is able to distinguish between the precursor protein and the mature form (Ma, Y. et al., 1996; Hinnah et al., 1997; Hinnah et al., 2002).

A number of TOC75 isoforms are present in *Arabidopsis thaliana*, Toc75-I, Toc75-III, Toc75-IV and Toc75-V. *At*Toc75-III is the most abundant outer envelope protein (Soll and Schleiff, 2004). One possibility for the existence of different AtToc75 genes is that they are differentially regulated and so represent the simplest means of achieving the necessary pattern of expression. An alternative explanation is that the different atToc75 proteins have different precursor recognition specificities (Jarvis and Soll, 2001).

TOC 64

Toc64 is a dynamically associated component of the Toc complex, since it cannot be co-isolated with the core complex (Sohrt and Soll, 2000; Schleiff et al, 2003b). The polypeptide is functionally divided into two modules. The N-terminal part exhibits homologies to amidases. However, the potential amidase function seems to be inactivated by a mutation of the central catalytic serine to glycine. The C-terminal part consists of three tetra-trico peptide repeats (TPR; Sohrt and Soll, 2000) which share homology to the TPR domains of Tom70 interacting with cytosolic chaperones (Young et al., 2003). Preliminary data indicate that the cytosolic guidance complex interacts specifically with Toc64. It is proposed that the preproteins arriving atToc64 as part of a guidance complex might pass to a receptor complex comprising Toc159, Toc 75 and Toc 34. Another possibility is that preproteins unload from the guidance complex on arriving at Toc 64, and then partition into the lipid bilayer prior to recognition by the other receptor proteins (Jarvis and Soll, 2001).

In *A.thaliana*, three genes coding for isoforms of Toc64 were identified (Soll and Schleiff, 2004). Interestingly, one of the isoforms does not represent a

chloroplast receptor but is assembled into the mitochondrial translocation system, replacing the Tom70 receptor. This underlies the close functional relationship between both receptor classes and the possibility that the function of Toc64 is similar to that of Tom70 (Truscott et al, 2003).

TIC APPARATUS

The import of pre-proteins proceeds from the outer envelope through the intermembrane space, across the inner envelope of chloroplasts engaging the Tic complex. To date seven components of this complex have been identified, namely Tic110, Tic62, Tic55, Tic40, Tic32, Tic22 and Tic20.

TIC110

The best studied of the Tic components; Tic110 is a 110kDa integral membrane protein of the inner envelope (Kessler and Blobel 1996; Lubeck *et al.* 1996). It contains one or two hydrophobic trans-membrane domains in its amino terminal region with the large hydrophilic domain (>90kDa) protruding into stromal compartment (Kessler and Blobel 1996; Jackson *et al.,* 1998). Tic110 was the first Tic component to be identified (Schnell *et al.*, 1994). On the inter-envelope-space side, it seems to be in the close vicinity of, or even in contact with, the Toc translocon. On the stromal side of the inner envelope, Tic110 could interact with molecular chaperones such as Hsp93 and chaperonin-60 (Kessler et al., 1996; Nielsen et al., 1997). Reconstitution experiments using heterologously expressed protein showed that Tic110 can form a cation-selective channel in lipid bilayers (Heins et al., 2002). The electrophysiological properties of this channel indicate a pore size of 15-20 Å, which is sufficient to allow the passage of a polypeptide chain.

Tic110 seems to act as a molecular workbench during the import process fulfilling three different functions on two different sites of the membrane leaflet, first it is involved in the formation of the contact sites to the Toc complex, second it recruits stromal chaperons to drive the translocation process and third it forms the protein conducting channel of the inner envelope membrane.

Circular dichroism spectra of heterologously expressed soluble tic 110 (residues 93-966) indicated a largely α-helical conformation (Inaba et al., 2003)

whereas CD spectra recorded after the refolding of a similar tic 110 domain following treatment with 8M urea indicated a high propensity for β-strands (Heins et al., 2002).

TIC62

Tic62 was found by blue native polyacrylamide gel electrophoresis to be complexed with Tic110 and Tic55 (Kuchler et al., 2002). This integral membrane protein contains a NAD-binding site and harbours a repetitive sequence motif interacting with ferredoxin-NADP oxidoreductase (FNR) on the stromal C-terminus of Tic62 (Kuchler et al., 2002). Since FNR transfers redox equivalents from ferrodoxin to NADP, it is conceivable that Tic62 and Tic55 functions in redox sensing and therefore in regulation of precursor translocation.

TIC55

Tic55 is an integral membrane protein and is partially exposed to the intermembrane space. Analysis of Tic55 protein structure revealed a predicted Rieske-type iron-sulfur cluster and a mononuclear iron-binding site suggesting that Tic55 might function as a regulator during protein import by responding to changes in redox status within the chloroplast (Caliebe *et al.*, 1997). *In vivo*, Tic55 might function specifically in the import of photosynthetic proteins that are critical for maintaining redox balance, (Kouranov *et al.*, 1998).

A single Tic55 homologue has been encoded in the *Arabidopsis* genome, but its role in chloroplast protein import remains elusive (Jarvis and Soll, 2001).

TIC40

Tic 40 is an integral membrane component of the Tic complex and it seems to be tightly associated with Tic 110 (Stahl et al., 1999; Chou et al., 2003). The carboxy terminal region is exposed on the stromal site of the inner envelope, and this region seems to have two functional domains. First it is homologous to the Hsp70 interacting proteins as well as Hsp70/hsp90 organizing proteins (Stahl et al., 1999) and second, it has a tetratricopeptide domain for protein-protein interactions (Chou et al., 2003). Immuno-precipitation experiments indicate not only a close association with Tic 110 but also with stromal hsp93. Tic40 is encoded by a single gene is *A.thaliana* (Chou et al., 2003).

TIC 22 and TIC20

Tic22 is a 22kDa protein that is located in the intermembrane space between the outer and inner envelope membranes and is peripherally associated with the outer face of the inner membrane. It is largely hydrophilic with no predicted transmembrane domains (Jarvis and Soll, 2001).

Since Tic22 forms a cross-linking product with the preprotein, which is not engaged with the Tic complex, its function as a chaperone is assumed. Tic22 is nuclear encoded and synthesized as a preprotein with a 50 amino acid N-terminal presequence. Import of Tic22 was stimulated by ATP and required the presence of protease sensitive components on the chloroplast surface. It is hypothesized that ATP might be necessary to stabilize Tic22 during or shortly after translocation (Kouranov *et al.*, 1999). In contrast to other transit peptides, the preTic22 presequence was unable to direct import to the stroma and the targeting of preTic22 to the intermembrane space was not competed by stromal preproteins. Thus, Tic22 import represents a new type of targeting signal specific for chloroplast intermembrane space (Kouranov *et al.*, 1999).

By contrast, Tic20 is predominantly hydrophobic and is predicted to have three α-helical transmembrane domains. It is resistant to alkali extraction and exogenously applied protease indicating that it is deeply embedded in the inner envelope membrane (Kouranov *et al.*, 1998; Chen *et al.*, 2002). Tic20 is proposed to function as a protein-conducting component of the Tic complex (Kouranov *et al.*, 1998). As shown by the level transfer cross-linking experiments, Tic20 and Tic22 are close to a chimeric preprotein.

Genes encoding two Tic22 homologues and at least two Tic20 homologues are present in the *Arabidopsis* genome, which help to establish the roles played by Tic22 and Tic20 in chloroplast protein import (Jarvis and Soll, 2001).

MOLECULAR CHAPERONS

Molecular chaperones are proteins of both prokaryotes and eukaryotes that bind to nascent or unfolded peptides and ensure correct folding or transport. Heat-shock proteins are an important subset of chaperones. Five major families are recognized, the chaperonines (GroEL and Hsp60), the Hsp70 family, the Hsp90

family, Hsp100 (Clp) family and the small Hsp family. Besides these major families there are other proteins with chaperone functions.

Two members of the Hsp70 chaperone family might participate in the translocation process at the outer envelope membrane. Com70, a Hsp70 homologue is associated with the Toc complex at the cytosolic site and generates crosslink products with precursor proteins during early stages of translocation (Kourtz and Ko, 1997). An Hsp70 in the intermembrane space, IAP70, was also identified as a component of the import machinery (Schnell *et al.*, 1994). Both chaperones might prevent folding and stabilize precursor proteins in an import competent form and so could maintain a productive transport cycle across the outer envelope membranes (May and Soll, 1999). The Hsp70 binding domain has been identified on transit peptides of pRSS (Ivey and Bruce, 2000) and FNR (Rial *et al.*, 2000). Rial *et al.* (2000) observed that the putative binding site for the chaperones is located at position 11 from the N-terminus of Transit peptide. The interaction of signal peptides with Hsp70 during import process is supported by biochemical studies; about 82.5% chloroplast transit peptides have an Hsp70 binding site (Zhang and Glaser, 2002). A member of Hsp100 chaperone family is identified in chloroplasts, which interacts with precursor proteins during import (Kessler and Blobel, 1996), may also function in assisting protein translocation.

STROMAL PROCESSING

Immediately upon arrival in the stroma, precursor proteins are proteolytically processed to remove their transit peptides. Newly imported proteins are then folded into an active conformation, either directly, or after further internal sorting, in case of thylakoid and envelope proteins (Keegstra and Cline, 1999; Jarvis and Soll, 2001). Transit peptide removal is catalyzed by an enzyme called stromal processing peptidase (SPP). The enzyme has been purified from pea chloroplast and has a signature zinc-binding motif (His-X-X-Glu-His). SPP initially releases the transit peptide intact upon precursor processing which is latter degraded by a second ATP- and metal-dependent protease in order to prevent their accumulation in the stroma (Jarvis and Soll, 2001).

MATERIALS AND METHODS

PLANT MATERIALS AND CHEMICALS:

Plant Material

Pea (*Pisum sativum*) seeds were used as experimental material. The seeds were obtained from Indian Agricultural Research Institute, New Delhi.

Chemicals

TNT-coupled SP_6 polymerase *in vitro* translation kit was purchased from Promega Corp., USA, 35 S methionine was purchased from Amersham Biosciences, Reverse Transcriptase for cDNA synthesis was purchased from Promega Corp., USA, and other chemicals were purchased from Amersham chemical company and Qualigens.

PLANT GROWTH CONDITIONS

The pea seeds were treated with 0.1% $HgCl_2$ solution for two minutes, washed with tap water several times. Seeds were grown in vermiculite in cool white fluorescent light (70 µmoles $m^{-2}s^{-1}$) at 25^0C. The plants were first grown for 8 days after which they were given salt stress. To induce the salt stress the rooting medium vermiculite was washed twice with 100mM or 200mmNaCl solution. After salt wash 100mM or 200mM NaCl water was added upto the level of the vermiculite. The water level was maintained by adding water at frequent intervals.

SODIUM DODECYL SULPHATE -POLYACRYLAMIDE GEL ELECTROPHORESIS

Materials

1. Monomer solution (30% T, 2.7% C)

Acrylamide	58.4g
Bis-acrylamide	1.6g

Distilled water added to make vol. 200ml

This stock solution was filtered and stored at 4^0C in amber bottle.

2. Separating gel buffer (4X)

Tris (1.5M) 36.3g

Final vol. made to 200ml with distilled water after adjusting pH to 8.8 with HCl. Stored at 4^0C.

3. Stacking gel buffer (4X)

   Tris (0.5M) 6.0g

Final vol. made to 100ml with distilled water after adjusting pH to 6.8 with HCl. Stored at 4^0C.

4. SDS (10%)
5. Sample buffer (2X)

Tris Cl (0.125M, pH 6.8)	2.5ml of stacking gel buffer
SDS (4%)	4ml of 10% SDS
β-mercaptoethanol (10%)	1ml
Glycerol (20%)	2ml

Distilled water added to make up the vol. to 10ml.

6. Tank buffer (4X)

Tris (0.025M)	15g
Glycine (0.192M)	72g
SDS (0.1%)	40ml of 10% SDS

Distilled water added to make up the vol. to 1L.

7. APS 10% (Initiator)- Always prepared fresh.
8. TEMED
9. β-mercaptoethanol

Recipes	**15% Separating gel**	**4.5% Stacking gel**
1. Monomer stock solution	12.0ml	1.7ml
2. Buffer	6.0ml (pH 8.8)	2.5ml (pH 6.8)
3. SDS (10%)	0.24ml	1.0ml
4. DW	5.6ml	4.8ml
5. TEMED	15µl	5µl
6. APS (10%)	400µl	150µl
Total vol.	**24ml**	**10ml**

PROTEIN IMPORT STUDIES

In vitro reconstitution of protein transport into chloroplast involves several different steps.

1. Production of ^{35}S-Met-labelled precursor proteins.
2. Isolation and purification of intact chloroplasts.
3. Incubation of precursor proteins and chloroplasts under conditions that would allow transport to occur.
4. Termination of transport reaction by separating the chloroplasts, containing import proteins, from the reaction mixture containing any residual precursors.
5. The extent of transport is determined by measuring the amount of mature protein that has accumulated inside the chloroplasts.

Preparation of precursor proteins

In vitro ^{35}S-Met-labelled precursor proteins (pRSS) were prepared by the TNT-coupled SP6 polymerase *in vitro* translation product following the manufacturer's protocol.

The kit constituents were taken out and kept in ice just before use. The reaction was set at room temperature.

The reaction mix contained the following components:

TNT wheat germ extract	25µl
TNT reaction buffer	2µl
TNT RNA polymerase	1µl
Amino acid mix minus Methionine (1mM)	1µl
RNAsin (40U/ml)	1µl
DNA template	~2µg
DEPC water	To make 50µl

The reaction mix was incubated at 30^{0}C for 1 h. The reaction was terminated by adding 50µl of cold methionine (60mM) in 2x import buffer and was kept in ice or frozen till further use.

Purification of intact chloroplast

Isolation was done as described by Tewari and Tripathy (1998).

Materials

1. 2x grinding buffer (2x GR), pH 7.5 with KOH

HEPES	0.1M
Sorbitol,	0.7M
$MgCl_2$	2mM
$MnCl_2$	2mM
Na_2EDTA	4mM
BSA	0.2%

1x GR was prepared by diluting with equal volume of water and adding isoascorbate (0.1gm in 200ml of 2x GR). 1x GR was prepared just before use in percoll gradient.

2. 2x Import buffer (2x IB), pH 8.0 with KOH

HEPES	0.1M
Sorbitol	0.7M

1x IB was prepared by adding equal amount of water to 2x IB.

3. Percoll gradient (50%)

15ml 2x GR

15ml 100% percoll

10mg D-isoascorbate (a pinch)

It was mixed by invertion.

4. Percoll gradient (40%)

2.5ml 2x IB

2ml; 100% percoll

0.5ml water.

Method

About 30gm of pea shoots were harvested into the grinding container by snipping with scissors. 3days after stress and 5 days after stress pea plants were taken.

Tissue was grinded in 7 vol of cold 1x GR using 2 bursts of 3 sec each. The brei was filtered through one layer of Miracloth and 8 layers of cheesecloth into the 500ml flask. The filtrate was centrifuged at 4000 rpm for 7 min in a Sorvall SS34 rotor at 4^0C with brake on. Supernatant was discarded, and the pellet was suspended in about 2ml of 1x GR using a paintbrush. The suspended pellets were added to the 50 % percoll gradient in a single centrifuge tube, and the contents were mixed gently by inversion, covering the tube with parafilm. The contents were centrifuged at 18,000 rpm for 22 min in a Sorvall SS34 rotor with brake off. Two bands were observed. The lower green band was collected and diluted with 3 fold 1x IB. It was centrifuged at 4000 rpm for 5 min with brake on. The supernatant was discarded. Pellet was suspended in 1ml of 1x IB. 20 µl was taken for assaying chlorophyll. The chloroplasts were pelleted at 3000 rpm for 5 min, and resuspended in 1x IB at a concentration of 1mg Chl/ml.

Transport reactions

1. The transport reaction was set at room temperature. The Reaction mix contained the following:

Precursor protein (Transport mixture)	10µl
100mM ATP	5µl
Chloroplast suspension (1mg chl/ml)	35µl
1x IB	to make 100µl.

It was incubated at 25^0C for 15 to 20 min, with gently agitating the reactions every 5min.

Termination

1ml of cold 1x IB was added to terminate the reaction. Intact plastids were pellet down by layering the reaction mix on the top of 1ml of 40% percoll in 1x IB and by centrifuging at 6,500 rpm for 6min in a microcentrifuge. The pellet was suspended in 1ml of cold 1x IB and was again pellet down for 5 min at 6000 rpm. The pellet was dissolved in 50 µl of 1x sample buffer, boiled for 3 min and analyzed by SDS-PAGE.

Gel Electrophoresis and Phosphorimaging

A 15% gel was run to analyze the Rubisco small subunit protein. Stock solutions and gel buffers were prepared as described earlier. Before loading, the

sample was spun briefly to pellet down the impurities. Gel was run for ~1.5hrs at a constant voltage of 90 volts. After the electrophoresis, gel was dried onto a filter paper and exposed overnight to imaging film at room temperature. The imaging plate was scanned by phosphorimager. The scanned image was quantified by using image gauge software.

ISOLATION OF TOTAL RNA

The Total RNA was isolated using TRI REAGENT from Sigma.

Materials

1. TRI REAGENT
2. Chloroform
3. Isopropanol
4. 75% Ethanol
5. Formamide

Method

This procedure is an improvement of the single step method reported by Chomczynski and Sacchi (1987). All the glassware used during RNA isolation were baked overnight at 200°C, the plastic ware were treated with DEPC and then autoclaved to avoid the possible contamination of RNase.

Leaves (100mg) were taken and homogenized in liquid N_2 in mortar and pestle. TRI REAGENT was added to the powdered leaves (1.0ml for 0.1g) while the leaf powder was in mortar and pestle and liquid nitrogen was still there. To ensure complete dissociation of nucleoprotein complexes, samples were allowed to stand for 5 minutes at room temperature. Then 0.2 ml of chloroform was added per ml of TRI REAGENT. The sample was then shaken vigorously for 15 seconds and allowed to stand at room temperature for 2-15 minutes at room temperature. Then the resulting mixture was centrifuged at 12,000 rpm for 15 minutes at 4^0C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing proteins), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). The aqueous phase is transferred to a fresh tube and 0.5ml of isopropanol was then added per ml of TRI REAGENT. Sample was allowed to stand for 5-10 minutes at room temperature and then centrifuged at 12,000 rpm for 10 minutes at 4^0C. The RNA precipitates at the bottom of the tube. RNA pellet was then washed by adding 1ml of 75% of ethanol per 1ml of TRI REAGENT. The sample was then centrifuged at 7,500

rpm for 5 minutes at 4^0C. The sample was then dried at room temperature. The pellet was dissolved in formamide by incubating at 65^0C.

Quantification of RNA

RNA concentration was measured by taking OD at 260 nm and OD at 280 nm in a Shimadzu UV-160 spectrophotometer. An OD of 1.0 corresponds approximately to 50 μg/ml for double-stranded DNA and 40 μg/ml for single-stranded DNA and RNA. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA or RNA have the values of 1.8 and 2.0 respectively.

First Strand Synthesis (cDNA synthesis) and Polymerase Chain Reaction

Total RNA was extracted from control and stressed plants as described above. For a total volume of 50μl, 4μg of RNA was taken along with 5μl of 10mM of dNTP mix, 3μl of oligodT (concentration 5picomole/μl for 50 μl reaction), 1μl of RNase inhibitor (40 units), 10 μl of 5Xbuffer and 20 units of AMV Reverse Transcriptase (Promega Corp.) and DEPC treated water to make up the volume. The mixture was then incubated for a period of 60 minutes at 37°C.

The total cDNA was then used in a 1/10 dilution for PCR. The PCR was carried out for a total volume of 20 μl containing 2μl of 1mM dNTP, 2μl of 10XTaq buffer, 3μl of both forward and reverse primer (15picomoles/ 20 μl reaction), 0.5 μl of Taq enzyme (1.5units) and 20ng of cDNA. The linear range for comparative PCR was determined to be 30 cycles. The product was detected by loading the mixture on 1% Agarose gel. The gene specific primers of pea have following sequences were used to amplify cDNA:

Actin F: 5'-CGG GAT CCA TGG CAG AAT CCG AAG ATA TT-3'
Actin R: 5'-CGG GAT CCT TAG AAG CAT TTC CTG TGT AC-3'
TOC34F: 5'-CGG GAT CCA TGG CTT CAC AAC AAC AAA CT-3'
TOC34R: 5'-GCT CTA GAT CAC TTC CGA TCA CCT ACA TCG CG-3'
TOC64F: 5'-GGG GTA CCA TGA AAT CAA TGG CTT CTC CGT CG-3'
TOC64R: 5'-CGG AAT TCC TAC TGA AAT AGT TTC CTC AAC CT-3'
TOC159intF: 5'-GCC TGG CAT ACA TTG TTA GAG GT-3'
TOC159 R : 5'-CGG GAT CCT TAA TAG ATG GAA TAG TTT TC-3'

TIC55F: 5'-GCT CTA GAA TGG CGT TGG CGT TGG CGT CGG CG-3'
TIC55R: 5'-GCT CTA GAT CAC AAT CTC CTA TGT ACC CT-3'
TIC62F: 5'-CGG GAT CCG GTA CCA TGG AAG GAA CTT GTT TTC TC-3'
TIC62R: 5'-CGG GAT CCG AAT TCC TAA TGA TTG GTG ACT GG-3'
TIC20F: 5'-CGG AAT TCA TGA TTC AAA ATG GTG GCA CT-3'
TIC20R: 5'-CGG GAT CCT TAC TCG TGT GGT ATT TGA AT-3'
TIC22F: 5'-CGG AAT TCA TGG AGT CTC AGG GAC AGT GG-3'
TIC22R: 5'-CGG GAT CCT TAA GCA ATA ACT CTC TGC AT-3'
TIC110intF: 5'-GCT TGA CGA AGG CCA GGG TTG AG-3'
TIC110 R : 5'-CGG GAT CCC TAG AAT ACA AAC TTC TCT TC-3'
HSP93F: 5'-GCA TGG CTA GAG TTT TGG CTC AGT CAC TT-3'
HSP93R: 5'-TTA TAT AGA AAG AGC CTC TGG TAA CGA CTC-3'
CPE F: 5'-CGG GAT CCA TGC CAA TGG CTG CTT CAA CT-3'
CPEintR: 5'-CGG GAT CCA GCT TAG GAA CTA GAA AAC TA-3'

Pulse Amplitude Modulation Measurements

F_o, F_v/F_m, Non-photochemical quenching, photochemical quenching, electron transport rate and quantum yield of photosystem II was measured at room temperature by a PAM-2100 Chl fluorimeter (Walz, Germany). Red actinic illumination (wavelength, 655 nm) was provided by five LEDS (H3000); Stanley, Irvine, CA, USA) focused onto the leaf surface (79 mm^2). Two other H-3000 LEDS, that emit 650-nm pulses, were used as measuring light. Leaf clip holder 2030-B, equipped with a microquantum sensor, monitored photosynthetically active radiation. Chl a fluorescence was detected by a photodiode (BYP 12; Siemens, Munich, Germany) that was shielded by a long-pass far-red filter (RG9; Schott, Southbidge, MA, USA) and a heat filter. All the PAM-2100 fluorescence data were recorded in a time span of 5 min. and 20 s.

RESULTS

The effect of salt stress on photochemical reactions and protein import into chloroplast were studied by taking pea (*Pisum sativum*) as a model plant. The pea plants were grown for 8 days under cool white fluorescent light (70μmoles $m^{-2}s^{-1}$) at 25°C and then were treated with 0 (control), 100mM and 200mM NaCl for 72 hours (3 days) and 120 hours (5 days).

Chloroplasts are the most sensitive organelles affected by stress. Any change in structure and function of chloroplasts affects photosynthesis and plant productivity. Chlorophyll a fluorescence may be used as a signature of photosynthetic reactions. Therefore, the chlorophyll a fluorescence has been used to study the F_o, F_v/F_m, Electron Transport Rate (ETR), Quantum Yield of Photosystem II (Yield), Photochemical Quenching (qP) and Non-photochemical Quenching (qN).

PHOTOCHEMICAL PARAMETERS

F_o and F_v/F_m

There was no significant effect of salt stress on F_o after 72 h of stress. However, when stress was prolonged to 96 h and 120 h the F_o values increased over that those of control samples (Fig 1).

The F_v/F_m values decreased in both 100mM and 200mM salt stressed plants after 72 h, 96 h and 120 h of stress. The F_v/F_m values were 70.85% and 21.24% of control in 100mM and 200mM salt-stressed plants respectively after 120 h of stress (Fig 2).

ELECTRON TRANSPORT RATE (ETR)

The electron transport rates are shown in figure 3. The electron transport rate of pea plants increased in response to PAR (Photosynthetically Active Radiation, μmol photons $m^{-2}s^{-1}$). The ETR of pea plants decreased due to salt treatment (100mM and 200mM). The maximum decline in ETR was observed after 120 h of salt-treatment. The electron transport rates were 48.64% and 42.07% of control in 100mM

and 200mM salt-stressed plants after 72 h of stress. ETR further declined after 120 h of stress *i.e.*, it was 21.97% and 6.3% of control in 100mM and 200mM salt-stressed plants.

QUANTUM YIELD OF PHOTOSYSTEM II (φPSII)

The quantum yield of photosystem II at 72 h and 120 h of stress are shown in figure 4. The quantum yield of PSII decreased in response to PAR (μmol photons $m^{-2}s^{-1}$). As compared to respective controls the yield decreased in the salt treated plants with increase in salt concentration and PAR. The yields were 47.97% and 41.66% of control in 100mM and 200mM salt-stressed plants respectively after 72 h of stress. After 120 h of stress it further decreased to 22.22% and 6.48% of control in 100mM and 200mM salt-stressed plants respectively.

PHOTOCHEMICAL QUENCHING (qP)

The photochemical quenching (qP) at 72 h and 120 h of stress are shown in figure 5. The photochemical quenching decreased in response to increase in PAR (μmol photons $m^{-2}s^{-1}$). The photochemical quenching decreased in the salt-treated plants (100mM and 200mM). After 72 h of stress the qP values were 82.81% and 53.12% of control in 100mM and 200mM salt-stressed plants. These values further declined to 42.85% and 30.14% of control in 100mM and 200mM salt-stressed plants after 120 h of stress.

NON-PHOTOCHEMICAL QUENCHING (qN)

The non-photochemical quenching (qN) increased in response to PAR (μmol photons $m^{-2}s^{-1}$). The qN substantially increased in response to salt treatment after 120 h of stress. As compared to control, qN of plants at 200mM and 100mM salt stress were 146.98% and 118.86 (Fig 6).

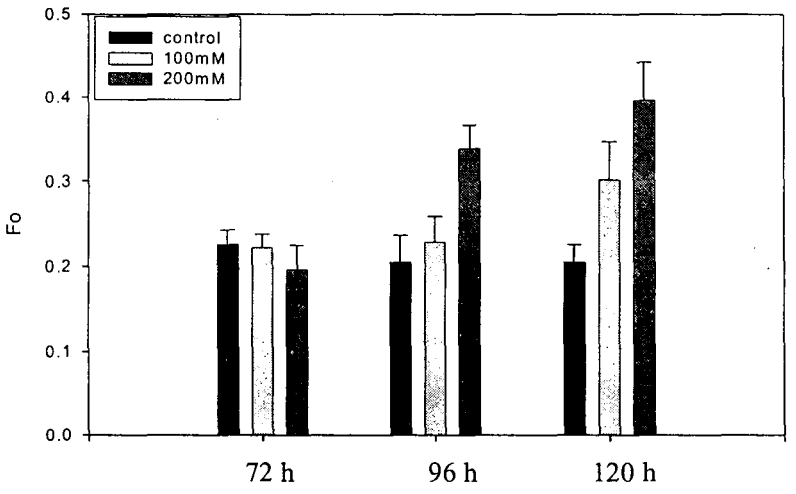


Figure 1. F_o values of control and salt stressed (100mM and 200mM) pea plants. The pea plants were grown for 8 days under cool white fluorescent light (70μmoles $m^{-2}s^{-1}$) at 25°C and then were treated with 100mM and 200mM NaCl for 72 h, 96 h and 120 h. F_o was measured at specific time points by PAM 2100 as described in materials and methods. Each data is average of three replicates. The error bar represents SD.

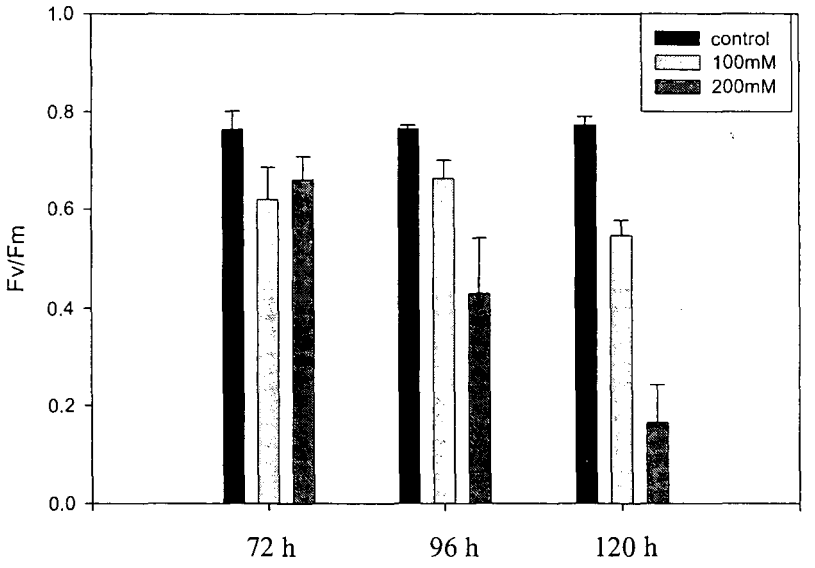


Figure 2. F_v/F_m values of control and salt stressed (100mM and 200mM) pea plants. The pea plants were grown for 8 days under cool white fluorescent light (70μmoles $m^{-2}s^{-1}$) at 25°C and then were treated with 100mM and 200mM NaCl for 72 h, 96 h and 120 h. Fv/Fm was measured at specific time points by PAM 2100 as described in materials and methods. Each data is average of three replicates. The error bar represents SD.

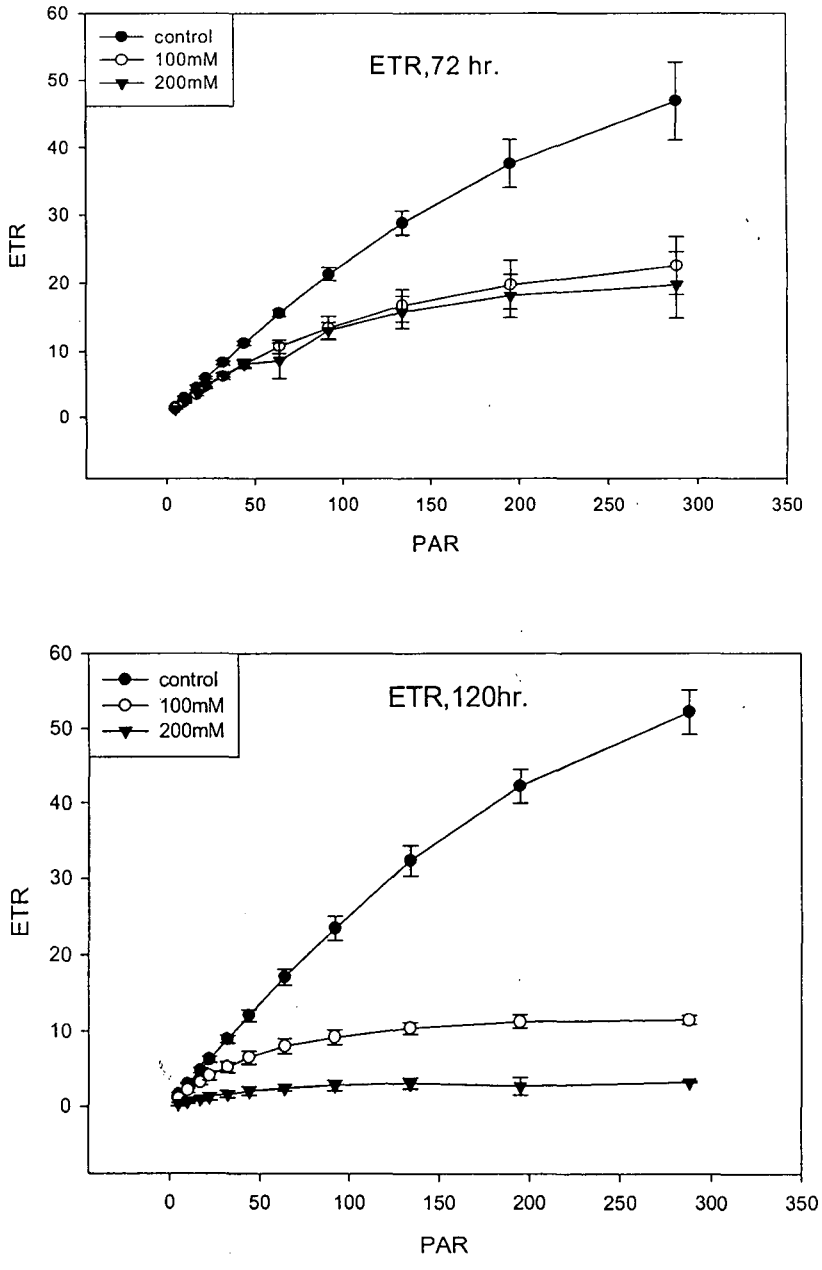


Figure 3. Electron Transport Rates (ETR) values of control and salt-stressed (100mM and 200mM) pea plants as measured by PAM 2100 at different time periods. Each data is average of three replicates. The error bar represents SD.

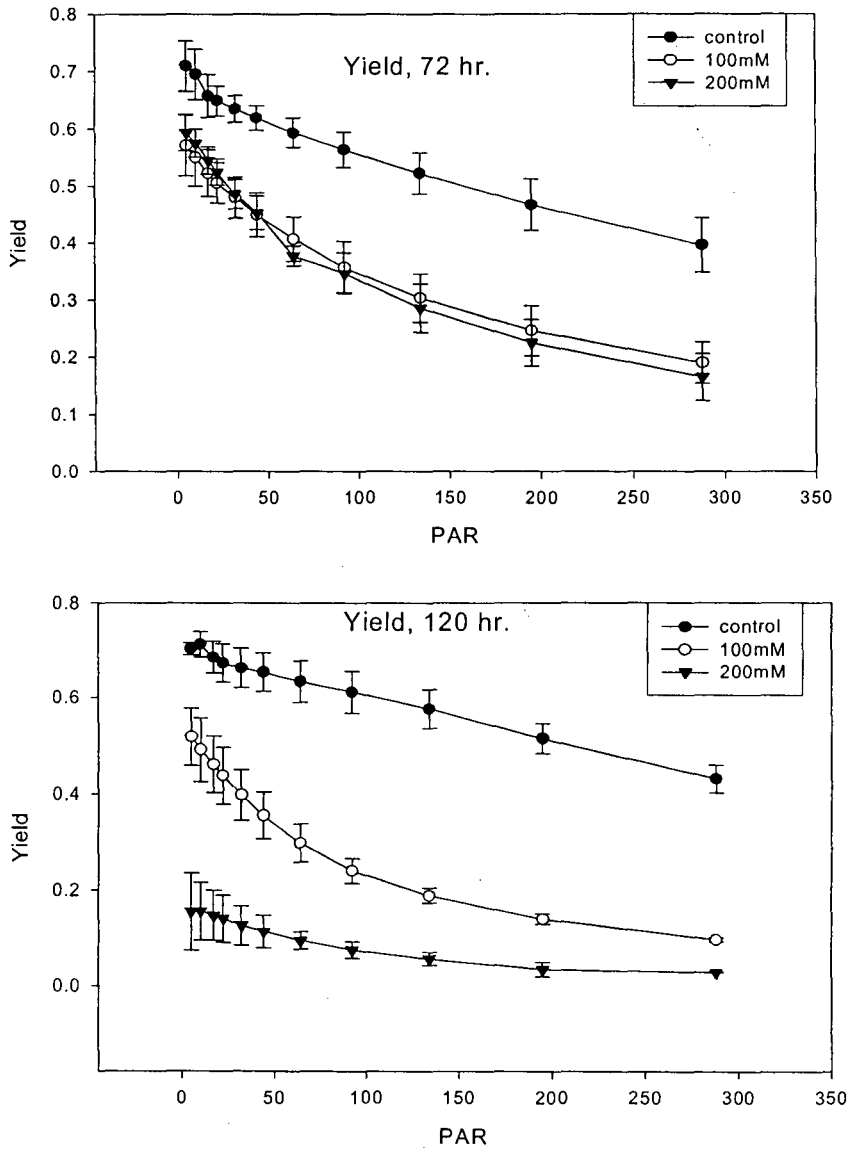


Figure 4. Quantum Yield of Photosystem II (φPSII) values of control and salt-stressed (100mM and 200mM) pea plants as measured by PAM 2100 at different time periods. Each data is average of three replicates. The error bar represents SD.

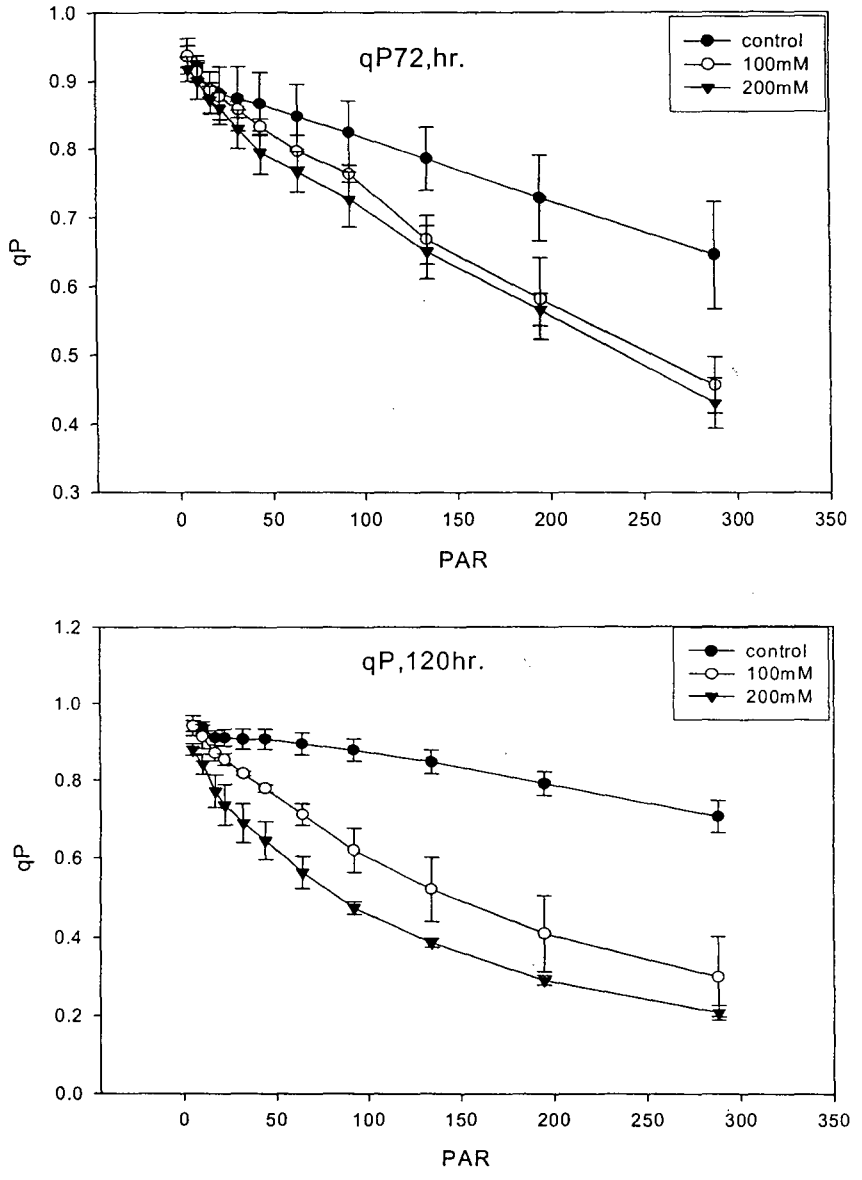


Figure 5. Photochemical Quenching (qP) values of control and salt-stressed (100mM and 200mM) pea plants as measured by PAM 2100 at different time periods. Each data is average of three replicates. The error bar represents SD.

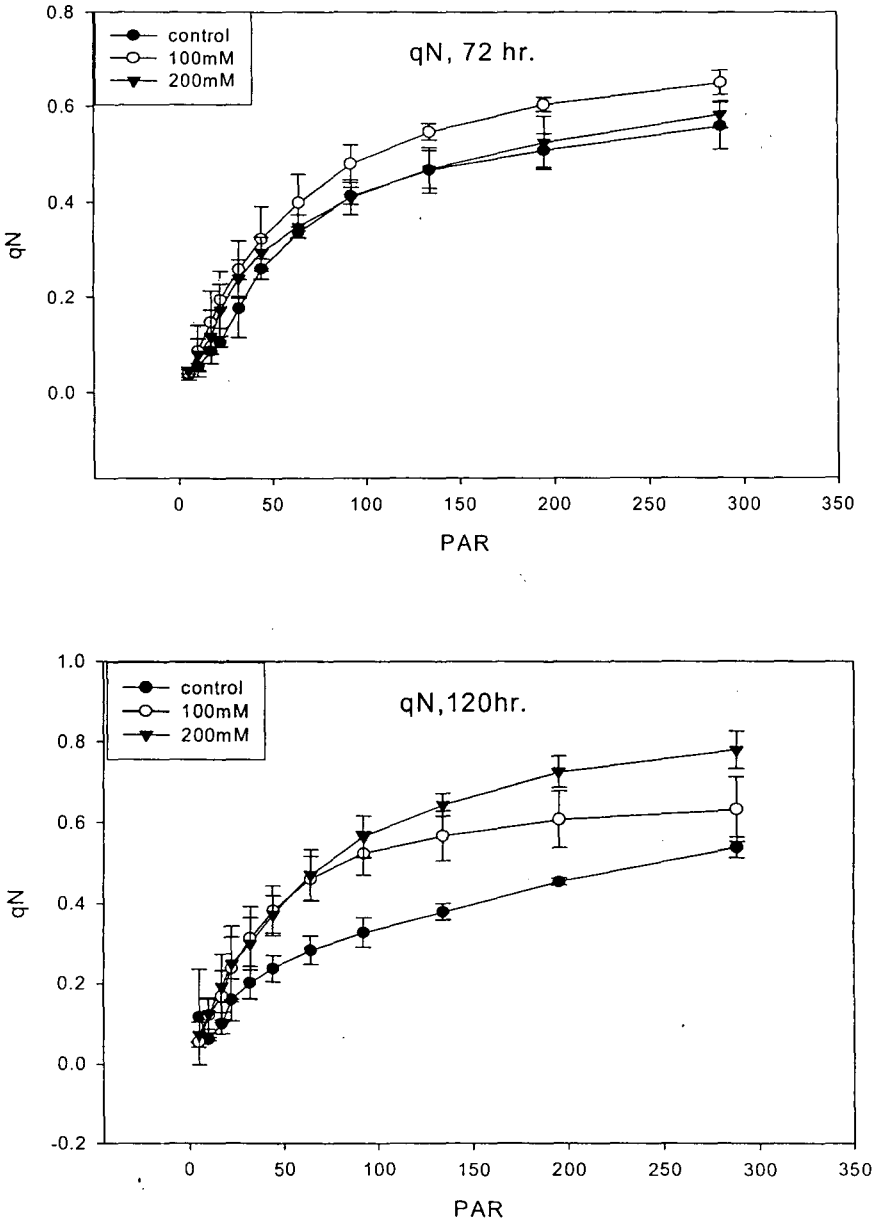


Figure 6. Non-Photochemical Quenching (qN) values of control and salt-stressed (100mM and 200mM) pea plants as measured by PAM 2100 at different time periods. Each data is average of three replicates. The error bar represents SD.

EFFECT OF SALT STRESS ON CHLOROPLAST PROTEIN IMPORT

The reduced efficiency of photosynthesis in salt stressed pea plants may be due to reduced photosynthetic gene expression, impaired protein synthesis or decline in import of chloroplastic protein synthesized in the cytoplasm. To elucidate the latter, protein import into chloroplast was studied *in vitro* in salt-stressed plants.

Figure 7(A) shows the autoradiogram demonstrating the *in vitro* translated pSSU protein precursor and its import into chloroplasts, isolated from pea plants treated with 0, 100mM and 200mM of NaCl for 72h. After the import assay the upper band at 20kDa refers to pSSU bound to the chloroplast envelope membrane and not imported into chloroplast. The lower band of 14kDa is the mature subunit (SSU) after imported into the stroma where the transit peptide is cleaved by the stromal processing peptidase. As the protease immediately degrades the transit peptide after it is cleaved by the processing enzyme, the 6kDa band is not seen in the autoradiogram. As compared to control the import efficiency of SSU (14kDa) was reduced by 30% and 65% in 100mM and 200mM NaCl-treated plants respectively (7B).

Figure 8(A) shows the import of pSSU into chloroplast isolated from pea plants treated with 0, 100mM and 200mM of NaCl for 120h. The import of pSSU into chloroplast was further reduced after 120 hours of stress. As compared to control the import efficiency of SSU (14kDa) was reduced by 60% and 80% in 100mM and 200mM NaCl-treated plants respectively (8B).

This inhibition of import into chloroplasts due to salt stress may be due to reduced efficiency of receptor pSSU interaction or due to damage to Toc and Tic complex of the protein import apparatus located in outer and inner envelope membrane respectively. The down regulation of stromal processing enzyme molecular chaperons that participate in protein import may contribute to reduced import in salt-stressed plants. To test if the down regulation of Toc-Tic complexes, stromal processing peptidase and molecular chaperone in salt-stressed plants is responsible for reduced import their gene expression was studied by RT-PCR.

RT-PCR results of Toc-Tic complex

The amplification of Toc159, Toc34, Toc64, Tic20, Tic22, Tic110, Tic55 and Tic62 was done by first isolating the total RNA from control and salt-stressed plants and then carrying out total cDNA synthesis after which the individual components

were amplified by using gene specific primers as described in materials and method. Actin was also amplified to serve as a loading control.

Toc Complex

Figure 9 shows the RT-PCR results of Toc complex from the plants treated with 0, 100mM and 200mM NaCl for 72 h and 120 h.

(i) Toc 159: After 72 h of stress the expression of Toc 159, the receptor protein on the outer envelope membrane, was partially reduced in both 100mM and 200mM salt-stressed plants. Its expression was further down regulated in both 100mM and 200mM plants after 120 h of stress.

(ii) Toc 34: After 72 h of stress the expression of Toc34 remained same as control in 100mM salt-stressed plants. However, its expression was reduced in 200mM plants. As compared to control, the expression of Toc34 reduced in both 100mM and 200mM plants after 120 h of stress.

(iii) Toc 64: The expression of Toc64 remained same in salt stressed plants as that of control after 72 h of stress. However, its expression was down regulated in both 100mM and 200mM plants after 120 h of stress.

Tic Complex

Figure 10 shows the RT-PCR results of Tic complex from the plants treated with 0, 100mM and 200mM NaCl for 72 h and 120 h.

(i) Tic 20: The Tic 20 expression in salt-stressed (100mM and 200mM) plants was only marginally reduced after 72 h and 120 h of treatment.

(ii) Tic 22: As compared to control, the expression of Tic22 increased in both 100mM and 200mM plants after 72 h of stress. Interestingly, its expression was severely reduced in both 100mM and 200mM plants after 120 h of stress.

(iii) Tic110: After 72 h of stress the expression of Tic110 remained same as control in 100mM salt-stressed plants. However, it was partially reduced in 200mM plants. Its expression was severely reduced in both 100mM and 200mM salt-treated plants after 120 h of stress.

(iv) Tic 55: The expression of Tic 55 is not affected very much in response to salt stress. The expression of Tic55 remained almost same in control and 100mM salt-stressed plants after 72h and 120 h of salt stress. However, its expression

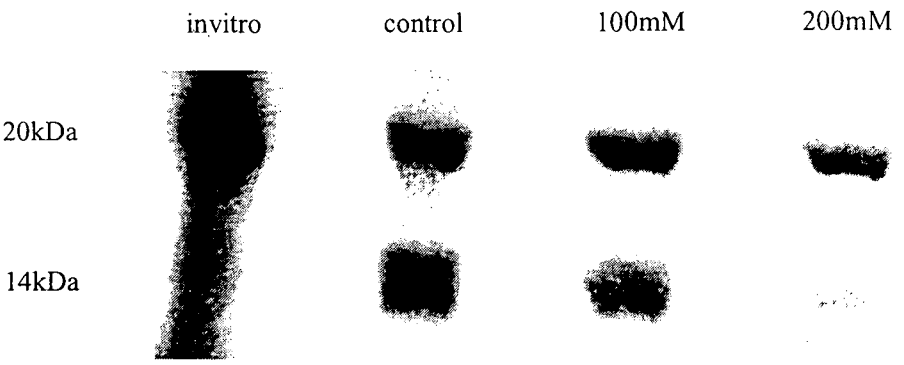
was partially reduced in 200mM plants after 72 h as well as 120 h of salt stress.

(v) Tic 62: The expression of Tic 62 remained almost same in control and 100mM plants but was reduced in 200mM plants after 72 h of salt stress. Its expression was further severely down regulated after 120 h of stress in both 100mM and 200mM plants.

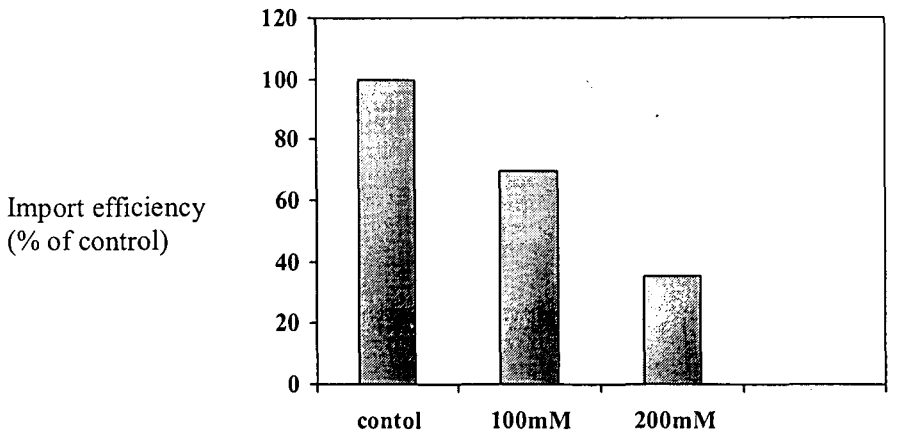
Expression of Chloroplast Processing Enzyme (CPE) and Hsp93

To test whether the expression of Chloroplast Processing Enzyme (CPE) was affected in salt-stressed conditions its expression level was studied. Figure 11 shows RT-PCR results of CPE from the plants treated with 0, 100mM and 200mM NaCl for 72 h and 120 h. After 72 hours of stress the expression of CPE remained unaffected in 100mM and was reduced in 200mM salt-stressed conditions. However, its expression was reduced in both 100mM and 200mM salt stressed plants after 120 h of stress.

The expression level of molecular chaperone Hsp93 was also studied. The expression was not affected in 100mM salt treated plants. In 200mM salt-treated plants its expression was partially reduced. However, its expression was down regulated in both the salt-stressed plants after 120 h of stress (Figure11).



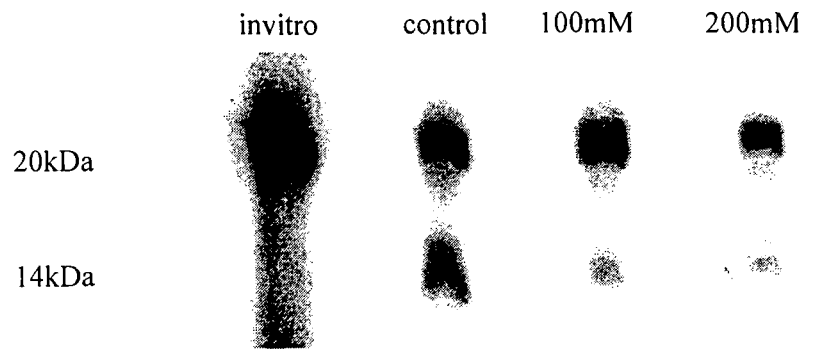
(A)



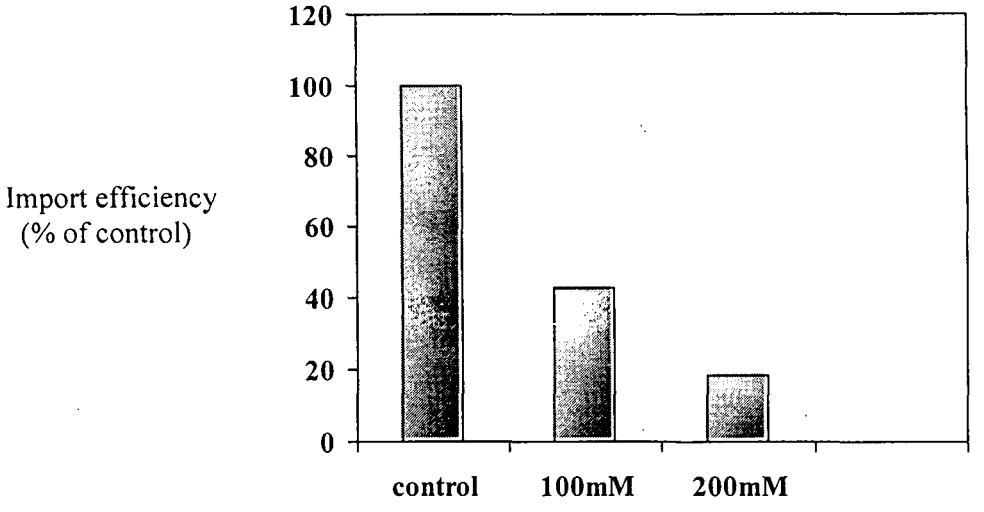
(B)

Figure 7. Import of pSSU into chloroplast isolated from control, salt-stressed pea plants. Plants were grown under cool-white fluorescent light (70μmoles $m^{-2}s^{-1}$) for 8 days and then were treated with 0, 100mM and 200mM NaCl for 72 hours. Intact chloroplast were isolated and import reactions were performed using invitro-translated radio labeled pSSU as described in materials and method.

(A)Autoradiogram showing the import of pSSU into chloroplasts isolated from control and salt stressed plants treated with 100mM and 200mm of NaCl. (B) Histogram showing the import efficiency as percent of control.



(A)



(B)

Figure 8. Import of pSSU into chloroplast isolated from control and salt stressed pea plants. Plants were grown under cool-white fluorescent light (70μmoles $m^{-2}s^{-1}$) for 8 days and then were treated with 0, 100mM and 200mM of NaCl for 120 hours. Intact chloroplast were isolated and import reactions were performed using invitro-translated radio labeled pSSU as described in materials and method.
(A)Autoradiogram showing the import of pSSU into chloroplasts isolated from control and salt stressed plants treated with 100mm and 200mM NaCl. (B) Histogram showing the import efficiency as percent of control

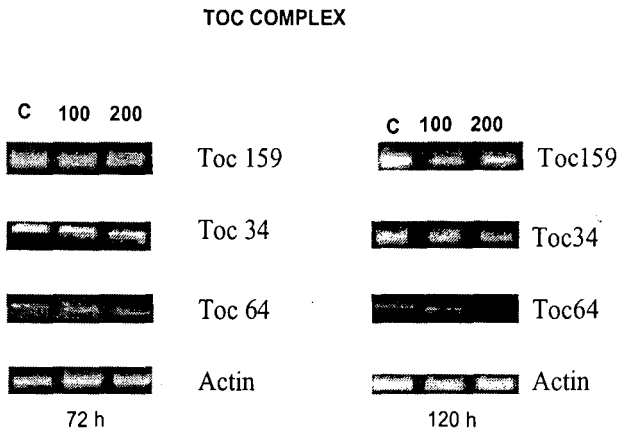


Figure 9. RT-PCR analysis of TOC components in control and salt stressed plants. Total RNA was isolated from control, 100mM and 200mM salt-stressed plants after 72 h and 120h of stress. First strand synthesis and PCR was done as described in materials and method.

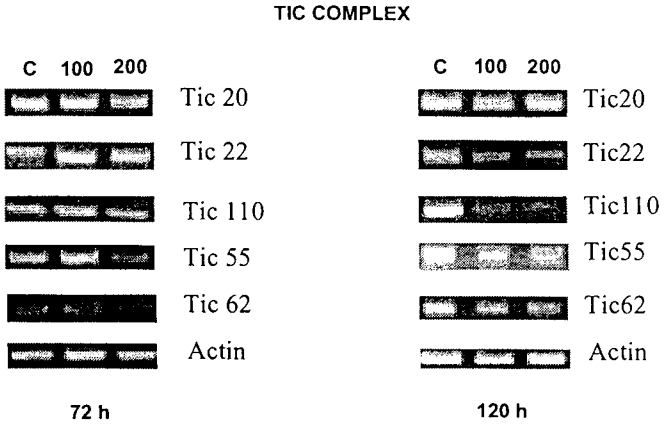


Figure 10. RT-PCR analysis of TIC components in control and salt stressed plants. Total RNA was isolated from control, 100mM and 200mM salt-stressed plants after 72 h and 120h of stress. First strand synthesis and PCR was done as described in materials and method.

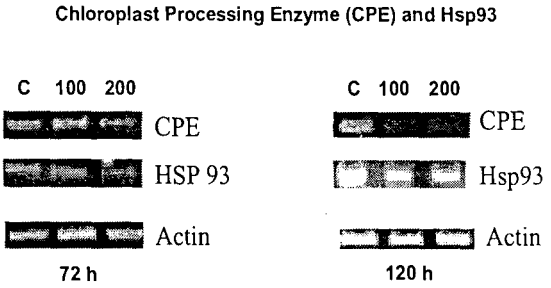


Figure 11. RT-PCR analysis of CPE and Hsp93 in control and salt stressed plants. Total RNA was isolated from control, 100mM and 200mM salt-stressed plants after 72 h and 120h of stress. First strand synthesis and PCR was done as described in materials and method.

DISCUSSION

Plants by their very nature, being embedded in the soil, are unable to escape exposure to the environmental extremes, and therefore must respond to survive. Microarray studies of gene expression following environmental stress suggest that while some common gene responses are observed, the majority of expression changes are specific to the stress applied (Seki et al., 2002). New proteins synthesized in response to these stresses may be targeted to various subcellular compartments in order to achieve appropriate response to stress.

Chlorophyll a fluorescence is a non-invasive and a good measure of the photosynthetic activities (Govindjee and Satoh, 1986; Krause and Weis, 1991; Govindjee, 1995). Fo is the fluorescence emitted by PSII when all the PS II complexes are open. There was no change in Fo values after 72 h of salt treatment. However, after 96 h and 120 h of salt treatment Fo values increased for both 100mM and 200mm NaCl. Rise in Fo was more in 200mm than 100mM of NaCl. This could be due to functional disconnection of LHCII from the PSII reaction centres, and also due to block of electron transport to Q_A (Schreiber and Armond 1978; Yamane et al 1995; Kraus and Weis 1991). Similar results pertaining to increase in Fo were reported in response to salt and high temperature (Psatenes and Horton 1999; Yamane et al 1997; Hong and Xu 1999; Misra et al 2001). The F_v/F_m, the maximum efficiency of PSII photochemistry, decreased in response to salt stress after 96 h and 120 h of stress. This clearly demonstrates the reduced quantum yield of photosynthesis.

The photosynthetic PSII electron transport rate (ETR) as measured by Chl a fluorescence quenching analysis, substantially declined after 72 h and 120 h of stress. The decline recorded at limiting light intensities (0-50 μmol photons $m^{-2}s^{-1}$) confirms the decline in F_v/F_m values. In the same vein the photosystem II yield (φPSII) and photochemical quenching (qP, a measure of the proportion of open PSII centres) decreased in response to increased photosynthetic active radiation (PAR) and salt stress. The decline in ETR, φPSII and qP was almost similar for 100mM and 200mM NaCl-treated plants after 72 h of stress. However, with increased duration of stress (120 h) a further decline in above parameters was observed, and this decline was more severe in 200mM plants as compared to 100mM plants. This indicates that pea plants can tolerate low (100mM) and high (200mM) salt stress to a similar extent for brief period only (*i.e.* 3 days only).

The non-photochemical quenching (qN) is a measure of the dissipation of excess energy that is not utilized in photochemical reaction as heat by zeaxanthine (Demming et al., 1996). In stress as photochemical reaction decreased more of absorbed energy is immediately dissipated as heat, through anthraxanthine-violaxanthine-zeaxanthine cycle (Niyogi et al., 1998) to prevent the conversion of unutilized light energy to active oxygen species by type I and type II photosensitization reaction. Exposure of seedlings to salt stress for 120 h increased the qN over that of control. This suggests that the adaptive mechanism of dissipation of unutilized light energy in salt stressed plants via zeaxanthine cycle remain intact even after long period of stress (120 h).

Autoradiogram results demonstrate that as salt concentration increases from 100mM to 200mM there is inhibition of protein import into chloroplast. The protein import was further reduced when the stress was prolonged from 72 h to 120 h. Therefore the reduction in photosynthetic efficiency as demonstrated by PAM may be due to reduced import of photosynthetic proteins into the chloroplast. The inhibition of protein import into chloroplasts isolated from salt-stressed plants may be due to impairment of binding of pSSU to the chloroplast envelope membrane i.e. reduced efficiency of receptor-pSSU interaction due to damage to Toc-Tic complex of the protein import apparatus located in outer and inner envelope membrane respectively. This could be due to down regulation in the activity of chloroplast processing enzyme (CPE) and stromal molecular chaperone (Hsp93).

Infact the results demonstrated that several Toc-Tic components are down regulated in response to salt stress. The expression of Toc and Tic complexes was constitutive (Toc64), up-regulated (Tic22) or down regulated (Toc 159, Toc34, Tic20, Tic55, Tic62, Tic110) after 72 h of stress. After 120 h of stress the expression of all the components is down regulated. There is reduction in the expression of Toc receptor protein Toc159 and 34. This could indicate the damage to Toc receptor complex and hence impairment in the binding of proteins to the membrane. Tic20 is proposed to function as a protein-conducting component of the inner envelope membrane. The up-regulated of Tic22 protein after 72 h of stress suggests that Tic22 may be one of the regulatory proteins of import machinery that could modulate itself in response to salt stress for efficient protein import. The *Tic22* gene may have salt stress responsive elements in its promoter.

The expression of Chloroplast Processing Enzyme (CPE) and stromal molecular chaperone Hsp93 was also reduced in response to stress showing that reduction in the activity of this enzyme is also responsible for the reduced amounts of mature protein in the organelle.

In conclusion, the reduced efficiency of photosynthesis may be due to reduction of protein import into chloroplast. The inhibition of import of precursor protein in isolated intact chloroplast could be due to damage to Toc-Tic complex and the reduced expression of CPE and Hsp93.

SUMMARY

Salt stress is the most typical abiotic stress affecting plants. Salt stress has great impact on chloroplast biogenesis and photosynthesis. It reduces photosynthesis in plants. Chloroplasts are organelles of endosymbiotic origin, and they transferred most of their genetic information to the host nucleus during this process. Therefore, the vast majority of chloroplast proteins are synthesized as precursor proteins (preproteins) in the cytosol and are imported post-translationally into the organelle. Hence, in the present study effect of salt stress was studied in chloroplast protein import.

In the present investigation pea plants were taken as a model. Pea plants were grown for 8 days and then were treated with 0, 100mM and 200mM NaCl for 72 and 120 h.

There was no significant effect of salt stress on F_o values after 72 h of stress. However, the F_o values increased when the stress was prolonged for 96 h and 120 h due to functional disconnection of LHCII from PSII and block in the electron transport to Q_A.

The F_v/F_m values decreased with the increase in salt concentration and duration of stress. The electron transport rate, quantum yield of PSII, photochemical quenching decreased with increase in salt concentration. The pea plants could tolerate the salt stress for brief period of time (3days) as the decline in the values for 100mM and200mM salt-treated plants for these parameters was almost same after 72 h of stress. Non-photochemical quenching (qN) increased with increase in salt concentration. This suggests that the adaptive mechanism of dissipation of unutilized light energy produced during salt stress via zeaxanthine cycle was intact even after long period of stress (120 h).

The import of *in vitro* translated protein into isolated intact chloroplast also decreased with increase in salt concentration and duration of stress. The expression of Toc-Tic components as studied by RT-PCR could be constitutive (Toc64), up-regulated (Tic 22), and down regulated (Toc 159, Toc34, Tic 20, Tic110, Tic55, Tic62) after 72 h of stress. However after 120 h of stress the expression of all the components was severely down regulated. The down regulation of receptor proteins Toc159 and Toc34 indicate to damage to Toc receptor complex and hence impairment in the binding of proteins to the membrane. The up-regulation of Tic 22 protein after 72 h of stress suggests regulatory role of the protein.

The expression of Chloroplast Processing Enzyme (CPE) and molecular chaperone was also affected in response to salt stress. The expression of CPE was only partially reduced after 72 h of stress but after 120 h of stress it was severely reduced. Similarly, the expression of Hsp93 also down regulated with response to salt stress.

In conclusion, the reduced efficiency of photosynthesis may be due to reduction of protein import into chloroplast. The inhibition of import of precursor protein in isolated intact chloroplast could be due to damage to Toc-Tic complex and the reduced expression of CPE and Hsp93.

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