Literature Review
Salt Stress

Plants are subjected to various abiotic stresses such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity during their life cycle among those, salinity stress is the most typical abiotic stress (Mahajan et al, 2005). It is estimated that 6% of the world’s total land and 30% of the world’s irrigated areas already suffer from salinity problems (Unesco Water Portal, 2007). Salinity is a soil condition characterized by a high concentration of soluble salts. Soils are classified as saline when the ECe is 4 dS/m or more (USDA-ARS.2008), which is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPa. This definition of salinity derives from the ECe that significantly reduces the yield of most crops (Munns and Tester, 2008). Because NaCl is the most soluble and widespread salt, it is not surprising that all plants have evolved mechanisms to regulate its accumulation and to select against it in favor of other nutrients commonly present in low concentrations, such as K+ and NO3 -. In most plants, Na+ and Cl- are effectively excluded by roots while water is taken up from the soil (Munns R, 2005). 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 hyper osmolarity and ion disequilibrium. High Salinity causes both hyperionic and hyper osmotic stress effects, and the consequence of these can be plant demise (Glenn, et al., 1999, Niu et al., 1995, Yeo; 1998). Most commonly, the stress is caused by high Na+ and Cl- concentrations in the soil solution. Altered water status most likely brings about initial growth reduction; however the precise contribution of subsequent process to inhibition of cell division and expansion and acceleration of cell death has not been well elucidated (Munns; 1993, Yeo, 1998). Membrane disorganization, reactive oxygen species, metabolic toxicity, inhibition of photosynthesis, and attenuated nutrient acquisition are factors that initiate more catastrophic events (Flowers et al., 1977, Greenway and Munns; 1980).

Salt Movement through Plants

Movement of salt into roots and to shoots is a product of the transpiration flux required to maintain the water status of the plant (Flowers and Yeo; 1992, Yeo; 1998). Unregulated transpiration can result in toxic levels of ion accumulation in the aerial parts of the plant. An

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immediate response to salinity, which mitigates ion flux to the shoot, is stomatal closure. However, because of the water potential difference between the atmosphere and leaf cells, and the need for carbon fixation, this is an untenable long-term strategy of tolerance (Munns and Termaat; 1986, Yeo; 1998).

To protect actively growing and metabolizing cells, plants regulate ion movement into tissues (Flowers and Yeo; 1992, Munns; 1993). One mode by which plants control salt flux to the shoot is the entry of ions into the xylem stream. Still debated is the extent to which symplastic ion transport through the epidermis and cortical cells contributes to a reduction in Na\(^+\) that is delivered to the xylem (Clarkson, 1991, Flowers and Yeo; 1992). The accumulation of large quantities of ions in mature and old leaves, which then dehisce, has often been observed under salt stress (Flowers and Yeo; 1992, Munns, 1993). The solutes content of tissues containing cells with little vacuolation (e.g. meristematic regions) predominated by organic osmolytes and in tissues with highly vacuolated cells by ions (Bizel et al. 1988; Wyn, 1981).

**Halophyte and Glycophyte Comparisons**

Halophyte for optimal growth require electrolyte (typically Na\(^+\) and Cl\(^-\)) in concentrations higher or much higher than those found in nonsaline soils. How and within which range of NaCl (roughly defined from 20 to 500 mM NaCl) these plants respond best is complex, and has led to a number of classification attempts (Flower et al., 1977, Glenn et al.; 1999, Greenway and Munns; 1980). Halophytes seem to lack unique metabolic machinery that is insensitive to or achieved by high Na\(^+\) and Cl\(^-\) (Flowers et al.; 1977, Nelson et. al; 1998, Niu et al.; 1995, Rhodes; 1987). Instead, plants ultimately survive and grow in saline environments because of osmotic adjustment through intracellular compartmentation that partitions toxicons away from the cytoplasm through the energy-dependent transport into the vacuole (Apse et al., 1999, Binzel et al., 1988). Some halophytes exclude Na\(^+\) and Cl\(^-\) through glands and bladders, which are specialized structure that seems to be evolutionarily late inventions by which halophytes gain an edge over glycophytes. Osmotic adjustment of both halophytes and glycophytes is also achieved through the accumulation of organic solutes in the cytosol, and the lumen, matrix, or stoma of organelles (Niu et al.; 1995, Rhodes and Samaras; 1994, Yeo; 1998).

A principal difference between halophytes and glycophytes is the capacity of the former to survive salt shock. This capacity allows halophytes to more readily establish metabolic steady
state for growth in saline environment (Braun et al.; 1986, Cacas et al.; 1991, Cushman et al.;
1990). Responsiveness to salinity and at least some ability to establish an adapted new steady
state is not unique, however, to halophytes in as much as both glycophyte cells and plants exhibit
substantial capacity for salt tolerance provided that stress imposition is gradual. (Amzallag et al.;
1990, Bressan et al., 1990) whereas glycophytes restrict ion movement to the shoot by
attempting control of ion influx into root xylem, halophytes tend more readily to take up Na+
such that roots typically much lower NaCl concentrations than the rest of plant. It seems that a
major advantage that halophytes have over glycophytes is not only more responsive Na+
partitioning but more effective capacity to co-ordinate this partitioning with processes
controlling growth, and ion flux across the plasma-membrane, in both cellular and organismal

Genetic Diversity for Salt Tolerance in Plants

Plants differ greatly in their tolerance of salinity, as reflected in their different growth responses.
Of the cereals, rice (Oryza sativa) is the most sensitive and barley (Hordeum vulgare) is the most

![Graph showing salinity tolerance of various plant species.](image)

**Fig.1**

tolerant (Figure 1). tolerant and durum wheat (Triticum turgidum ssp. durum) is less so. Tall
wheat grass (Thinopyrum ponticum, syn. Agropyron elongatum) is a halophytic relative of wheat

and is one of the most tolerant of the monocotyledonous species (Figure 1); its growth proceeds at concentrations of salt as high as in seawater. The variation in salinity tolerance in dicotyledonous species is even greater than in monocotyledonous species. Some legumes are very sensitive, even more sensitive than rice (L'au.chli A, 1984); alfalfa or lucerne (Medicago sativa) is very tolerant, and halophytes such as saltbush (Atriplex spp.) continue to grow well at salinities greater than that of seawater (Figure 1). Many dicotyledonous halophytes require a quite high concentration of NaCl (100–200 mM) for optimum growth (Flowers et al, 1977). Arabidopsis, when compared with other species under similar conditions of light and humidity (that is, at high transpiration rates), is a salt-sensitive species (Figure 1). This sensitive plant may provide limited insights into mechanisms of salinity tolerance unless it is compared with a tolerant relative such as Thellungiella halophila. The differences between these two species are highlighted by their responses to 100 mM NaCl under conditions of high transpiration. Continued exposure to 100 mM does not allow Arabidopsis to complete its life cycle (Sickler CM et al, 2007), but has little effect on the growth rate of Thellungiella (James RA et al, 2006). Plant Bread wheat (Triticum aestivum) is moderately tolerant of salt because it exists in plant taxa is distributed over numerous genera (Flowers et al.; 1986; Greenway and Munns, 1980). Most crops are salt sensitive or hypersensitive plants (glycophytes) in contrast to halophytes, which are native flora of saline environments. Some halophytes have the capacity to accommodate extreme salinity because of very special anatomical and morphological adaptations or avoidance mechanisms (Flowers et al.; 1986). However these are rather unique characteristics for which genes are not likely to be easily introgressed into plants. Research of recent decades has established that most halophytes and glycophytes tolerate salinity by rather similar strategies often using analogous tactical process (Hasegawa et al.; 2000b). The cytotoxic ions in saline environments, typically Na⁺ and Cl⁻, are compartmentalized into vacuoles and used as solutes (Blumwald et al.; 2000; Niu et al.; 1995). It follows then that many of rare molecular entities that mediate ion homeostasis and salt stress signaling are similar in all plants (Hasegawa et al.; 2000b).
Mechanism of Salt Stress Tolerance in Plants

The mechanisms of salinity tolerance fall into three categories

Tolerance to osmotic stress: The osmotic stress immediately reduces cell expansion in root tips and young leaves, and causes stomatal closure. A reduced response to the osmotic stress would result in greater leaf growth and stomatal conductance, but the resulting increased leaf area would benefit only plants that have sufficient soil water. Greater leaf area expansion would be productive when a supply of water is ensured such as in irrigated food production systems, but could be undesirable in water-limited systems, and cause the High salinity causes hyper osmotic stress and ion disequilibrium that produce secondary effects or pathologies (Hasegawa et al., 2000; Zhu, 2001). Fundamentally, plants cope by either avoiding or tolerating salt stress. That is plants are either dormant during the salt episode or there must be cellular adjustment to tolerate the saline environment. Tolerance mechanism can be categorized as those that function to minimize osmotic stress or ion disequilibrium or alleviate the consequent secondary effects caused by these stresses. The chemical potential of the Saline soil initially establishes a water potential imbalance between the apoplast and symplast that leads to turgor decrease, which if severe enough can cause growth reduction (Bohnert et al, 1995). Soil water to be used up before the grain is fully matured (Abebe T, 2003).

Na\(^+\) exclusion from leaf blades: Na\(^+\) exclusion by roots ensures that Na does not accumulate to toxic concentrations within leaves. A failure in Na\(^+\) exclusion manifests its toxic effect after days or weeks, depending on the species, and causes premature death of older leaves (Munns R, 2008).

Tissue tolerance, i.e., tolerance of tissue to accumulated Na\(^+\), or in some species, to Cl\(^-\): Tolerance requires compartmentalization of Na\(^+\) and Cl\(^-\) at the cellular and intracellular level to avoid toxic concentrations within the cytoplasm, especially in mesophyll cells in the leaf. Toxicity occurs with time, after leaf Na\(^+\) increases to high concentrations in the older leaves (Munns R, 2008).

Growth cessation occurs when turgor is reduced below the yield threshold of the cell wall. Cellular dehydration begins when the potential difference is greater than can be compensated for by turgor loss. (Taiz and Zeiger, 1998).
The cellular response to turgor reduction is osmotic adjustment. The cytosolic and organellar machinery of glycophyte and halophyte is equivalently Na⁺ and Cl⁻ sensitive; so osmotic adjustment is achieved in these compartments by accumulation of compatible osmolytes and osmoprotectants (Bohnert et al., 1995, Bohnert and Jenson, 1996). However, Na⁺ and Cl⁻ are energetically efficient osmolytes for osmotic adjustment and are compartmentalized into the vacuole to minimize cytotoxicity (Blumwald et al., 2000; Niu et al., 1995). Movement of ions into the vacuole might occur directly from the apoplast into the vacuole through membrane vesiculization or a cytological process that juxtaposes the plasma membrane to the tonoplast (Hasegawa et al., 2000). Then compartmentalization would be achieved with minimal or no exposure of the cytosol or toxic ions. The bulk of Na⁺ and Cl⁻ movement from the apoplast to the vacuole likely is mediated through ion transport systems located in the plasma membrane and tonoplast. Presumably, tight coordinate regulation of these ion transport system is required in order to control net influx across the plasma membrane and vacuolar compartmentalization. The SOS signal pathway is a pivotal regulator of; at least some, key transport systems required for ion homeostasis (Hasegawa et al., 2000; Sanders, 2000; Zhu 2000). CaMV 35S promoter driven overexpression of the Arabidopsis thaliana SOS1 gene, which encodes a plasma membrane Na⁺/H⁺ antiporter, improves plant salt tolerance in A. thaliana (Shi et al., 2003).

**Osmolytes and Osmoprotectants**

Salt tolerance requires that compatible solutes accumulate in the cytosol and organelles where these function in osmotic adjustment and osmoprotection (Rhoder and Hanson, 1993). Some compatible osmolytes are essential elemental ions such as K⁺, but the majorities are organic solutes. A major category of organic osmotic solutes consists of simple sugars (mainly fructose and glucose), sugar alcohols (glycerol and methylated inositols) and complex sugars (trehalose, raffinose and fructans) (Bohnert and Jensen, 1996). The addition of glucosylglycerol (GG) to the culture medium protected cyanobacterium *Synechocystis* sp. PCC 6803 cells against salt stress and reversed the adverse effects of NaCl on cell division and cell size (Ferjanl et al., 2003). Trehalose-producing, transgenic rice (*Oryza sativa*) plants showed increased tolerance to drought, salt, and cold, as shown by chlorophyll fluorescence and growth inhibition analyses (Jang et al., 2003). Others osmolytes include quaternary amino acid derivatives (proline, glycine betaine, β-alanine betaine, proline betaine), tertiary amines (1, 4, 5, 6- tetrahydro-2-methyl-4-
corboxyl pyrimidine), and sulfonium compounds (choline o-sulfate, dimethyl sulfonium propionate) (Nuccio et al.; 1999). NaCl alleviated the inhibitory effect of UV-B on PSII activity. Proline accumulated under salt stress conditions might be one of the reasons for the observed tolerance of barley seedlings to UV-B radiation (Fedina et al., 2003). Many osmolytes are presumed to be osmoprotectants, as their levels of accumulation are insufficient to facilitate osmotic adjustment. Glycine betaine preserves thylakoid and plasma membrane integrity after exposure to saline solutions or to freezing or high temperatures (Rhodes and Hanson, 1993). A common feature of compatible solutes is that these compounds can accumulate to high levels without disturbing intracellular biochemistry (Bohnert and Jensen, 1996).

**Ion Homeostasis**

Since NaCl is the principal soil salinity stress, a research focus has been the transport system that is involved in utilization of Na\(^+\) as an osmotic solute (Blumwald et al., 2000; Hasegawa et al., 2000; Niu et al., 1995). Research of more than 30 years previously, established that Ca\(^{2+}\) modulates intracellular Na\(^+\) homeostasis and salt tolerance. High [Na\(^+\)]\(_{ext}\) negatively affects K\(^+\) acquisition (Rains and Epstein, 1967). Na\(^+\) competes with K\(^+\) for uptake through common transport system and does this effectively since the [Na\(^+\)]\(_{ext}\) in saline environments is usually considerably greater than [K\(^+\)]\(_{ext}\). Ca\(^{2+}\) enhances K\(^+\)/Na\(^+\) selective intracellular accumulation. (Maathuis et al, 1996; Rains and Epstein, 1967). Recently, the SOS stress signaling pathway was identified to be pivotal regulator of plant ion homeostasis and salt tolerance (Hagegawa et al.; 2000; Sanders, 2000). This signaling pathway functionally resembles that yeast calcineurin cascade that controls Na\(^+\) influx and efflux across the plasma membrane (Bressan et al.; 1998).

**Ion transport Systems that mediate Na\(^+\) homeostasis**

**H\(^+\)-pumps.** H\(^+\)-Pumps in the plasma membrane and tonoplast energize solute transport necessary to compartmentalize cytotoxic ions away from the cytoplasm and to facilitate the functions of ions as signal determinants (Maeshima, 2000; Maeshima, 2001; Morsomme and Boutry, 2000; Ratajczak, 2000). These pumps provide the driving force (H\(^+\) electrochemical potential) for secondary active transport and function to establish membrane potential gradients that facilitate electrophoretic ion flux. The plasma membrane localized H\(^+\) pump is a P-type ATPase and is primarily responsible for the large (pH and membrane potential gradient across this membrane (Morsomme and Boutry, 2000). A vacuolar H\(^+\)-ATPase and vacuolar
pyrophosphates generate the ΔpH and membrane potential across the tonoplast (Drozdowicz and Rea, 2001; Maeshima 2001). The activity of these H⁺ pumps is increased by salt treatment and induced gene expression may account for some of the up regulation (Hasegawa et al., 2000b; Maeshima, 2001).

**Na⁺ influx and Efflux across the Plasma Membrane**

Recently, much insight has been gained about Na⁺ transport system that is involved in the net flux of the cation across the plasma membrane (Amtman and Sanders; 1999; Blumwald et al.; 2000; Hasegawa et al., 2000). Transport systems with greater selectivity for K⁺ are presumed to facilitate Na⁺ “Leakage” into cells. Specifically, Na⁺ is a competitor for uptake through plasma membrane K⁺ inward rectifying channels, such as those that are in Shaker type family, e.g. AKT1 (Schachtman, 2000). K⁺ outward rectifying channels also may facilitate Na⁺ influx (Schachtman, 2000). Recently the properties of HKT proteins from Arabidopsis (Kato et al., 2001; Uozumi et al., 2000), rice (Horie et al.; 2001) and eukalyptus (Fairbairn et al., 2000) have been characterized. At HKT1 is the only member of the Arabidopsis gene family while both rice and eukalyptus have at least two genes. (Uozumi et al.. 2000). Rice (Oryza sativa L. India) OsHKT1 and OsHKT2 were identified based on sequence similarity with wheat HKT (Horie et al. 2001). OsHKT1 and 2 transcripts accumulate in response to low K⁺ but their steady state abundance is reduced by treatment with 30mm NaCl. Identification of sos3-1 hkt1 double mutation in Arabidopsis has confirmed the existence of a Na⁺ entry system(s) different than HKT1 that functions in Planta. (Amtmann and Sanders, 1999). Energy-dependent Na⁺ transport across the plasma membrane of plant cells is mediated by the secondary active Na⁺ / H⁺ antiporter SOS1. Phylogenetically, SOS1 is similar to SOD2 of Saccharomyces pombe, NHA1 of S. cerevisiae and Nha A and Nha P of Pseudomonas aeruginosa (Shi et al.; 2000).

**Na⁺ Vacuolar Compartimentalization**

A Na⁺/H⁺ antiporter that is energized by the DPH across the tonoplast facilities vacuolar compartmentalization of the cation. The Arabidopsis AtNHX1 was isolated by functional genetic complementation of yeast mutant detected for the endosomal Na⁺/H⁺ antiporter yeast (ScNHX1) and has sequence similarity to mammalian NHE transporters (Apse et al., 1999; Gaxiola et al., 1999, Quintero et al., 2000). Transgenic Arabidopsis and tomato plants that overexpress AtNHX1 accumulate abundant quantities of the transporter in the tonoplast and exhibit
substantially enhanced salt tolerance (Apse et al.; 1999; Quintero et al., 2000; Zhang and Blum Wald, 2001).

**Ca$^{2+}$ Signaling and SOS signal Transduction Pathway**

Jian-Kang Zhu and Currosbess identified three genetically linked *Arabidopsis* loci (SOS1, SOS2 and SOS3), which are components of a stress signaling pathway that controls ion homeostasis and salt tolerance (Hasegawa et al. 2000; Sanders, 2000; Zhu, 2000; 2001). Genetic analysis of Na$^+$/Li$^+$ sensitivity established that sos1 is epistatic to sos2 and sos3 (Zhu, 2000). SOS3 encodes a Ca$^{2+}$ binding protein with sequence similarity to the regulatory B subunit of calcineurin and neuronal Ca$^{2+}$ sensors (Ishitani et al., 2000, Liu and Zhu, 1998). SOS codes for a serine / threonine kinase (446 amino acids) which has a 267 amino acid N-terminal catalytic domain that is similar in sequence to yeast SNF1 (Sucrose non-fermenting) kinase and the mammalian AMPK (AMP-activated protein kinase (Liu et al.; 2000; Zhu, 2000). Kinase activity of SOS2 is essential for its salt tolerance determinant function (Zhu, 2000) Ca$^{2+}$ binds to SOS3, which leads to interaction with SOS2 and activation of kinase. The plasma membrane sited Na$^+$/H$^+$ antiportor SOS1 is controlled by the SOS pathway at the transcriptional and post transcriptional level (Guo et al., 2001; Zhu, 2001).

**Cell Signaling during Salt Stress**

High salinity, low temperature and drought are common stress conditions that adversely affect plant growth and crop production. The cellular and molecular responses of plants to environmental stress have been studied intensively (Thomashow, 1999; Hasegawa et al.; 2000). Signal transduction during salt stress is not completely understood. A generic signal transduction pathway starts with signal perception, followed by the generation of second messengers (e.g., inositol phosphates and reactive oxygen species [ROS]. Second messengers can modulate intracellular Ca$^{2+}$ levels, often initiating a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transduction factors controlling specific set of stress related genes. Products of these genes may participate in the generation of regulatory molecules like plant hormones abscisic acid (ABA), ethylene, and salicylic acid (SA). These regulatory molecules can, in turn initiate a second round of signaling that may follow the same pathway, although different components are often involved (Xiong and Zhu, 2002). Signal transduction requires the proper spatial and temporal co-ordination of all signaling molecules.
Thus there are certain molecules that participate in the modification, delivery, or assembly of signaling components, but do not directly relay the signal. These are protein modifiers (e.g. enzymes for protein lipidation, methylation, glycosylation and ubiquitation), scaffolds and adapters (Xiong and Zhu, 2001).

Chloroplast biogenesis: A brief overview

Chloroplast is the hallmark organelle of plant. It performs photosynthesis and is therefore required for photoautotrophic plant growth. Chloroplasts are double membrane organelles which originated when a photosynthetic prokaryote was engulfed by the non photosynthetic eukaryotic ancestor of green and red algae and land plants. (Dyall et al, 2004; Martin et al, 2002). Chloroplast not only the site of photosynthesis but also carry out other non photosynthetic functions no less important than photosynthesis. They manufacture fatty acids, aromatic and nonaromatic amino acids (essential for protein synthesis, but also for a vast array of plant secondary metabolites), purine and pyrimidine bases, isoprenoids (like carotenoids and sterols) and tetrapyrroles (like haem and chlorophyll). Most of these functions are essential for every cell type, and chloroplasts have integrated into cellular development pathways by differentiating into a variety of other, interconvertible, non-photosynthetic plastid types (Waters et al, 2004).

Thylakoids are dominating structure inside fully mature chloroplasts. In dark grown plants in the absence of light proplastids turn into etioplasts which contain very few internal membranes but a characteristic prolemellar body. Prolemellar body is paracrystalline structure consisting of lipids and essentially a single protein, the NADPH-dependent protochlorophyllide oxidoreductase. Shortly after the onset of illumination the prolemellar body is dispersed and thylakoids begin to form (Vothknecht and Westhoff, 2001). Chloroplast biogenesis is a complex process and is co-ordinated by both nuclear and plastid genome. Only 5-10 % proteins of chloroplast are encoded by its own genome remaining proteins are nuclear encoded so translated into cytoplasm and then imported into chloroplast. (Jarvis and Soll, 2002) So the chloroplast biogenesis involves the biosynthesis of many lipids, proteins and pigments including chlorophyll. Pigments and proteins unite to form dominating complexes of thylakoids, Photosystem I and Photosystem II and their associated light harvesting antenna, the cytochrome b6f complex and the proton translocating ATP synthase (Vothknecht and Westhoff, 2001).
Chlorophyll biosynthesis in higher plants: A brief overview

The Chl biosynthesis pathway in higher plants is much more complex and the metabolic flux is regulated by 17 enzymes (Fig. 2). However the formation of Chlorophyll can be subdivided into three parts, (i) formation of 5-aminolevulinic acid (ALA), the committed step for all tetrapyrroles, (ii) formation of protoporphyrin IX (Proto) from eight molecules of ALA, and (iii) formation of Chl in the magnesium branch. Enzymes catalyzing early steps in the synthesis are highly soluble and located mostly in the chloroplast stroma, whereas enzymes of the late steps are associated with thylakoid or inner envelope membranes. All of the enzymes of the pathway are encoded by nuclear genes and are synthesized in the cytoplasm as precursor polypeptides with amino-terminal extensions (transit peptides) that enable them to pass through the double membrane of the chloroplast envelope and to their site of function within the organelle. The only molecule required for Chl biosynthesis that is synthesized within the organelle is tRNA^{Glu}, which is encoded on the chloroplast genome (Eckhardt et al, 2004; Tanaka and Tanaka, 2007).

ALA formation from glutamate:
δ-Aminolevulinic acid (ALA), the first universal tetrapyrrole precursor, can be formed by two different pathways. Members of the α proteobacterial group (which includes photosynthetic bacteria of the Rhodobacter and Rhodopseudomonas, and Rhodospirillum genera as well as the nonphotosynthetic genera Agrobacterium, Rhizobium and Bradyrhizobium), and all eukaryotic organisms that do not contain chloroplasts (animals, yeasts, fungi), form ALA by condensation of succinyl-coenzyme A with glycine in a reaction catalyzed by the pyridoxal-P-containing enzyme ALA synthase (EC 2.3.1.37) (Gibson et al. 1958; Kikuchi et al. 1958). In contrast, all plants and algae, and all bacteria that are not in the α proteobacterial group, including cyanobacteria, many photosynthetic bacteria, and archaea, form ALA by a different route that begins with the five-carbon precursor, glutamate (Beale and Castelfranco 1974; Beale et al. 1975; Meller et al. 1975), which is activated ligation to tRNAGlu (Kannangara et al. 1984), followed by reduction of the α carboxyl group of the activated glutamate to form glutamate 1-semialdehyde (GSA) (Pontoppidan and Kannangara 1994), and transamination of GSA to form ALA (Kannangara and Gough 1978). The fact that ALA biosynthesis in all plants and most bacteria differs from that in animals suggests that it may be possible to develop herbicides and
Figure 2 A scheme of tetrapyrrole biosynthetic pathway (Smith and Griffiths, 1993, modified)
Table A. List of enzymes involved in tetrapyrrole biosynthesis and their respective gene names and localization in the cell. M: Mitochondria, P: Plastid

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme name</th>
<th>Localization</th>
<th>Gene name(s)</th>
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<tbody>
<tr>
<td>1</td>
<td>Glutamyl-tRNA synthase</td>
<td>P</td>
<td>GluRS</td>
</tr>
<tr>
<td>2</td>
<td>Glutamyl-tRNA reductase</td>
<td>P</td>
<td>HEMA1, HEMA2, HEMA3</td>
</tr>
<tr>
<td>3</td>
<td>Glutamate-1-semialdehyde aminotransferase</td>
<td>P</td>
<td>GSAT1, GSAT2</td>
</tr>
<tr>
<td>4</td>
<td>Porphobilinogen synthase</td>
<td></td>
<td>AlaD/HEMB, HEMB1, HEMB2</td>
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<tr>
<td>5</td>
<td>Porphobilinogen deaminase</td>
<td>P</td>
<td>PbgD/HEMC</td>
</tr>
<tr>
<td>6</td>
<td>Uroporphyrinogen III synthase</td>
<td>P</td>
<td>UroS/HEMD</td>
</tr>
<tr>
<td>7</td>
<td>Uroporphyrinogen III decarboxylase</td>
<td>P</td>
<td>UroD/HEME, HEME1, HEME2</td>
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<td>Coproporphyrinogen oxidase</td>
<td>P</td>
<td>COP/HEMF, HEMF1, HEMF2</td>
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<td>Protoporphyrinogen oxidase</td>
<td>P/M</td>
<td>PPX/HEMG, HEMG1, HEMG2</td>
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<td>Mg-chelatase</td>
<td>P</td>
<td>CHL D, CHLH, CHL1, CHL2</td>
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<td>11</td>
<td>Mg-protoporphyrin IX methyltransferase</td>
<td>P</td>
<td>CHLM</td>
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<td>12</td>
<td>Mg-protoporphyrin IX monomethyl ester cyclase</td>
<td>P</td>
<td>CRD1</td>
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<td>13</td>
<td>Divinyl reductase</td>
<td>P</td>
<td>DVR</td>
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<td>NADPH-protoclorophyllide oxidoreductase</td>
<td>P</td>
<td>PORA, PORB, PORC</td>
</tr>
<tr>
<td>15</td>
<td>Geranylgeranyl reductase</td>
<td>P</td>
<td>Chl P</td>
</tr>
<tr>
<td>16</td>
<td>Chlorophyll synthase</td>
<td>P</td>
<td>Chl G</td>
</tr>
<tr>
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<td>Chlorophyllide a oxygenase</td>
<td>P</td>
<td>CAO</td>
</tr>
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antibiotics that specifically target steps in the five-carbon ALA biosynthetic pathway and that are nontoxic to animals.

**Glutamyl t-RNA synthetase:**
Glutamyl-tRNA synthetase (EC 6.1.1.17) has been studied in connection with its role in protein synthesis. Like all aminoacyl-tRNA synthetases, the enzyme requires the cognate amino acid and tRNA as substrates, and the reaction requires the energy of ATP hydrolysis. There is no evidence to suggest that the glutamyl-tRNA synthetase that charges tRNAGlu for ALA biosynthesis differs from the one involved in protein synthesis. The single glutamyltRNA synthetase found in barley chloroplasts has a subunit molecular mass of 54 kDa (Beale S I, 1999). Glutamyl-tRNA (Glu-tRNA) synthetase (GluRS), which forms Glu-tRNA by the esterification of glutamate to the 3' end of tRNA, is found in different organisms in two forms: a discriminating enzyme (D-GluRS) that recognizes exclusively tRNAGlu and a relaxed specificity nondiscriminating enzyme that recognizes both tRNAGln and tRNAGlu (ND-GluRS). This ND-GluRS produces Glu-tRNAGln in the many organisms (most bacteria and all archaea and eukaryotic organelles) that lack GluRS; the mischarged Glu-tRNAGln is then transformed by a tRNA-dependent amidotransferase to Gln-tRNAGln. Genomewide analysis revealed that many bacteria contain duplicated GluRSs (GluRS1, GluRS2). In addition, some organisms contain a truncated version of GluRS (GluQRS) that is involved in the modification of tRNAAsp (Levican et al, 2007).

**Glutamyl-tRNA reductase**
Glutamyl-tRNA reductase, the second enzyme of the pathway, reduces the activated α-carboxyl group of glutamyl-tRNA (Glu-tRNA) in the presence of NADPH and releases glutamate 1-semialdehyde (GSA). Pyridine nucleotides are required for this reaction (Hoober et al., 1988). In addition, the enzyme is subject to feedback regulation by heme and appears to be a major control point of porphyrin biosynthesis (Wang et al., 1987; Kannangara et al., 1988). Micromolar concentrations of Zn$^{2+}$, Cu$^{2+}$ and Cd$^{2+}$ inhibit barley glutamyl-tRNA reductase (Pontoppidan and Kannangara, 1994). In green barley plants, this enzyme is stimulated by GTP (Kannagara et al., 1988).

accumulated in response to demand of Chl synthesis in photosynthesising tissues, whereas hemA2 mRNA was expressed in response to the demand of synthesis of porphyrins other than chlorophylls. Arabidopsis plants expressing antisense *HEMAI* mRNA, showed less chlorophyll, decreased ALA levels and decreased glutamyl-tRNA levels (Kumar and Soll, 2000). In Arabidopsis GluTR interacts with FLU, a negative regulator of chlorophyll biosynthesis pathway (Meskauskiene et al., 2001, 2002).

**Glutamate 1-semialdehyde aminotransferase**

The formation of δ-aminolevulinate from GSA is catalyzed by glutamate 1-semialdehyde aminotransferase. This enzyme is functionally an aminomutase, which transfers the amino group on carbon 2 of the glutamate- semialdehde to the neighbouring carbon atom i.e., carbon 5 of ALA. During the conversion of GSA to ALA, amino group from pyridoxamine phosphate is donated to GSA, leading to formation of an intermediate, 4, 5-diaminoovalerate. The enzyme then releases amino group from position-4 of this intermediate, releasing 5-ALA. The enzyme is inhibited rapidly by gabaculine (Gough et al., 1992). Tolerant GABA mutant of GSA-AT, resulted from a point mutation, Met-248-Ile, in the middle of the polypeptide chain accompanied by a deletion of three amino acids (serine, proline, and phenylalanine) close to the NH₂ terminus but can also be affected by the point mutation alone (Grimm et al., 1991; Smith & Grimm, 1992).

Light is reported to stimulate transcription of the gene encoding the enzyme in Arabidopsis (Ilag et al., 1994) and Chlamydomonas reinhardtii (Matters and Beale, 1994). The gene has been isolated from *Hordeum vulgare* (Kannangara et al., 1994), *Pisum sativum* (Pugh et al., 1992), *E.coli*, *Synechococcus* (Grimm et al., 1991), *Salmonella* (Elliot et al., 1990), *Arabidopsis* (gsa1and gsa2) (Ilag et al., 1994), *Glycine max* (Sangwan and O'Brien, 1993), *Nicotiana tabacum* (Hofgen et al., 1994) and *Brasicca napus* (Tsang et al., 2003). The gene have also been cloned in *Bacillus subtilis* and purified and crystallized from *E. coli* (Lv et al., 2006). The amino acid sequences of these genes show extensive homology among themselves.

**5-Aminolevulinic acid dehydratase**

This enzyme is also known as PBG synthase. It is a homooolameric metalloenzyme that catalyzes the condensation of two 5-aminolevulinic acid molecules to form the tetrapyrrrole
precursor porphobilinogen. The mechanism of action of ALAD was first proposed by (Shemin, 1976). The aldol condensation between two ALA molecules involves the initial binding of two substrate molecules and a five membered heterocyclic ring of PBG is formed with the help of a lysine and a histidine residue (Nandi, 1978; Jordan and Shemin, 1980; Spencer and Jordan, 1994, 1995). Pea ALAD has a distinct metal binding domain based on aspartate and an active site domain of lysine, which is highly conserved (Boese et al., 1991).

This enzyme has been isolated from wheat (Nandi and Waygood, 1967), tobacco (Shetty and Miller, 1969), radish (Shibata and Ochiai, 1977), spinach (Liedgens et al., 1980) and tomato (Polking, 1995). In pea, the ALAD expression was high in dark grown tissues as compared to light grown samples (Li et al., 1991). ALA dehydratase was detectable in embryonic leaves whether the plants were grown in darkness or under continuous white-light illumination. In the leaves of dark-grown seedlings, the highest levels of message accumulation were observed at approximately 8 to 10 d postgermination, and, thereafter, a steady decline in mRNA levels was observed. In the leaves of light-grown seedlings, steady-state levels of mRNA encoding the three chlorophyll biosynthetic enzymes were inversely correlated with leaf age (He et al., 1994). In constrast, light is not essential for expression of ALAD in leaves of dark-grown plants of soybean (Kaczor et al., 1994).

**Porphobilinogen deaminase**
The enzyme, which is a soluble chloroplast protein (Castelfracco et al., 1988) catalyses the formation of the linear tetrapyrrole, hydroxymethylbilane, from four molecules of Porphobilinogen (PBG). The tetrapyrrole is either converted to uroporphyrinogen III by uroporphyrinogen cosynthetase or non-enzymatically cyclized to uroporphyrinogen I (Frydman and Frydman, 1978a, b). Porphobilinogen deaminase enzyme has been purified from A. thaliana, which is a monomer of 35 kDa. It is inactivated by arginine-, histidine-, lysine-specific reagents and also by the substrate analogue 2-bromoporphobilinogen (Jones and Jordan, 1994).

A cDNA clone for porphobilinogen deaminase from Arabidopsis encodes a precursor protein of 382 residues, which is then imported to the isolated chloroplasts and processed to a mature protein (Lim et al., 1994). The enzyme was encoded by a single gene and is expressed in both leaves and roots (Witty et al., 1996). Northern blot analysis has shown that the PsPBGD is expressed in chlorophyll-containing tissues and is subjected to light induction. The enzyme has
an acidic isoelectric point and is a single polypeptide showing different levels of sensitivity to
divalent cations, being most sensitive to Fe$^{2+}$. The synthesis of PBGD is regulated by light
(Smith, 1988; Spano and Timko, 1991; Shashidhara and Smith, 1991; He et al., 1994).
In Euglena gracilis, PBGD transcript levels regulated at the post-transcriptional level
(Vacula et al., 2001).

**Uroporphyrinogen III cosynthetase**
This enzyme catalyses formation of uroporphyrinogen III from hydroxymethylbilane. The
enzyme has been isolated from E. gracilis (Hart and Battersby, 1985) and wheat germ (Higuchi
and Bogorad, 1975). The enzyme was found to be heat labile and Na$^+$ and K$^+$ enhanced its
activity. The enzyme PBGD and cosynthase may be present as a complex (Tsai et al., 1987).
HemD gene encodes for the enzyme and has been cloned and isolated from various organisms.
The enzyme from A. nidulans has an inferred amino acid sequence which shows 43-50%
homology to that from B. subtilis, E. coli (Jones et al., 1994). The inferred amino acid sequence
has conserved arginine residue at codon 146, which has been implicated to be important for
cosynthase activity (Hansson et al., 1991). Gene for uroporphyrinogen synthase has been
identified and characterized in A.thaliana. A precursor protein of 34 kDa when was imported to
chloroplast produce mature protein of 29 kDa (Tan 2008). The first gene encoding UROS was
isolated from Escherichia coli (Sasarman et al, 1987) with those from human (Tsai et al 1988)
Bacillus subtilis (Hansson et al 1991) Pseudomonas aeruginosa (Mohr et al 1994) Anacystis
nidulans R2 (now reclassified as Synechococcus PCC 7942) (Jones et al 1994) mouse
(Bensidhoum et al 1994) and budding yeast Saccharomyces cerevisiae (Amillet et al 1995).

**Coproporphyrinogen oxidase**
This enzyme catalyses the oxidative decarboxylation of propionate side chains on ring A and B
to yield protoporphyrinogen IX. In aerobic organisms, oxygen is utilized as the sole electron
acceptor for enzymatic activity while in anaerobic organisms NADP$^+$ is used (Seehra et al.,
1983). The coprogen oxidase was first purified from tobacco (Hsu and Miller, 1970). Yeast
enzyme is a homodimer of 70 kDa (Camadro et al., 1986). It has shown that tobacco plants
containing antisense RNA for coprogen oxidase are more resistant to tobacco mosaic virus
(Mock et al., 1999). Tobacco plants containing antisense coprogen oxidase RNA showed
decreased enzyme levels of coprogen oxidase and were characterized by growth retardation and
necrosis, showing that these plants were damaged due to oxidative stress (Kruse et al., 1995a). The full-length cDNA from barley and tobacco were cloned and found that these encode for precursor proteins of 43.6 and 44.9 kDa respectively (Kruse et al., 1995b). These proteins were processed to 39 kDa after import into pea chloroplast and accumulated in the stroma.

There are two isoforms of coprogen oxidase (Cpx1 and Cpx2) was found in maize. The Cpx1 fused with GFP showed that it was localized in plastid, whereas in case of Cpx2, it appeared to localize to mitochondria (Williams et al., 2006). Mutants defective in the enzyme have been isolated from *R. sphaeroides* (Coomber et al., 1982), defective in two genes, *hemN* and *hemF*, which encode alternative forms of coprogen oxidase (Xu et al., 1992). An *Arabidopsis* mutant defective LIN2 gene encoding coprogen oxidase develops lesions on leaves, in a developmentally regulated and light dependent manner (Ishikawa et al., 2001).

**Protoporphyrinogen oxidase**

Protoporphyrinogen oxidase catalyzes the final reaction of the common branch of the heme and chlorophyll biosynthesis pathways, i.e., conversion of Protoporphyrinogen IX (Protogen) to Protoporphyrin IX (Proto IX) in plants. Protogen is unstable and spontaneously undergoes oxidation in presence of oxygen and its oxidation is enhanced by light (Jacobs and Jacobs, 1979). Enzyme catalyzed reaction mechanism of Protogen oxidation consists of three consecutive dehydrogenations and a subsequent tautomerization yielding the porphyrin through the stereospecific loss of the fourth *meso* hydrogen as a proton (Akhtar, 1994). Protox is active only if there are no polar groups on ring A and B and is quite stable towards acids and bases.

Protox was isolated from yeast mitochondrial membranes (Camadro et al., 1994), from spinach, where protox was both found in chloroplast and mitochondria respectively (Watanabe et al., 2001), from *Arabidopsis* where transcripts of plastidal protox were very high in leaves, where as it was low in roots and floral buds (Narita et al., 1996), from tobacco (*PPXI* and *PPX2*) where transcripts of both genes were expressed synchronously during tobacco plant development and diurnal and circadian growth (Lermontova et al., 1997), purified from barley etioplasts (Jacobs and Jacobs, 1987), localized in the envelope (stromal side) and thylakoid membranes (stromal side) of chloroplasts (Matringe et al., 1992a; Che et al., 2000). Dailey et al., 1994 have expressed *hemY* gene of the *B. subtilis* in *E.coli* and found the protein was capable of oxidizing coprogen III and protogen IX. Yamato et al., 1995 have shown that amino acid sequences of
protox purified from tobacco-cultured cells have shown homology to acid/base catalysis and heme binding regions of plant peroxidases. When human protoporphyrinogen IX oxidase was overexpressed in rice resulted in severe necrotic leaf and growth retardation. Tetrapyrrole-induced photooxidation was confirmed by increased lipid peroxidation and subsequent cell death (Jung et al 2008).

**Mg-chelatase**

The chelation of Mg$^{2+}$ into protoporphyrin IX, which is catalyzed by the enzyme Mg-chelatase, is the key reaction unique to chlorophyll biosynthesis. In photosynthetic organisms, Mg-chelatase is a three component enzyme and catalyses the insertion of Mg$^{2+}$ in two steps, with an ATP-dependent activation followed by an ATP-dependent chelation step (Walker and Willows 1997; Walker and Weinstein, 1994). The optimal ATP concentration for activation is found to be higher than that of chelation step. The activation step requires interaction of subunits D and I. Since D subunit occurs as an aggregate, this step involves dissociation of a single D subunit from the aggregate. ATP and a kinase-mediated phosphorylation are also involved in this step. The second step (chelation) of catalysis is Mg$^{2+}$ insertion, and involves the H subunit along with the I$_2$.D complex. The subunit of I$_2$.D complex then drives the release of a water molecule from the Mg$^{2+}$ ion's co-ordination sphere, by using water as a substrate for hydrolysis of ATP. The released Mg$^{2+}$, co-coordinated to a histidine residue on the H subunit, promotes dissociation of hydrogen ions from the pyrrole nitrogen atoms and is then itself co-coordinated into porphyrin macrocycle (Walker and Willows, 1997). The stoichiometry of H: I$_2$.D complex has been shown as 4:1 (Willows et al., 1996). In tobacco, only a 110 amino acid long part of ChlH is required for interaction with partner subunits and maintenance of the enzymatic activity (Grafe et al., 1999).

In the purple bacteria *R. capsulatus* and *R. sphaeroides*, Mg-chelatase is encoded by the *bchD, I* and *H* genes (Gibson et al., 1995; Willows et al., 1996). The *bchH* and *bchD* gene pair have been expressed separately in *E. coli* and insertion of Mg$^{2+}$ into proto IX was obtained by combining soluble extracts from induced cells and supplying substrate, ATP and Mg$^{2+}$ (Willows et al., 1996). BchlH (140 kDa) binds the substrate protoporphyrin IX in a molar ratio of 1:1, while Bchl with its ATP binding domain functions as a homodimer. BchD participates as a polymer with an apparent Mr of 550 kDa. The complex catalyses an activation step requiring
BchI, BchD and Mg-ATP and a metal insertion step involving protoporphyrin IX and BchH (Gibson et al., 1995). Structural analyses have shown that BchI forms hexamers and belongs to ATPases associated with various (AAA (+) families of proteins. AAA (+) proteins are Mg (2+)-dependent ATPases that normally form oligomeric ring structures in the presence of ATP. It has been suggested that ATP hyrolysis of each BchI within the hexamer causes a conformational change of the hexamer as a whole (Hansson et al., 2002).

Jung et al., 2003 isolated the chlH mutant from rice, where they have shown that ChlH is light inducible and the mutant is defective in thylakoid development. Depending upon the concentration of Mg\(^{2+}\) in lysis buffer, the ChlH migrated between stroma and the envelope membrane and was localized in the envelope membrane at very higher concentrations of Mg\(^{2+}\) (above 5mM), indicating that the activity of Mg-chelatase was regulated by the expression and subchloroplastidic localisation of ChlH protein (Nakayama et al., 1998).

Transgenic tobacco plants expressing antisense RNA for Mg-chelatase ChlH were chlorophyll deficient. In these plants, less protoporphyrin IX and heme accumulated, and a decrease in 5-aminolevulinate synthesizing capacity was seen (Papenbrock et al., 2000b). Virus-induced gene silencing of ChlH in tobacco led to lowering of ChlD and ChlI mRNAs along with less chlorophyll content (Hiriart et al., 2002). Mochizuki et al., 2001 have isolated one Mg-chelatase subunit H mutant of Arabidopsis, which they named GUN5 (Genome Uncoupled). Mutation in this gene had repressed expression of lhcb whereas those with mutations in Chll, ChlD gene did not show any lhcb1 repression. This comparison suggests a specific function for ChlH protein in the plastid-signaling pathway (Mochizuki et al., 2001; Strand et al., 2003).

The Chll gene was cloned from soybean by Nakayama et al., 1995. It is localized in stroma and has an ATP-binding motif. The Chll mRNA was reversibly induced by light in cell cultures of soybean. In Arabidopsis, a second Chll gene, Chll-2 has been identified that supports limited chlorophyll synthesis in a T-DNA knockout mutant of the chlorina locus (chlI) (Rissler et al., 2002). Using transformants of tobacco with sense and antisense mRNA for Chll, it has shown that both elevated and decreased levels of Chll mRNA and Chll protein led to reduced Mg-chelatase activity and in these plants chlorophyll synthesis was also reduced (Papenbrock et al., 2000a). In arabidopsis double-knockout mutant of chli/chli1 chli2/chli2 was albino. Comparison with the pale-green phenotype of a chli1/chli1 single-knockout mutant indicates that
CHL2 could support some chlorophyll biosynthesis in the complete absence of CHLI (Huang and Li, 2009). The tobacco ChlD cDNA sequence was isolated and cloned (Papenbrock et al., 1997). The amino terminal half of ChlD cDNA was 46% homologous to that of ChlI, indicating gene duplication from an ancestral gene. Reconstitution experiments using yeast protein extract expressing the three subunits of tobacco Mg-chelatase showed additional requirement of ATP (Papenbrock et al., 1997). ChlD in pea is associated with the membranes in the presence of MgCl₂. It was 89 kDa protein expressed in soluble form and was active in a Mg-chelatase reconstitution assay (Luo et al., 1999). CHLH gene encoding the H subunit of Mg-chelatase was induced by light under all conditions with an initial peak after 2-4 h light. The other Mg-chelatase subunit genes CHLI and CHLD genes were not strongly regulated at the level of transcript abundance (Stephenson and Terry, 2008).

The chlH subunit of Synechocystis stimulates magnesium protoporphyrin methyltransferase (ChIM) activity (Shepherd et al., 2005). The ATPase activity of recombinant CHLII in Arabidopsis thaliana was found to be fully inactivated by oxidation and easily recovered by thioredoxin-assisted reduction, suggesting that CHLII is a target protein of thioredoxin (Ikegami et al, 2007).

S-adenosyl-l-methionine: Mg-protoIX methyltransferase

This enzyme catalyzes the conversion of Mg-protoporphyrin IX to Mg-protoporphyrin monomethyl ester (MPE) by transferring of a methyl group to the carboxyl group of the C13-propionate side chain of MgProto (Gibson et al., 1963) where, SAM acts as a methyl group donor. This enzyme belongs to the broad family of S-Adenosyl-L-Methionine (SAM)-dependent methyltransferases (Kagan and Clarke, 1994), which contains the S-adenosyl- methionine (SAM)-binding domain, a seven-stranded b-sheet (Jones, 1999). The Synechocystis PCC 6803 chlM was over expressed in E. coli and the protein has been purified which is a 20 kDa protein (Shepherd et al., 2003). Taking the purified protein steady state kinetic assays were performed using Magnesium deuteroporphyrin IX (MgD), a substrate analogue of Magnesium protoporphyrin IX where the initial rate studies showed that the reaction proceeds via a ternary complex, a rapid binding of the substrate and the enzyme preceded by slower isomerization of the enzyme (Shepherd et al., 2004). Co-expression of Mg-chelatase (all subunits) and chlM from Synechocystis in E. Coli yielded soluble protein extracts that converted protoporphyrin IX to Mg-protoporphyrin IX monomethyl ester (Jensen et al., 1999).
In *Arabidopsis* and spinach, this protein has a dual localization in chloroplast membranes as well as thylakoids. Averina *et al.*, 2002 showed that SAM-MgPixMT was located not only in prothylakoids but also in prolammener bodies of barley containing photoactive Pchlide. *ChlM* from tobacco encodes a 35-kDa protein. The amount of ALA, Mgproto and heme were reduced in antisense *chlM* plants of tobacco. The Mg chelatase activity was reduced in antisense plants where as the Fe-chelatase activity was increased. The gene expression of *chlH, GluTR, GSAT* was less in antisense plants and more in sense plants. Western analysis of these corresponding proteins revealed a direct corellation between RNA levels and protein amounts (Alawady and Grimm, 2005).

**Ferrochelatase**

The chelation of Fe$^{2+}$ to make heme is a crucial branch point of the tetrapyrrole synthesis pathway, which is catalyzed by ferrochelatase where Fe$^{2+}$ gets inserted into protoporphyrin IX to generate protoheme. The ferrochelatase gene has been isolated from *Arabidopsis* (Simth *et al.*, 1994), barley & cucumber (Miyamoto *et al.*, 1994), soybean & *E. coli* (Kanjo *et al.*, 2001), and tobacco (Papenbrock *et al.*, 2001).

Though plastids are the major site of heme biosynthesis, mitochondria have also the capacity for heme production (Cornah *et al.*, 2002). In higher plants, ferrochelatase activity has been detected in bean cotyledons, oat seedlings and spinach leaves (Jones 1968; Porra and Lascelles, 1968) and barley etiolated seedlings where the ferrochelatase activity was found to be associated with mitochondria, etioplasts and plasma membranes (Little and Jones, 1976; Jacobs and Jacobs, 1995). In pea chloroplasts, the activity was shown to be associated with thylakoid membranes (Matringe *et al.*, 1994). Suzuki *et al.*, 2000 have shown that CsFeCl protein from cucumber was present in hypocotyls and roots but not in cotyledons and targeted both to chloroplast and mitochondria (Masuda *et al.*, 2003) and CsFeC2 is localized predominantly in thylakoids and in very minor quantity in envelope membrane. It is detected in all tissues and was light responsive in cotyledons. Chow *et al.*, 1998 have shown the presence of two types (*AtFC-I, AtFC-II*) of ferrochelatases in *Arabidopsis*. One form was shown to be expressed in leaves, stems, roots and flowers and imported into chloroplasts and mitochondria whereas the other one was expressed in leaves, stems and flowers and targeted solely to chloroplasts (Lister *et al.*, 2001). *AtFC-I* was found to induce in response to TMV infection suggesting the requirement of
heme synthesis as part of defence response (Singh et al., 2002). Impaired expression of plastidic ferrochelatase led to necrotic phenotype of antisense RNA transformed tobacco plants indicating that it plays a role in Chl biosynthesis (Papenbrock et al., 2001). PIF1, members of a basic helix-loop-helix family of transcription factors regulates expression of a ferrochelatase FeChII in dark (Moon et al., 2008). Expression of ferrochelatase upregulated in light whereas downregulated in chill and heat stress (Mohanty et al., 2006).

**Vinyl reductase**

The 8-vinyl reductase reduces the 8-vinyl group on the tetrapyrrole to an ethyl group (Parham and Rebeiz, 1995) using NADPH as the reductant. This activity has been detected with isolated plastid membranes, in intact chloroplasts (Parham and Rebeiz, 1995; Tripathy and Rebeiz, 1988) from Cucumber (Cucumis sativus) and Barley respectively and also in solubilized crude extracts (Kolossov and Rebeiz, 2001).

Nagata et al., 2005 followed by Nakanishi et al., 2005 isolated the mutant of Arabidopsis, which accumulates divinyl chlorophyll. The mutant is pale green and the chlorophyll a/b ratio varies in between 6 and 10 depending on the developmental stage and growth conditions (the chlorophyll a/b ratios of the wild type were between 3 and 3.8). This mutant is capable of photosynthesizing and growing under low-light conditions (70 to 90 μmole m⁻² s⁻¹); but rapidly died under highlight conditions (1000 μmole m⁻² s⁻¹) (Nagata et al., 2005). The thylakoid membranes were in a disorderly fashion having no distinct grana stacks in the mutant but no distinct differences in the size and the number of chloroplasts between the wild type and the mutant. Starch granules were not found in the mutant chloroplasts, suggesting the reduction of photosynthetic activity in the mutant (Nakanishi et al., 2005). The transcript level of DVR expression is high in leaves, stems and flower buds, and low in roots and localized in mesophyll cells of the chloroplast.

Though DVR genes were found in both higher plants and green algae, complete genomic sequence data from the unicellular red alga Cyanidioschyzon merolae (Matsuzaki et al., 2004), which accumulates monovinyl chlorophylls, suggested that it lacks DVR homologs. These data suggest that another type of enzyme is involved in the reduction of the 8-divinyl groups in this organism. In cyanobacteria, a DVR gene was found in Synechococcus sp WH1802 but was not found in other cyanobacterial lineages. The knock-out mutant of gene Slr1923 lost its ability to
synthesize monovinyl chlorophyll and accumulated 3,8-divinyl chlorophyll instead. It was concluded that Srl1923 encodes the vinyl reductase or a subunit essential for monovinyl chlorophyll synthesis (Ito et al., 2008). [3, 8-DV]-Chlide a is the major substrate of DVR. Accordingly, name of the enzyme was from [3, 8-DV]-Pchlide a 8-vinyl reductase to [3, 8-DV]-Chlide a 8-vinyl reductase (Nagata 2007).

**Protochlorophyllide oxidoreductase**

It is the first light-requiring enzyme of the chlorophyll biosynthesis pathway. It catalyses the conversion of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) by using light as a substrate along with protochlorophyllide (Pchlide) and NADPH as a cofactor. As a result of its requirement for light, this reaction is an important regulatory step in chlorophyll biosynthesis pathway and subsequently assembly of the photosynthetic apparatus. The initial binding of NADPH involves three distinct steps, which appear to be necessary for the optimal alignment of the cofactor in the enzyme active site. This is followed by the binding of the Pchlide substrate and subsequent substrate-induced conformational changes within the enzyme that occur prior to the formation of the final "poised" conformational state. These studies, which provide important information on the formation of the reactive conformation, reveal that ternary complex formation is the rate-limiting step in the overall reaction and is controlled by slow conformational changes in the protein (Heyes et al., 2008). The role of conformational changes in explaining the huge catalytic power of enzymes has been solved using using the chlorophyll biosynthetic enzyme NADPH: protochlorophyllide (Pchlide) oxidoreductase, which catalyses a unique light-driven reaction involving hydride and proton transfers (Sytina et al., 2008). Pchlide reduction occurs by dynamically coupled nuclear quantum tunneling of a hydride anion followed by a proton on the microsecond time scale in the Pchlide excited and ground states, respectively (Heyes et al., 2009). POR is nuclear encoded, translated as a precursor protein in the cytosol and ultimately transported into plastids (Apel, 1981). POR converts Pchlide to Chlide, by adding two hydrogen atoms at C17 and C18 on ring D (Figure II). The fact that POR is light-activated means the enzyme-substrate complex can be formed in the dark, removing the diffusive components out of the reaction. This has recently been exploited by studying Pchlide reduction at low temperatures to trap intermediates in the reaction pathway (Heyes et al., 2002, 2003; Heyes and Hunter, 2004). As a result, the reaction has been shown to consist of at least three distinct steps: an initial light-
driven step, followed by a series of ‘dark’ reactions. An initial photochemical step can occur below 200 K (Heyes et al., 2002), whereas two ‘dark’ steps were identified for Synechocystis POR, which can only occur close to or above the ‘glass transition’ temperature of proteins (Heyes et al., 2003). This implies a role for protein motions during these stages of the catalytic mechanism. A thermophilic form of the enzyme has been used to identify two additional ‘dark’ steps, which were shown to represent a series of ordered product release and cofactor binding events. First, NADP⁺ is released from the enzyme and then replaced by NADPH, before release of the Chlide product and subsequent binding of Pchlide have taken place (Heyes and Hunter, 2004). Monovinyl protochlorophyllide (MV-Pchlide) and Divinyl protochlorophyllide (Dv-Pchlide) don’t influence differentially the enzyme kinetics or the steps involved in the reaction pathway (Heyes et al., 2006). The POR activity in different species is also temperature dependent. In Synechocystis the optimum temperature for POR activity is 30°C, whereas in thermophilic cyanobacterium Thermosynechococcus elongates the optimum temperature for POR activity is in between 50°C-55°C and is much less active at room temperature (McFarlane et al., 2005).

The absorption of light by the tetrapyrrole may produce torsional strain in the molecule providing favorable conditions for hydride/hydrogen transfer from NADPH. Tyr and Lys are absolutely conserved among all members of the short chain alcohol dehydrogenase family and these residues are important for catalysis. The Tyr may be deprotonated, acting as a general acid to facilitate hydride transfer to or from NAD (P)⁺H (Bohren et al., 1994). The proton at the C-18 position of Pchlide is derived from Tyr-275 (numbering of Pea POR) and the hydride transferred to the C-17 position is derived from the pro-S face of NADPH. The proximity of the Lys residue may be important for lowering the apparent pKa of the phenolic group (around 10) of the Tyr to facilitate deprotonation (Figure III). It may also play a role in ensuring that the nicotinamide ring of NAD (P)⁺H is in the correct orientation for pro-S hydride transfer (Varughese et al., 1994).

Spectroscopic studies of the dark grown bean seedlings gave the idea about two forms of protochlorophyllide, a main component with a red absorption band at 650 nm and a minor component absorbing at 636 nm (Shibata, 1957). Again by fluorescence study it has been shown that there are two main forms of protochlorophyllide absorbing at 650 nm and 638 nm and a minor form which absorbs at 628 nm. The 650-nm and 638-nm forms are photoconvertible to chlorophyll at room temperature but the 628-nm form was not photoconvertible. Griffiths, 1975
observed in the barley-etiolated membranes, the non-photoactive protochlorophyllide could be converted into photoactive form (P638/652) by an NADPH requiring reaction.

At least three different spectral forms of Pchlide are recognized in intact tissues based on their fluorescence emission maximum (in nm): Pchlide F631 (due to the pigment structural arrangements), Pchlide F644 (due to association of POR), and Pchlide F655 (due to localization in PLBs and/or prothylakoids) (Böddi et al., 1992, 1993). Fluorescence lifetime of Pchlide measured in plants showed that short- and long-wavelength Pchlide forms have fast (0.3 to 0.8 ns) and slow (5.1 to 7.1 ns) components with different proportions depending on plant species (Mysliwa-Kurdziel et al., 2003). Short-wavelength Pchlide forms are thought to be located in the prothylakoids, bound in a monomeric form to proteins other than POR (Böddi et al., 1998; Kis-Petik et al., 1999). The main photoactive form present in etiolated plants is Pchlide F655, which after illumination, is converted to Chlide and subsequently to Chl (F682) through the formation of several, long wavelength intermediates (Böddi and Franck, 1997; Schoefs et al., 2000a). After flash illumination, photoactive Pchlide complex can be regenerated by reloading with non-photoactive Pchlide on a fast time-scale with concomitant release of Chlide (Franck et al., 1999; Schoefs et al., 2000b).

Full-length cDNA clones of POR were isolated from Barley (Schulz et al., 1989; Holtorf et al., 1995), from Oat (Darrah et al., 1990), from Pea (Spano et al., 1992), from Wheat (Teakle and Griffiths, 1993), from Arabidopsis (Benli et al., 1991; Armstrong et al., 1995; Oosawa et al., 2000), cyanobacterium Plectonema boryanum and Phormidium laminosum (Fujita et al., 1998; Rowe and Griffiths, 1995), from Liverwort (Takio et al., 1998), from Chlamydomonas (Li and Timko, 1996), Syneccystis (Suzuki and Bower, 1995), Tobacco (Masuda et al., 2002), Cucumber (Kuroda et al., 2000) and from Banana (Coemans et al., 2005). The high degree of sequence similarity among PORs from different taxonomic group implies a common mechanism of the enzyme action.

A characteristic feature of POR accumulating in dark is its sensitivity to illumination. The POR mRNA expression was also decreased (Santel and Apel, 1981). Red and far-red light treatment also inhibits POR mRNA expression indicating that POR expression was controlled by phytochrome (Apel, 1981; Batschauer and Apel 1984; Mosinger et al., 1985). The negative effect of light on POR enzyme and its mRNA was observed in different dicotyledons like Bean,
Pea, Tomato, Arabidopsis (Forreiter et al., 1991; Spano et al., 1992; Armstrong et al., 1995) and in monocotyledonous plants maize and barley (Forreiter et al., 1991; Holtorf et al., 1995). However, some flowering plants have isoforms of POR. In Arabidopsis (Armstrong et al., 1995; Oosawa et al., 2000; Pattanayak and Tripathy, 2002; Su et al., 2001), in barley (Holtorf et al., 1996a) and in Tobacco (Masuda et al., 2002) there are different PORs present. In Arabidopsis, there are three isoforms of POR, namely PORA, PORB, and PORC. These three isoforms are differentially regulated by light. The level of porA mRNA and protein decreases on illumination of etiolated plants (Holtroff and Apel, 1996b) while that of porC increases and is dominantly expressed in both mature and immature tissues (Oosawa et al., 2000). PorB transcript and protein levels remain constant in both dark and on illumination (Armstrong et al., 1995, Holtrof et al., 1995; Holtrof and Apel, 1996a). Both porB and porC of Arabidopsis thalina exhibited diurnal fluctuation but only the porB mRNA of Arabidopsis exhibits circadian regulation (Su et al., 2001). PorC mRNA and protein expression also increased under high light intensity (Su et al., 2001; Masuda et al., 2003). In cucumber, the levels of the POR mRNA increased etiolated cotyledons when they were illuminated with continuous light and even matured leaves (Kuroda et al., 1995, 2000; Fusada et al., 1995) via transcriptional activation (Fusada et al., 1995). In Tobacco, two POR isoforms have been isolated, the expression of which was not negatively regulated by light, persisted in mature green tissue and showed diurnal fluctuations with a similar oscillation phase (Masuda et al., 2002). The plant hormone cytokinin also regulates cucumber POR gene expression by binding to the cis-elements present at 5' region of the POR promoter.

Degradation of PORA is specific and controlled by nuclear-encoded proteases. The mechanism of light-activation of protease expression is unknown. Mapleston and Griffiths, 1980 observes that POR activity decreased after illumination. Reinbothe et al., 1995d showed that barley pPORA-Pchlide complex was resistant to protease treatment independent of the presence or absence of NADPH. In contrast, pPORA-Chlide complex was rapidly degraded. The naked pPORA without its substrate or products was less sensitive to proteolysis than the pPORA-Chlide complex suggesting that both substrate binding and product formation had caused differential changes in protein conformation (Reinbothe et al., 1995d). PORB was not degraded by the protease. The PORA degrading protein is assumed to be nuclear encoded, energy dependent and plastid localized protein in barley (Reinbothe et al., 1995d). But the study of post-import degradation of radiolabeled barley pPORA and pPORB on incubation with stroma
enriched fractions from etiolated and light grown barley or wheat did not show any protease activity (Dahlin et al., 2000).

The import of the precursor of barley PORA (pPORA) into chloroplasts was totally dependent on envelope bound Pchlide, the substrate for the enzyme’s catalytic activity (Reinbothe et al., 1995 b, c). During the light induced transformation of etioplasts into chloroplasts, the concentration of Pchlide dramatically declined, and chloroplasts rapidly lost the ability to import pPORA. However ALA feeding restored the capacity to import pPORA. Exogenously applied Pchlide competitively released the envelope-bound pPORA into the cytosol (Reinbothe et al., 1996c). The role of the transit peptide of pPORA was further analyzed by production of chimeric constructs, in which the transit sequences of pPORA and pPORB were exchanged and fused to either their cognate mature polypeptides or to a reporter protein of cytosolic dihydrofolate reductase (DHFR) from mouse. The transit peptide of pPORA conferred Pchlide-dependent import of both the mature PORB and DHFR, while that of pPORB directed the mature PORA and DHFR into chloroplasts even without the endogenous Pchlide (Reinbothe et al., 1997), suggesting that the transit peptide of pPORA directly interacts with Pchlide in the plastid envelope for translocation. The Pchlide-dependent import of the transit peptide of barley pPORA and DHFR chimeric construct was also shown in chloroplasts of tobacco, Arabidopsis, and five other tested monocotyledonous and dicotyledonous plant species (Reinbothe et al., 2000). pPORA also interacts with Toc33/34, one 16 kDa plastid envelop protein (ORP16) and Pchlide a oxygenase during its posttranslational import into isolated barley chloroplasts (Reinbothe et al., 2004a,b). Again Reinbothe et al., 2005 showed that Plastids of the ppi1 mutant of Arabidopsis lacking Toc33, were unable to import pPORA in darkness but imported the small subunit precursor of ribulose-1, 5-bisphosphate carboxylase/oxygenase (pSSU), precursor ferredoxin (pFd) as well as pPORB. In white light, photooxidative membrane damage, induced by excess Pchlide accumulating in ppi1 chloroplasts because of the lack of pPORA import, to be the cause of the general drop of protein import. Kim and Apel, 2004  also showed the POR import is substrate dependent. They found that the homozygous xantha2 (xantha2 is devoid of Pchlide and even also after ALA feeding in dark no Pchlide accumulation) mutants (where GFP fusion protein was stably expressed) failed to accumulate PORA-GFP, which may be caused by the lack of Pchlide. Using transgenic flu mutants that express the various GFP fusion proteins tested the uptake of PORA into plastids further. In cotyledons of flu and wild-type seedlings kept
under continuous light, SSU-GFP and PORB-GFP, but not PORA-GFP, accumulated within chloroplasts. Soon after these seedlings were transferred to the dark, flu started to accumulate Pchlide and PORA-GFP began to appear in plastids of this mutant, whereas in wild-type control seedlings, the fusion protein was not detected. However the Pchlide dependent PORA import is confined to the cotyledons but not to the matured green leaves. Again Kim et al., 2005 showed, PORA and PORB import into plastids of intact seedlings (mutant lines of Toc34, Toc33, Toc159) revealed an unexpected multiplicity of import routes that differed by their substrate, cell, tissue and organ specificities.

On the contrary, several reports indicated that Pchlide is not required for the import of POR in vitro (Teakle and Griffiths, 1993 in wheat), (Dahlin et al., 1995; Aronsson et al., 2000, 2001b, 2003a in pea), (Jarvis et al., 1998; Jarvis and Soll, 2001 in Arabidopsis). The absence or presence of Pchlide did not significantly affect the capacity to import pPORA and pPORB in barley (Dahlin et al., 1995; Aronsson et al., 2000).

POR disrupted mutant has been constructed and characterized in Synechocystis sp. PCC 6803, (Wu and Vermaas, 1995) and P. boryanum (Fujita et al., 1998). Although DPOR contribute to Chl synthesis in Synechocystis, but POR plays the major role in the cells grown under strong light (Fujita et al., 1998). The repression of POR gene expression in A. tricolor resulted in loss of Chl synthesis activity (Iwamoto et al., 2001).

**Light-independent protochlorophyllide oxidoreductase**

Light-independent chlorophyll (Chl) biosynthesis is a prerequisite for the assembly of photosynthetic pigment–protein complexes in the dark (Demko V et al, 2009). In contrast to angiosperms’ dependence on light for Pchlide photoreduction, nonflowering land plants, algae and photosynthetic bacteria have a distinct enzyme known as light-independent protochlorophyllide reductase (DPOR) that catalyzes reduction of Pchlide in the dark. Spectral investigation of chlorophyll a formation during regreening process in chlL(-) and chlN(-) mutants and downregulation of chlL and chlN in green light in wild type strain of cyanobacteria suggests that the regulation of dark-operative protochlorophyllide oxidoreductase (DPOR) in the transcriptional level is essential for cyanobacteria to synthesize appropriate chlorophyll for acclimating in various light colour environments (Gao Y et al, 2009). The light-independent
protochlorophyllide reductase genes have been isolated from different species i.e. from *R. capsulatus* (*bchN, bchB* and *bchL*), from cyanobacteria (*chlN, chlB* and *chlL*) and several gymnosperms (Choquet *et al.*, 1992; Li *et al.*, 1993). *ChlN* was identified coding for a 545 amino acid protein, involved in reduction of Pchlide to Chlide (Choquet *et al.*, 1992). The protein product of *C. reinhardtii* chloroplast gene *chlL* has 53% homology to *bchlL* gene product in *Rhodobacter* (Suzuki and Bauer, 1992). Dark POR activity was dependent on the presence of all three subunits, ATP and the reductant dithionite in *R. capsulatus* (Fujita and Bauer, 2000). In *R. capsulatus*, ferredoxin functioned as an electron donor to DPOR for its activity (Nomata *et al.*, 2005). In *Rhodobacter capsulatus*, mutations in genes known to completely block reduction of protochlorophyllide to chlorophyllide (*bchN, bchB, bchL*) accumulate a pool of monovinyl and divinyl forms of protochlorophyllide; but, *bchJ*-disrupted strains accumulate reduced levels of bacteriochlorophyll concomitant with the accumulation of divinyl Pchlide and thus have an altered ratio of monovinyl to divinyl Pchlide (Suzuki and Bauer, 1995).

Studies in yellow-in-the-dark mutants of *Chlamydomonas* indicated that synthesis of *ChlL* subunit is controlled by light intensity whereas the synthesis of *ChlB* and *ChlN* seem not to be influenced by light intensity (Cahoon and Timko, 2000). *ChlB* gene encoding one of the subunits of light-independent POR was cloned by Richard *et al.*, 1994; from *Ginkgo biloba*. *ChlB* mRNA starts to accumulate at the beginning of each dark phase (Richard *et al.*, 1994). The cyanobacterium *Plectonema boryanum* devoid of the *chlL* gene is unable to synthesize Chl in the dark (Kada *et al.*, 2003). There is a decrease in PSI activity in this mutant, however the PSII activity remained unchanged.

**Chlorophyllide a oxygenase**

Chlorophyll b is one of the major light-harvesting pigments produced by land plants, green algae and several cyanobacterial species. It is synthesized from chlorophyll a by chlorophyllide a oxygenase (CAO), which in higher plants consists of three domains, namely, A, B, and C (Kanematsu *et al.*, 2008). The transgenic plants overexpressing the A-domain-deleted CAO accumulated an excess amount of chlorophyll b during greening. The transgenic plants which lacked the A domain either died or were obviously retarded when they were exposed to continuous light immediately after etiolation (Yamasato *et al.*, 2008). The A domain of CAO is essential in the control of chlorophyll biosynthesis and in the survival of seedlings during de-
etiolation especially under strong illumination (Yamasato et al, 2008). B domain is not involved in the regulation of CAO protein level as there was no effect on protein level in transgenics overexpressing deleted CAO in chlorophyll b less mutants of Arabidopsis (Sakuraba et al, 2007). Tanaka et al., 1998 isolated and characterized a gene from Chlamydomonas reinhardtii using insertional mutational studies, which they found to be encoding CAO enzyme, an enzyme involved in conversion of Chl a to b. The enzyme was found to be similar to methyl monooxygenases and its coding sequence of 463 amino acids was found to contain domains for a [2Fe-2S] Rieske center and for a mononuclear nonheme iron-binding site. Chl b is synthesized by oxidation/ conversion of methyl group on the D ring of the porphyrin molecule to a formyl group at that position. During conversion of Chl a to Chl b the electron is transferred from Rieske centre to the mononuclear iron with subsequent activation of molecular oxygen for oxygenation of the Chl a methyl group (Beale and Weinstein, 1990; Porra et al., 1993). Since CAO is chloroplast localized and present in both thylakoid and envelop membrane (Eggink et al., 2004), it could accept electrons from ferredoxin-NADPH oxidoreductase or from ferredoxin itself. Using the conserved motifs of CAO of Chlamydomonas as probe Espineda et al., 1999 isolated the same gene from Arabidopsis that has 8 introns and coding sequence of 537 aminoacids. The hydropathic profiles of AtCAO suggested that there were no transmembrane helices. Tomitani et al., 1999 isolated Chl b-synthesizing genes (CAO) from two prochlorophytes and from some major chlorophytes. The alignment of the deduced amino acid sequences showed 51 % to 83 % identity among the sequences and all the sequences shared typical motifs for [2Fe-2S] centre and for the mononuclear iron-binding site.

Oster et al., 2000 have shown that, a recombinant CAO enzyme produced in E. coli catalyses an unusual two-step oxygenase reaction that is the ‘missing link’ in the chlorophyll cycle of higher plants. Overexpression of the CAO gene in Arabidopsis led to an increase in the Chl b level leading to reduction of the Chl a: b ratio from 2.85 to 2.65 in full green rosette leaves and at the same time there is 10-20 % increase in antenna size (Tanaka et al., 2001). Expression of the CAO gene from Arabidopsis in Synechocystis sp. resulted in production of Chl b up to about 10 % of total Chl in this organism and these expressed chl-b pigments efficiently incorporated into the P700-chlorophyll a-protein- complex (Satoh et al., 2001). Duncan et al., 2003 also showed that chl b can bind to CP43 protein. Chl b up to 80 % of total Chl was produced in Synechocystis sp. when higher plant gene coding for LHCP II was incorporated into
its genome together with the CAO gene although LHC II did not accumulate (Xu et al., 2001). These studies indicate that there must be a communication between regulation of Chl b synthesis and synthesis of Chl b- binding proteins. Masuda et al., 2002, 2003 showed that CAO gene responded to the level of irradiance. After a high light to low light shift of the Dunaliella salina culture, both the CAO and lhcb transcripts were rapidly induced. In higher plants light plays a major role in regulating the CAO gene and protein expression. Both transcript and protein level of CAO increased when Arabidopsis plants were transferred from moderate to shade light (Harper et al., 2004). Over expression of CAO in tobacco plants resulted in 20% increase in Chl b in low light where as 72% increase in Chl b under high light. The Chl a/b ratio decreased from 3.38 in WT plants to 2.33 in transgenic plants when grown in high-light (Pattanayak et al., 2005). In Arabidopsis plants also there is decrease in Chl a/b ratio (Tanaka and Tanaka, 2005). The CAO sequence has been classified into 4 parts, the N-terminal sequence predicted to be a transit peptide, the successive conserved sequence unique in land plants (A-domain), a less-conserved sequence (B-domain) and the C-terminal conserved sequence common in chlorophytes and prochlorophytes (C-domain) (Nagata et al., 2004). The C-domain is sufficient for catalytic activity and the N-terminal A domain confers protein instability by sensing the presence of Chl b and regulate the accumulation of CAO protein (Yamasato et al., 2005). Lee et al., 2005 isolated and characterized two isoforms (OsCAO1, OsCAO2) from Rice where OsCAO1 transcript is less in dark and induced by light. OsCAO2 mRNA levels are higher under dark conditions, and its expression is down regulated by exposure to light.

Geranyl-geranyl reductase
This enzyme catalyses the reduction of geranylgeranyl diphosphate to phytyl diphosphate. The cDNA encoding a pre-geranyl-geranyl reductase (mature protein is of 47 kDa) from Arabidopsis thaliana was isolated and characterized. This gene when expressed in E.coli sequentially catalyzed the reduction of geranyl-geranyl-chlorophyll a into phytyl-chlorophyll a as well as the reduction of free geranyl-geranyl diphosphate into phytyl diphosphate, suggesting that this is a multifunctional gene. The transcript level is up-regulated during etioplast to chloroplast and chloroplast to chromoplast development (Keller et al., 1998).

The decrease in CHLP activity affects the chlorophyll and tocopherol contents (Tanaka et al., 1999). Using the transgenic Tobacco plants expressing antisense RNA for geranyl geranyl
reductase (1.51 kb, mature protein of 47kDa) they have shown that chlorophyll content decreases in high light-intensity. They have also shown that CHLP provides phytol for both chlorophyll and tocopherol synthesis and is present at a branchpoint of tocopherol and chlorophyll biosynthesis. The reduced chlorophyll content in chlP antisense plants resulted in the reduction of electron transport chains per leaf area without a concomitant effect on the stoichiometry, composition and activity of both photosystems. However the reduced tochopherol content of the thylakoid membrane is a limiting factor for defensive reactions to photo-oxidative stress (Grasses et al., 2001). PSII was severely photo-inhibited in mature leaves of all chlP antisense tobacco plants. Lipid peroxidation was also more in antisense plants and accumulation of xanthophylls cycle pigments was observed which could be a compensatory mechanism for tochopherol deficiency (Havaux et al., 2003). A bchP gene coding for chlP has been detected from Rhodobacter capsulatus as a part of photosynthetic gene cluster (Mars, 1981; Zsebo and Hearst, 1984). Addlesee et al., 1996 cloned and sequenced chlP (1.224 kb, 44.87 kDa) from Synechocystis sp. PCC 6803 and complement the bchP mutant of R. sphaeroides. Inactivation of the chlP in Synechocystis sp. PCC 6803 resulted in decreased Chl and carotenoid contents. The mutant is also unable to grow photoautotropically due to instability and rapid degradation of photosystems because of photooxidative stress caused by the accumulation of geranylgeranylated chlorophyll a. (Shpilyov et al., 2005)

Chlorophyll synthetase
Chlorophylls are esterified with a long chain C-20 alcohol, phytol. The reaction is catalyzed by enzyme chlorophyll synthetase, where both Chlide a and Chlide b were equally esterified (Rüdiger et al., 1980). The PChlide and Bchlide (bacterial chlorophyllide) are not accepted by the plant enzyme indicating that reduction of the 17, 18 double bond on the ring D was essential but reduction of 7, 8 double bond on ring B was unacceptable (Benz and Rüdiger, 1981b). Helfrich et al., 1994 conclude that compounds which have the 13 (2)-carbomethoxy group at the same side of the macrocycle as the propionic side chain of ring D are neither substrates nor competitive inhibitors. Only compounds having the 13(2)-carbomethoxy group at the opposite site are substrates for the enzyme. Domanskii et al., 2003 and Schmid et al., 2002 showed that esterification kinetics of chlorophyllide is a rapid phase, leading to esterification of 15% of total chlorophyllide within 15-30 sec, followed by a lag-phase of nearly 2 min and a subsequent main phase.
In etioplasts, geranyl geranyl pyrophosphate (GGPP) is used as a substrate (Rudiger et al., 1980), while in chloroplasts; the preferential substrate is phytanyl diphosphate (PhPP) (Soll et al., 1983). Chlorophyll synthetase in chloroplast thylakoid membranes incorporates phytol in presence of ATP and a stromal kinase (Benz and Rudiger, 1981a). The chlorophyll synthetase was found to be present in heat-bleached, ribosome-deficient plastids from rye and oat (Hess et al., 1992), indicating its nuclear origin and synthesis by cytoplasmic ribosomes. The enzyme was not affected by the developmental stage of the plastids. In etiolated wheat, the enzyme was found in latent form in prolamellar bodies (Lindsten et al., 1990). In photosynthetic bacteria Rhodobacter, the essential loci involved in bacteriochlorophyll a biosynthesis are clustered in a 46 kb region of the chromosomes (Bauer et al., 1993). BchG in bacteria (Bollivar et al., 1994b) and G4 (Gaubier et al., 1995) in A. thaliana encode for the enzyme and show a homology of 60-75% in amino acid sequences.

The final product of the reaction is Chl a, which differs from Chl b only by the presence of a methyl group at pyrrole ring II in place of formyl group (Beale and Weinstein, 1990). In C. vulgaris and greening maize respectively (Schneegurt and Beale, 1992; Porra et al., 1993, 1994), the 7-formyl oxygen of Chl b is derived from O₂ by an oxygenase mechanism and these oxygenase reactions are irreversible (Hayaishi O., 1987). Despite the irreversibility the 7-formyl group, Chl b can be reduced to a 7-methyl group leading to synthesis of Chl a (Ito et al., 1996; Ohtsuka et al. 1997).

It was shown that the conversion of Chlide to chlorophyll is a four-step process including three intermediates i.e., Chlide geranylgeraniol, Chlide dihydrogeranylgeraniol and Chlide tetrahydrogeranylgeraniol before the formation of Chlide phytol or chlorophyll (Shoefs and Bertrand, 2000).

**Regulation of chlorophyll biosynthesis pathway**

Chlorophyll, the most abundant tetrapyrrole in plants, responsible for harvesting and trapping light during photosynthesis are ubiquitously distributed in all plant species and perform a multitude of functions throughout development. The genes responsible for the pathway have been identified and the enzymatic steps of the pathways are well characterized. The steady-state levels of transcripts and the encoded enzymes were determined in many organisms with respect to response to environmental stimuli, tissue specific and developmental program and revealed
novel insights into the variation of the expression profiles in this pathway. All enzymes of the Chl biosynthetic pathway are nuclear encoded and so a tight regulation is expected at various levels of gene expression (Smith and Griffiths, 1993; Beale, 1999). Chl synthesis is highly synchronized with the formation of other pigments like heme, carotenoid, quinines etc and pigment-binding proteins to ensure the coordination between the different organelles in plants.

There are three main reasons for higher plants to control the chlorophyll biosynthesis pathway:

1. Since other tetrapyrroles except chlorophyll molecules are present in different parts of plants and essential for plant development, regulation at the branch point particularly at the Mg$^{2+}$ insertion step is essential.

2. Chlorophyll biosynthetic pathway intermediates, the porphyrin molecules which are photodynamic in nature generate singlet oxygen species when they accumulate during skotomorphogenesis to photomorphogenesis. The singlet oxygen endogenously inhibits many metabolic pathways of the plants. The major control is at the production of the initial precursor ALA.

3. Since chloroplast development is a complex process and depends upon a lot of proteins, co-factors along with the chlorophyll pigments, a careful co-ordination between the synthesis of chlorophyll pigments and apoproteins should be occurred. The coupling of pigment synthesis and the assembly should be tightly regulated so that there won’t be any free chlorophyll molecules, which are phototoxic.

The initial part of the pathway i.e. ALA formation is crucial for metabolic flow through the pathway and is generally accepted as the rate limiting in Chl synthesis. In maize, a lag-phase of about 3h for ALA synthesis exists prior to the formation of porphyrins suggesting an inhibition of ALA synthesis by Pchlide (Stobart and Ameen-bukhari, 1984) or heme.

GluTR, one of the enzymes of ALA production from glutamyl-tRNA is the major regulatory point in the pathway. The gene encoding for GluTR, that is; HEMA is regulated by hormones, the circadian clock (Kruse et al., 1997), by light, through the action of phytochrome and cryptochrome (McCormac and Terry, 2002; McCormac et al., 2001), and sugars (Ujwal et al., 2002). Thus multiple external factors can influence the flux through the tetrapyrrole pathway, by the synthesis of the initial precursor.
There is also evidence that heme, whose synthesis may be enhanced in the dark by the accumulation of pathway intermediates behind the block at POR, acts to repress ALA synthesis, since GluTR activity is inhibited by haem (Vothknecht et al., 1996). This is supported by the observation that phytochrome chromophore-deficient mutants, such as hyl and hy2, have reduced protochlorophyllide and ALA synthesis in the dark (Papenbrock et al., 2001). The reduced ability to turnover haem may cause an increase in its levels. Tobacco plants transformed with antisense ferrochelatase RNA exhibited necrotic lesions induced by an accumulation of protoporphyrin IX (Papenbrock et al., 1999), whereas tobacco plants antisense for subunits of magnesium chelatase had a decrease in chlorophyll content in leaves, but did not show necrotic lesions, suggesting that porphyrin accumulation was prevented by haem feedback (Papenbrock et al., 2000a, 2000b).

The Arabidopsis flu mutant accumulates very high levels of Pchlide in the dark, and if the plants are exposed to white light, photobleaching is observed due to the phototoxicity of Pchlide (Goslings et al., 2004; Danon et al., 2005). Subsequently, FLU was shown to interact directly with GluTR in the yeast two-hybrid system (Goslings et al., 2004) and was specific for the GluTR. ulf3, a suppressor of flu gene was isolated which reduced ALA synthesis and Pchlide accumulation. It was found to be allelic to hyl; supporting the model that haem antagonizes the effect of the flu mutation by inhibiting GluTR independently (Goslings et al., 2004).

The branch point for proto IX represents another regulatory step at which the quantitative distribution of intermediate is controlled in the direction of Chl and heme. In plastids, the insertion of Mg^{2+} or Fe^{2+} into the porphyrin ring determines the flux to either Chl or heme. Magnesium chelatase has a higher affinity for proto IX than Fe-chelatase (Guo et al., 1998). It suggested that while both the chelatases contribute to a coordinated allocation of proto IX, the excessive flow of proto IX is towards Chl synthesis in irradiated plants. Ferrochelatase is inhibited by ATP (Cornah et al., 2002), so in the light, when ATP levels are higher, the magnesium branch of the pathway would be favored; conversely, in the dark, magnesium chelation would be reduced. The steady-state level of total heme increases in tobacco plants during the dark period and corresponds to the Fe-chelatase activity (Papenbrock et al., 1999). In Arabidopsis, the magnesium chelatase subunit ChlH reaches a peak at the beginning of the light
phase and ferrochelatase reaches a peak at the end of the light phase, indicating a diurnal rhythm (Harmer et al., 2000).

The most important external modulator of the tetrapyrrole pathway in plants is light. In angiosperms, it plays a direct role in the chlorophyll branch, because POR requires light for activity. Although plants require freshly synthesized Chl molecules throughout their lifetimes to meet the demands of growth and pigment turnover, light exerts a rapid and dramatic negative regulation on POR-mediated light-dependent Pchlide reduction at the level of enzyme activity and protein accumulation (Forreiter et al., 1990). Different POR isoforms express differently in light, porA is present in dark but absent in light, porB is constitutively expressed both in dark and light but porC is absent in dark and light inducible (Armstrong et al., 1995; Oosawa et al., 2000; Su et al., 2001). The light-activated reduction of Pchiide to Chlide and the simultaneous photoconversion of Pr form of phytochrome to the Pfr form trigger plant photomorphogenesis which includes changes in gene expression, formation of chloroplasts, cotyledon expansion, leaf development and inhibition of stem elongation (Chory, 1991).

Light also strongly regulates POR mRNA level during greening of most of the monocot plants (Darrah et al., 1990). POR-A is rapidly degraded in etiolated seedlings upon illumination but if complexed with its product, Chlide, it becomes highly sensitive to proteolysis (Reinbothe et al., 1995a, 1995b). A light-induced plastid protease activity degrades the POR-Chlide complex. This POR-degrading protease, composed of both Asp-type and Cys-type proteinases, is not found in etioplasts but is highly active in chloroplasts (Reinbothe et al., 1995a, 1995b), as some of its constituents are encoded by light-responsive nuclear genes (Reinbothe et al., 1995a).

Regulation of tetrapyrrole biosynthesis in higher plants has been attributed to negative feedback control of steps prior to delta-aminolevulinic acid (ALA) formation. One of the first mutants with a defect in this control had been identified in barley. The tigrina (tig) d mutant accumulates 10-15-fold higher amounts of Pchlide than wild type, when grown in the dark. The identity of the TIGRINA d protein and its mode of action are not known yet. Initially this protein had been proposed to act as a repressor of genes that encode enzymes involved in early steps ofALA formation. The TIGRINA d gene of barley is an ortholog of the FLU gene of Arabidopsis thaliana (Lee et al., 2003). Phytochrome-interacting factor1 (PIF1) negatively regulates Chl biosynthesis in dark and its activity is negatively regulated by lighty (Huq et al., 2004). pifi
mutant seedlings accumulate excess free protochlorophyllide when grown in the dark, with consequent lethal bleaching upon exposure to light.

In *Synechocystis* sp PCC 6803, a Chl binding 'chelator' protein binds to newly synthesized Chl and provides Chl for new photosynthetic reaction centers and antenna molecules (Wu and Vermaas, 1995). The FLP (flu like protein) proteins act as regulators of chlorophyll synthesis in response to light and plastid signals in Chlamydomonas. Reduction of the FLP proteins by RNA interference leads to the accumulation of several porphyrin intermediates and to photobleaching when cells are transferred from the dark to the light (Falciatore *et al.*, 2005).

Nuclear genes control plastid differentiation in response to developmental and environmental signals. Chlorophyll biosynthesis pathway intermediates plays crucial role in plastid development and signaling between chloroplast to nucleus. Susek and Chory, 1992 isolated mutants which do not repress *Lhcb* transcription completely in *Arabidopsis* seedlings, in which chloroplast development is prevented due to photooxidative damage by norflurazon treatment. This group of mutants is referred to as *gun* for genomes uncoupled (Susek *et al.*, 1993; Mochizuki *et al.*, 2001). The *lhb1* expression is not repressed in norflurazon treated GUN5 mutant, as compared to wild type (Mochizuki *et al.*, 2001). Interestingly transgenic *Arabidopsis* lines overexpressing *porA* or *porB* restored the loss of nuclear gene expression due to the norflurazon treatment, indicating that the transgenic lines phenotypically resemble *gun* mutants (McCormac and Terry 2002, 2004).

In return, plastids emit signals that are essential for proper expression of many nuclear encoded photosynthetic proteins. Accumulation of Mg-protoporphyrin IX is a plastid signal (Strand *et al.*, 2003) that represses nuclear transcription through a signaling pathway in *Arabidopsis* that requires the GUN4 gene. GUN4 binds the product and substrate of Mg-chelatase and activates it, thus participating in plastid-to-nucleus signaling by regulating Mg-proto synthesis or trafficking (Larkin *et al.*, 2003). Cyanobacterial gun4 mutant cells exhibit lower Chl contents, accumulate protoporphyrin IX and show less activity not only of Mg-chelatase but also of Fe-chelatase (Wilde *et al.*, 2004).
Oxidative stress and reactive oxygen species

There are many potential sources of ROIs (Reactive oxygen intermediates) in plants. Some are reactions involved in normal metabolism, such as photosynthesis and respiration. These are in line with the traditional concept, considering ROIs as unavoidable byproducts of aerobic metabolism (Asada and Takahashi, 1987). Other sources of ROIs belong to pathways enhanced during abiotic stresses, such as glycolate oxidase in peroxisomes during photorespiration. However, in recent years, new sources of ROIs have been identified in plants, including NADPH oxidases, amine oxidases and cell-wall-bound peroxidases. These are tightly regulated and participate in the production of ROIs during processes such as programmed cell death (PCD) and pathogen defense (Dat et al, 2000; Hammond-Kosack and Jones, 1996; Polle, 2001). Whereas, under normal growth conditions, the production of ROIs in cells is low (240 μM s−1O2 − and a steady-state level of 0.5 μM H2O2 in chloroplasts) (Polle, 2001), many stresses that disrupt the cellular homeostasis of cells enhance the production of ROIs (240–720 μM s−1 O2 − and a steady-state level of 5–15 μM H2O2) (Polle, 2001). These include drought stress and desiccation, salt stress, chilling, heat shock, heavy metals, ultraviolet radiation, air pollutants such as ozone and SO2, mechanical stress, nutrient deprivation, pathogen attack and high light stress (Dat et al, 2000; Bowler et al, 1992; Orozco-Cardenas and Ryan, 1999). The production of ROIs during these stresses results from pathways such as photorespiration, from the photosynthetic apparatus and from mitochondrial respiration. In addition, pathogens and wounding or environmental stresses (e.g. drought or osmotic stress) have been shown to trigger the active production of ROIs by NADPH oxidases (Hammond-Kosack and Jones, 1996; Orozco-Cardenas and Ryan, 1999; Pei et al, 2000). The enhanced production of ROIs during stress can pose a threat to cells but it is also thought that ROIs act as signals for the activation of stress-response and defense pathways (Desikin et al, 2001; Knight and Knight, 2001). Thus, ROIs can be viewed as cellular indicators of stress and as secondary messengers involved in the stress-response signal transduction pathway. Although the steady-state level of ROIs can be used by plants to monitor their intracellular level of stress, this level has to be kept under tight control because over-accumulation of ROIs can result in cell death (Dat et al, 2000; Hammond-Kosack and Jones, 1996; Polle, 2001). ROI-induced cell death can result from oxidative processes such as membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage (the traditional concept). Alternatively, enhanced levels of ROIs can activate a PCD pathway, as
was recently demonstrated by the inhibition of oxidative stress (paraquat)-induced cell death in tobacco by anti-apoptotic genes (Mitsuhara et al, 1999). Because ROIs are toxic but also participate in signaling events, plant cells require at least two different mechanisms to regulate their intracellular ROI concentrations by scavenging of ROIs: one that will enable the fine modulation of low levels of ROIs for signaling purposes, and one that will enable the detoxification of excess ROIs, especially during stress. In addition, the types of ROIs produced and the balance between the steady-state levels of different ROIs can also be important. These are determined by the interplay between different ROI-producing and ROI scavenging mechanisms, and can change drastically depending upon the physiological condition of the plant and the integration of different environmental, developmental and biochemical stimuli.

Chl a Fluorescence and OJIP transient
Chlorophyll a (Chl a) fluorescence induction observed in plants, algae and cyanobacteria, known also as the fluorescence transient or Kautsky effect (Stirbet et al., 1998), has been extensively studied (Govindjee & Papageorgiou, 1971; Fork & Mohanty, 1986; Govindjee, 1995). It consists of light intensity dependent polyphasic changes in Chl a fluorescence emission when a dark-adapted leaf, or a suspension of isolated chloroplasts or intact photosynthetic cells, is illuminated with the continuous light. In the first phase of the transient (time scale from zero to one or several seconds, depending on the light intensity) the fluorescence intensity rises quickly from an initial low value, F0 (The O level), to a higher one Fp (The P level). Under low light fluorescence rises to an intermediary step denoted as Fp1. But under high light, typically over 500 Wm⁻², two intermediary step denoted as Fj (the “J” or I₁, level) and F₁ (the “J” or I₂ level) normally appear (Strasser & Govindjee, 1991, 1992; Neubauer & Schreiber, 1987). The Fp level becomes saturated from 100 Wm⁻² and is then denoted as Fm. A dip between I and P is sometimes present in the fluorescence transient and is denoted as D (Munday & Govindjee, 1969).

The variable fluorescence Fv (t) [defined as F (t)-F₀, where F (t) is the fluorescence intensity at any time t] is related mainly to the fluorescence of antenna chlorophyll of photosystem II, and is due to the variation of the quantum yield of fluorescence emission (between 2 and 10 %) (Latimer et al, 1956). Fluorescence induction measurements are now frequently used in different in vivo studies of plants, such as stress, pollution and productivity (Scheriber & Bilger, 1993). Chl a fluorescence emission in vivo comes from both PSI and PSII
photosystems, the contribution of PS1 being smaller [10-25% of Fo] (Lavergne & Trissl, 1995). PSI does not contribute to the variable fluorescence (Butler, 1978). The first phase of Chl a fluorescence induction is mainly related to the photochemical process and charge separation reactions that take place at the PSII level (Dau, 1994; Govindjee, 1995; Joshi and Mohanty, 1995). The PSII unit catalyses the light-induced electron transport from water to the plastoquinone (PQ) pool molecules (mobile electron carriers between PSI & PSII). The electron transport system at PSII level starts with the excitation trapping by the photochemical reaction centre chlorophyll a (P 680) which induces the primary charge separation: P 680+ Phe– (Greenfield & Wasielewski, 1996) This primary radical pair can decay by several pathways: transfer of the electron from the Phe– to QA (the secondary charge separation), recombination and reformation of singlet excited state of the photochemical reaction centre P 680*, formation of the P 680 triplet state, and other non-radiative pathways.

**Non-photochemical quenching and photochemical quenching.**

Chlorophyll fluorescence quenching is measured with a commercial fluorometer that can measure fluorescence yield in the presence of varying background white light as shown in Fig.III. Over a wide range of light intensities, plants are able to maintain a low steady state fluorescence yield and chl* yield due to a combination of qP and NPQ. Thus qP and NPQ help to minimize production of O2* in the PSII antenna. The quenching due solely to NPQ can be determined periodically by measuring the fluorescence during a brief (<1s) pulse of light that saturates photochemistry so that there is no more quenching due to qP (Muller et al, 2001). Non-photochemical quenching can be divided into at least three components according to their relaxation kinetics in darkness following a period of illumination, as well as their response to different inhibitors (Horton and Hague 1998). The major and most rapid component in most algae and plants is the pH or energy dependent component, qE. A second component, qT, relaxes within minutes and is more important in algae, but rather negligible in most plants during exposure to excess light. This component is due to the phenomenon of state transition, the uncoupling of LHCs from PSII. The third component of NPQ shows the slowest relaxation and is the least defined. It is related to the photoinhibition of photosynthesis and is therefore called qI (Muller et al, 2001).
Components of Thylakoids membranes:
Thylakoids membranes are unique structure present in chloroplast. Sunlight is trapped by a photosystem unit of thylakoids membranes (Fig. 3) consisting of PSI, PSII, Cytb6f complex and converted into chemical energy producing reducing power in the form of NADPH which in turn utilized for CO2 fixation (Baker 2008).

Photosystem II complex

![Diagram of Photosystem II complex]


Fig. 3

Photosystem II contains at least nine different redox components (chlorophyll, pheophytin, plastoquinone, tyrosine, manganese, iron, cytochrome b559, carotenoid and histidine), which have been shown to undergo light induced electron transfer. However, only five of these redox
components are known to be involved in transferring electrons from water to plastoquinone pool: the water oxidizing manganese cluster (Mn)$_4$, the amino acid tyrosine (Yz), the reaction centre chlorophyll (P680), pheophytin and the two plastoquinone molecules, QA and QB. (Whitmarsh and Govindjee, 2002). PS II, a multisubunit complex consisting of 25 different polypeptides, is embedded in the thylakoid membrane of higher plants, algae and cyanobacteria (Hankamer et al., 1997). It uses light energy to catalyze a series of electron transfer reactions resulting in the splitting of water into molecular oxygen and reduction of PQ for which it is also known as water-plastoquinone oxidoreductase. The polypeptide composition of the oxygen evolving PSII complex with their associated functions is shown in table 1. All PSII proteins are believed to be present as one copy per P680 reaction center (RC). The primary photochemical reaction that generates redox potential to oxidize water and reduce PQ occurs in PSII RC. The RC consists of two highly hydrophobic proteins D1 and D2, which are conserved in higher plants species and have a significant degree of homology with the primary sequence of the L and M subunits of purple bacteria (Nanba & Satoh, 1987; Satoh, 1996). The excitation energy derived from the absorption of light by chls is used to convert the primary oxidant, P680$^*$ to P680 (Nanba & Satoh, 1987; Satoh, 1996). P680 is thought to consist of two chlorophyll molecules ligated to D1 and D2 proteins (Durrant et al., 1995). The excited state, P680$^*$, donates a single high-energy unpaired electron to a molecule of pheophytin (Pheo), thereby forming the radical pair, P680$^+$ Pheo$^-$ (Nanba & Satoh, 1987; Satoh, 1996). Each time P680$^+$ is formed, it accepts an electron from a specific amino acid residue (D1-Tyr$^{161}$) and therefore is reduced to P680 (Babcock, 1987; Britt, 1996). As D1-Tyr$^{161}$ donates an electron to P680$^+$, it accepts another electron from water via a four atoms manganese cluster associated with the luminal side of PSII (Joliot et al., 1969; Kok et al., 1970). Pheo, after accepting electrons from P680 passes them onto a PQ molecule (QA), tightly bound to D2 protein (Trebst, 1986; Diner et al., 1991). QA$^-$ passes its electron onto a second PQ molecules associated with the Q$_B$ site of D1 protein. (Trebst, 1986; Dostani et al., 1988; Diner et al., 1991). This electron transfer is aided by the presence of a nonheme iron localized between QA and Q$_B$ (Diner et al., 1991). Each PQ associated with the Q$_B$ site can accept two electrons derived from water and two protons from stroma before being released into the lipid matrix in the form of reduced PQ.

The electron extraction from water required for the reduction of D1-Tyr$^{161}$ is facilitated by involvement of four manganese associated with the luminal surface of PSII. The process of
Water oxidation is known to involve the accumulation of four oxidizing equivalents on manganese leading to four electron oxidation of water (Joliot et al., 1969; Kok et al., 1970). These manganese are stabilized by three extrinsic proteins associated with the PSII. The 33 kDa subunit stabilizes the manganese cluster while the 23 and 17 kDa subunits allow PSII to evolve oxygen under Ca\(^{2+}\) and Cl limiting conditions respectively (Ghanotakis et al., 1984; Miyao et al., 1984a; 1984b).

**Photosystem I complex**

PSI complex consists minimum of 13 different polypeptides in chloroplast of higher plants (Chitnis, 1996). It uses light energy to catalyze the photo oxidation of plastocyanin, a copper protein present in the lumen of thylakoid membrane and the photo reduction of Fd, a [2Fe-2S] protein in chloroplast stroma. The polypeptide composition of PSI complex with their associated functions is shown in table. All proteins are believed to be present as one copy per P700 RC. The co-factors of PSI are bound to the PsaA, PsaB, and PsaC proteins (Chitnis et al., 1995; Chitnis, 1996).

The primary photochemical reaction that generates redox potential to oxidize plastocyanin and reduce Fd occurs in PSI RC. The excitation energy absorbed by the chls is transferred to the primary electron donor P700 that is a dimmer of chl a molecules (Norris et al., 1971). Excitation of P700 leads to the charge separation, which is stabilized by spatial displacement of electrons through a series of redox centers. In forward reaction, an electron from the excited P700 is transferred to the primary acceptor \(A_0\), a chl a monomer (Shuvalov et al., 1986) and then to an intermediate acceptor \(A_1\), which is one of the two phylloquinone molecules found in PSI (Thurnauer & Gast, 1985). The next acceptor of electrons in the sequence is the [4Fe-4S] cluster Fx. The subsequent path of electrons through \(F_A/F_B\) remains an unresolved area in the electron transfer pathway in PSI (Jung et al., 1995; Mannen et al., 1996). The electrons may travel in a series: Fx to \(F_{B/A}\) to \(F_{A/B}\) to Fd. Alternatively, the electrons from Fx may be transferred to \(F_A\) or \(F_B\) and then one or two of these reduced clusters can donate electrons to Fd. The electron lost by P700 is gained by oxidizing plastocyanin.

**Cytochrome b\(_6\)f complex**

The cyt b\(_6\)f complex is the simplest and the best characterized of the multisubunit complex that catalyses the light reaction of photosynthesis in the thylakoid membrane. It mediates the transfer
of electrons from plastoquinone to plastocyanin, and is involved in noncyclic electron flow from PSII to PSI (Wood & Bendall, 1976; Hurt & Hauska, 1981) as well as in cyclic electron flow around PSI (Lam & Malkin, 1982).

The precise manner in which cyt b₆f complex transfer electrons from reduced PQ to plastocyanin is yet to be worked out, but the most popular models are based on the Q-cycle scheme of Mitchel (1975). It is believed to contain two PQ-binding sites C and site Z, which are located near the two surfaces of the membrane. It seems that first electron is passed to the Rieske center, which then reduces cyt f. The second electron can be passed either to the Rieske center or donated to the low potential form of cyt b₆f, since the semiquinone species is a strong reductant. If the later occurs, the electron on cyt b₆LP (Em = -150 mV) is probably passed to the high potential cyt b₆HP (Em = -50 mV) and then then used to reduce a bound plastoquinol to a semiquinone at site C. Further reduction of this semiquinone bound at site C could occur in several different ways, either another plastoquinol molecule is reduced at the Z site, making a second electron available via a reduction of cyt b₆HP, or electron donation may come directly from PSII or indirectly from PSI via reduced Fd. If site C is near the outer surface, then the effect of the cyt b₆-catalysed Q cycle is to pump protons across the membrane and shift the proton/electron stoichiometry from 1 to 2 for plastoquinol oxidation.

**Effect of salt stress on chloroplast biogenesis and photosynthesis**

Chloroplasts are the most sensitive organelles affected by the salt stress (Lapiana & Popov, 1970). Any change in structure and function of the chloroplast will affect the function of the organelle, which in turn affects the ultimate yield of the plants. Genes or proteins associated with photosynthetic pathways were in general not among the most altered by the stress. For example, in Thellungiella (a stress-tolerant plant), photosynthesis genes correspond to 15% of all genes down-regulated (Wong et al., 2006), while in rice alterations in photosynthesis-related enes are mostly associated with stress recovery (Zhou et al., 2007). The effect of salinity and drought on genes associated with photosynthesis (AraCyc Pathways, www.arabidopsis.org/tools/aracyc) was checked in arabidopsis seedlings using the expression profiles available (generated with the Affymetrix ATH1 chip; Kilian et al., 2007). The imposed stresses were evaluated for 24 h. Although no information on the physiological status of the seedlings was given, the imposed stresses may be considered as mild. Of the 139 photosynthesis-related genes (AraCyc Pathways)
the AtGenExpress salt and drought microarray has information for 102 genes. As a general trend, both stresses led to gene down-regulation, most of the changes being small possibly reflecting the mild stress (Stitt et al, 2007).

**Pigment contents:** Various pigments contents are affected differently by the salinity stress depending upon the halophytic or glycophytic nature of the plants and algae. Photosynthetic pigments are reported to change with the plant genotypes, the system used and the time period of the stress imposed (Misra et al., 1995, 1996). Chlorophyll decreased in salt susceptible crops but increased in salt toletrant crops (Misra et al., 1997; Lapiana & Popov, 1970). Ma et al. (1977) have shown that in *P.euphratica* Olive with increasing salt levels chlorophyll a contents and chlorophyll a/b reatio increased, while chlorophyll b and carotenoid content decreased. According to Lu et al. (2002) salt in halophyte *Suaeda salsa* does not affect neoxanthin, leutin, β-carotene, violaxanthin, antheraxanthin, and chlorophyll a and b contents. Tezara et al. (2003) have shown that salt stress has no significant effect on chlorophyll content in *Lycium nodosum* while according to Kovach et al. (1992) chlorophylla/b ratio increased with salinity treatment in *Hydrophila Polysperma* (Roxb.) Anders. (Acanthaceae). Singh & Kshatriya et al. (2002) have shown that graded concentrations of NaCl (20-200 mM) show decrease in the chlorophyll 'a' contents of *Anabaena* with increasing concentration of NaCl except at extremely low concentration of NaCl (5-20 mM).

**Chloroplast structure:** High salinity causes ion imbalance and osmotic stress in plants. Plastids from *Triticum aestivum* cv. Giza 168 had swollen prothylakoids however the prolamellar bodies were regular (Abdelkader, 2007). According to Barhoumi et al., 2007 NaCl affected the ultrastructure of the chloroplast, especially in the mesophyll. The chloroplasts of mesophyll cells from control plants were discoidal and exhibited well compartmentalised grana stacks. Little or no starch was present. Salinity caused the development of an undulating thylakoid system and numerous plastoglobuli in most of the mesophyll chloroplasts. The thylakoids were often greatly distended, leading to the appearance of vesicles in the stroma. In some cases, the disorganisation of thylakoid membranes was accompanied by the disappearance of grana stacking. Occasionally, the chloroplast envelope was disrupted in several places and the stroma very lightly stained. Salinity affects the chloroplast ultrastructure (Boyer, 1976). According to Hernandez et al. (1995) in *Pisum sativum* L. salt treated leaves showed disorganized thylakoid structure of the chloroplast, an increase in the number and size of plastoglobule in sensitive plants and decrease
in starch content in salt tolerant plants. Sairam & Srivastava (2002) have shown that membrane
stability index (MSI) decreased with salt stress but decline was more in wheat genotype HD2687
(susceptible) than in Kharchia 65 (tolerant). NaCl-induced changes in the thylakoid membrane of
wild-type *Anabaena variabilis* and its NaCl(r) mutant strain as indicated by difference in its
absorption and fluorescence spectra at different wavelength (Chauhan et al., 2000).

**Reactive oxygen species and super-oxide detoxifying enzymes:** Salt stress affects enzymatic
activities of chloroplast enzymes. According to Hernandez et al. (1995) in salt treated *Pisum
sativum* L., chloroplast from tolerant plants showed significant increase of Cu Zn-SODII and
ascorbate peroxidase activities as well as in ascorbate content, while those in from sensitive
plants showed increase in H$_2$O$_2$ content and lipid peroxidation. In Lotus japonicas gene
expression of dehydroascorbate reductase, superoxide dismutase and glutathione peroxidase
increased in response to NaCl stress. (Rubio MC et al, 2008)

Superoxide dismutase (SOD) is one of the crucial enzymes that protect cells against the
oxidative damages (Raychqudhuri and Deng, 2000; Fridovich et al, 1986). There are three main
isoforms SODs in eukaryotic cells: Mn-SOD in mitochondria, Cu/Zn-SOD in chloroplast and
cytoplasm, and Fe-SOD in plastid. Unlike Cu/Zn-SOD and Fe-SOD, Mn-SOD, whose activity is
not inhibited by H$_2$O$_2$ and cyanide, exists only in mitochondria (Bowler et al, 1992, 1994), in
which the over-produced ROS could cause mitochondrial diseases and aging (Wei and Lee,
2002). The roles of SODs under environmental stresses have been studied extensively
(Raychqudhuri and Deng, 2000; Yu and Rengel, 1999). Mn-SOD overexpressed in *Arabidopsis*
played a pivotal role in preventing the over accumulation of ROS and protecting the cells against
ROS caused by salt stress, as a result, enhanced salt-tolerance of the transgenic plants
(Wang et al, 2006).

Two cytosolic (OsAPx1 and OsAPx2), two peroxisomal (OsAPx3 and OsAPx4), and
four chloroplastic (OsAPx5, OsAPx6, OsAPx7, and OsAPx8) isoforms identified in the rice
genome. NaCl at 150 mM and 200 mM increased the expression of OsAPx8 and the activities of
APx, but had no effect on the expression of OsAPx1, OsAPx2, OsAPx3, OsAPx4, OsAPx5,
OsAPx6, and OsAPx7 in rice roots. However, NaCl at 300 mM up-regulated OsAPx8
expression, increased APx activity, and down-regulated OsAPx7 expression, but had no effect on
the expression of OsAPx1, OsAPx2, OsAPx3, OsAPx4, OsAPx5, and OsAPx6
(Hong et al, 2007).
Wheat genotype Kharachia 65(tolerant) exhibited less decrease in Ascorbic acid content, less increase in $\text{H}_2\text{O}_2$, thiobarbituric (TBARS) measure of lipid peroxidation and higher increase in superoxide dismutase (SOD) and its isozymes, ascorbate peroxidase (APOX) and glutathione reductase (GR) in all subcellular fraction than salt sensitive HD2687 in response to salinity stress. Chloroplast fraction showed higher total SOD, APOX and GR activity than mitochondrial and and cytosolic fraction. Cu/Zn-SOD and Fe-SOD were observed in all the subcellular fractions; however the activities were higher in chloroplastic fractions for both the isoforms. Susceptibility of HD2687 to long term salinity stress seems to be due to less induction of SOD enzymes, no induction in chloroplastic and mitochondrial APOX and the cytosolic GR and decrease in chloroplastic and GR under salt stress resulting in higher oxidative stress in the form of $\text{H}_2\text{O}_2$ and TBARS contents (Sairam & Srivastava, 2002). Separation of the isoforms of leaf SOD, APX and CAT by polyacrylamide gel electrophoresis followed by in-gel activity staining revealed that the salt-induced activities of APX and CAT were the result of increases in activities of all the isoenzymes in *P. euphratica* (Wang et al., 2008).

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 glutathione 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
over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit.

**Photosynthesis:** Photosynthetic response to drought and salinity stress is highly complex. It involves the interplay of limitations taking place at different sites of the cell/leaf and at different time scales in relation to plant development. The intensity, duration and rate of progression of the stress will influence plant responses to water scarcity and salinity, because these factors will dictate whether mitigation processes associated with acclimation will occur or not. Acclimation responses to salinity include synthesis of compatible solutes as well as adjustments in ion transport (such as uptake, extrusion and sequestration of ions). These responses will eventually lead to restoration of cellular homeostasis, detoxification and therefore survival under stress. (Chaves et al., 2009).

Chloroplast is essential for photosynthetic function. Chloroplast biogenesis and photosynthesis are interrelated. Photosynthesis is highly affected by salinity stress. Saline irrigation in tropical spiny shrub *Lycium nodosum* causes more than 80% reduction in photosynthetic rate, carboxylation efficiency and stomatal conductance (Tizara et al., 2003). According to Kovach et al. (1992) salt stressed plants of *Hygrophila polysperma* grew longer, had more total internodes, mature nodes, leaves and roots but have a slight lower level of photosynthesis than control plants.

Salt stress greatly enhanced phosphoenolpyruvate carboxylase-kinase (PEPCase-k) activity in leaves of sorghum. Salinization also increased the phosphorylation state of PEPCase-k in darkened sorghum leaves. This fact, together with increased malate production during the dark period, suggests a shift towards mixed C4 plants and Crassulacean acid metabolism types of photosynthesis in response to salt stress. (Garcia-Maurino et al., 2002). According to Adams et al. (1992) Common ice plant (*Mesembryanthemum crystallinum*) shows developmentally programmed inducibility for a switch from C3-photosynthesis to CAM (Crassulacean Acid Metabolism). This metabolic switch is enhanced by salinity. CAM induction is dependent on organized leaf tissue and cannot be elicited by salt stress in suspension culture cells. It suggests that cells in culture mimic only the partly the stress response mechanism of intact plants and communication between different tissue is required to mount a complete environmental response. According to Iglesias et al. (2004) salt stress reduced total plant biomass by 27-38%, whereas potassium nitrate supplementation partially counteracted this effect by increasing dry matter and
new leaf area. Salinized *Carrizo citrange* had the greatest response to nitrate supplementation, whereas the effects on salinized *Cleopatra mandarin* and *C. macrophylla* were less apparent. Nitrogen and chlorophyll contents and photosynthetic activity also increased in leaves of the nitrate-supplemented salinized plants. In salinized plants, nitrate supplementation reduced leaf abscission, stimulated photosynthetic activity and increased growth of new leaves.

Effect of salt stress on photosynthesis and salt protection of plant is mediated by various enzymes. 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, dependent on the conditions, polyamine contents 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 100 mM NaCl before transfer to 125 mM NaCl, but not in plants that were directly transferred to 125 mM NaCl without prior treatment with 100 mM 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. Morant-Manceau et al. (2004) have shown that salinity decreased the net photosynthesis and transpiration rates in wheat as compared to control plants, but induced no significant change in chlorophyll a fluorescence parameters.

**Photochemical parameters:** The growth of the plant is reduced by salinity stress although plant species differ in their tolerance to salinity (Munns & Termaat, 1986). The decline in growth observed in many plants subjected to salinity stress is often associated with decrease in their photosynthetic capacity. 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; Mishra et al., 1991; Masojidek & Hall, 1992; 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; Morales et al., 1992; Abdia et al., 1999). Under natural conditions more than one environmental stresses co-occur frequently. Thus plants grown under natural conditions are subjected to the interaction of multiple environmental...
stresses. It has been reported that responses of the plants to several simultaneous stresses are complex and usually not predictable by a single factor analysis and that a combination of different environmental stress factors can result in intensification, overlapping or antagonistic effects (Osmond et al., 1986). The effects of interaction of salinity stress and high light on PSII have shown that salinity stress predisposes PSII photoinhibitory damage (Mishra at al., 1991; Masojidek & Hall, 1992).

High salt concentration lead to stomatal closure, inhibition of PSII and CO2 fixation, increased non-photochemical quenching in A. thaliana. However gas exchange and PSI was unaffected (Stepien and Johnson, 2009). Chl and carotenoids contents decreased in halophyte Cakile maritime at extreme salt concentration (Debez et al, 2008). However anthocyanin content increased with increasing salt concentration. Net photosynthetic rate (A), stomatal conductance, maximum quantum efficiency of PSII and quantum yield increased at lower salt concentration but decreased at higher (≥200 mM) salt concentration and it was accompanied with increase in non-photochemical quenching (Debez et al, 2008). Moderate salinity stress (200 mM) increases plant growth, PSII and carboxylation efficiency in halophyte A. portulacoides (Redondo-Gómez, 2007).

Salinity treatment shows no effects on PSII photochemistry, photochemical quenching (qP) and non-photochemical quenching (NPQ), but increases the resistance of photosystem II to heat stress in halophyte Suaeda salsa. Salt stress (400 mM NaCl) resulted in a significant accumulation of sodium and chloride in leaves of halophyte Suaeda salsa and neither effect on the maximal efficiency PSII (Φ PSII). Also S.salsa showed high resistance not only to salinity stress but also to photoinhibition even when treated with high salinity as high as 400 mM NaCl and exposed to full sunlight (Lu et al., 2002, 2003). According to Delfine et al. (1998) PSII is unaffected by the low level of salt accumulatin in spinach leaves but is likely to be more sensitive than PSI when salt accumulation is high. Chen et al. (2004) have shown that NaCl treatment in Rumex leaves alone had no effect on the maximal photochemistry of PII or the polyphasic rise of chlorophyll fluorescence but prompted heat resistance of the O2-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). According to Allakhvedev et al. (2000) salt stress modified 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). According to Meloni et al. (2003) photochemical efficiency of PSII was not affected in either of the cotton cultivar with salt stress.

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). In *Lycium nodosum* no evidence of chronic photoinhibition due to salinity was observed, since maximum quantum yield of PSII, Fv/Fm did not change due to salinity. Nevertheless, saline irrigation causes decrease in photochemical quenching (qP) coefficients relative to controls (Tizara et al., 2003). In *Spirulina platensis* cells Salinity stress induced a decrease in oxygen evolution activity, which correlated with the decrease in the quantum yield of PSII electron transport (Φ PSII) and induced an increase in non-photochemical quenching (qN) and a decrease in photochemical quenching (qP) (Lu & Vonshak, 2002).