CHLOROPHYLL METABOLISM DURING RICE (ORYZA SATIVA) LEAF SENESCENCE

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CERTIFICATE

The research work embodied in this thesis entitled "Chlorophyll metabolism during Rice (*Oryza sativa*) leaf senescence" has been carried out in the school of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or full for any other degree or diploma of any other university.

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ABBERIVIATIONS

^0C	Degree celsius
ALA	5-aminolevulinic acid
APS	Ammonium persulphate
ATP	Adenosine-5`-triphosphate
BAP	6-Benzylaminopurine
BCIP	5-bromo-4-chloro-3indolyl phosphate
BSA	Bovine Serum Albumine
CAO	Chlorophyllide A oxygenase
CBB	Coomassie brilliant blue
CDTT	Dithiotreitol
CLH	Chlorophyllase
Chl	Chlorophyll
Chlide	Chlorophyllide
CPO	Coproporphyronogen III
D	Dark
EDTA	Ethylenediaminetetraacetic acid
EtBR	Ethidium bromide
FC1	Ferrochelatase 1
FCC	Fluorescent chlorophyll catabolite
Fig	Figure
GluTR	Glutamyl tRNA reductase
GSA	Glutamate-1-semialdehyde
GSAT	Glutamate-1-semialdehyde aminotransferrase
HEAR	Hexane extracted acetone residue
kb	Kilobase
kDa	Kilodalton
L	Liter
LHCP	Light-Harvesting-Chlorophyll-Protein Complex
m^{-2} s^{-1}	meter square per second
MCS	Metal chelating substance
Mg-Proto IX	Mg-protoporphyrin
min	Minute
ml	mililiter
MOPS	3-[N-Morpholino] propane sulphonic acid
MP (E)	Magnesium protoporphyrin IX monomethyl (ester)
MS	Murishage and Skoog
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate

NBT	Nitroblue tetrazolium
NCC	Nonfluorescent chlorophyll catabolite
nm	Nanometer
P_{680}	Reaction center of PSII
P_{700}	Reaction center of PSI
PAGE	Polyacrylamide gel electrophoresis
PaO	Pheide a oxygenase
Pchlide	Protochlorophyllide
POR	Protochlorophyllide oxidoreductase
Proto IX	Protoporphyrin IX
Protox	Protoporphyrinogen oxidase
PS I	Photosystem I
PS II	Photosystem II
RCC	Red chlorophyll catabolite
RCCR	Red chlorophyll catabolite reductase
rpm	revolutions per minute
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TBST	Tris buffer saline with Tween-20
TCA	Trichloroacetic acid
TE	Tris EDTA
Tris	Tris (hydroxymethyl) aminomethane
UroD	Uroprophyrinogen decarboxylase
Urogen	Uroporphyrinogen III
Vol	Volume
β-ME	β –mercaptoethanol
μg	Microgram
μl	Microliter

CONTENTS

INTRODUCTION

Earth has been termed the "green planet" emphasizing the major role of plants in sustaining life. The major role of chlorophyll in the physiology of plants is as one of the central players in harvesting light energy for photosynthesis. In its functional context as part of the protein-pigment photosynthetic complex, the capture of a photon by chlorophyll is the first step in converting light energy into chemical energy and carbon assimilation.

Senescence leads to loss of chlorophyll pigments, desiccation and eventual abscission. Leaf senescence is a major determinant of yield in many crops .Although senescence occurs in an age dependent manner, its initiation and progression can be modulated by a variety of environmental factors such as temp, mineral deficiency as well as by internal factors such as plant growth regulators. Exogenous application of kinetin inhibits degradation of chlorophyll and of proteins of the photosynthetic apparatus. Kinetin controls the degradation of chlorophyll by suppressing the action of chlorophyll catabolizing enzymes.

Chlorophyll metabolism involves both anabolism (biosynthesis) and catabolism (degradation). It is estimated that billions of tonnes of chlorophyll are synthesized every year leading to the surge in chlorophyll levels in spring in temperate climatic zones and equal amount of it is degraded leading to loss of green pigmentation during fruit ripening and autumnal deciduous leaves in temperate zones. The seasonal appearance and disappearance of green pigments are probably only biochemical process that can be observed from outer space.

Although the title of thesis is Chlorophyll metabolism during Rice (*Oryza sativa*) leaf senescence, present investigation deals with Chl biosynthetic reactions during senescence.

The objectives of present investigation are:

1. Estimation of chlorophyll, carotenoid and protein content during dark-induced leaf senescence of excised leaves.
2. Estimation of protochlorophyllide (Pchlide) contents.
3. Determination of the functional status of two early enzymes of Chl biosynthetic pathway i.e. 5-Aminolevulinic acid (ALA) dehydratase and porphobilinogen (PBG) deaminase.
4. Immunoblot analysis of chlorophyll biosynthetic enzymes and photosynthetic proteins.

LITERATURE REVIEW

LEAF SENESCENCE:

Leaf senescence, encompassing a period from maturation to attrition, is a unique developmental process. Unlike other developmental processes, which are composed primarily of cell division, cell differentiation, and /or cell expansion, leaf senescence involves massive programmed cell death (*Gan and Amasino, 1997 Yuehui and Susheng, 2002*). During senescence leaves lose their photosynthetic and other anabolic capacity, and catabolism of chlorophyll, lipids, proteins and nucleic acids predominates. It is the sequential degradation that leads to a massive mobilization and export of nitrogen and minerals and eventually to leaf death (*Buchanan and Wolleston, 1997 Congming etal 2001)*, the released nutrients are mobilized to growing leaves, developing seed, or storage tissues. Therefore, leaf senescence also is a nutrient-mining and recycling process that is distinct from many other developmental programs (*Nooden, 1988: Buchanan –Wollaston, 1997: Quirino etal., 2000)* event in leaf senescence is the disassembly of the photosynthetic apparatus within chloroplasts and thus the concomitant decrease in photosynthetic activity *(Woolhouse, 1984; Grover and Mohanty, 1992).*

The onset of leaf senescence can be regulated by an array of endogenous and external factors. Environmental cues such as drought stress, extreme temperature, nutrient deficiency, and pathogen infection readily trigger leaf senescence. Among the internal factors are reproduction and plant growth regulators such as abscisic acid, ethylene, jasmonic acid, and salicylic acid (Smart, 1994). In the absence of stress factors, leaf senescence is believed to be initiated by age (Hensel etal., 1993; Jiang etal., 1993).

CHLOROPHYLL METABOLISM:

Chlorophyll metabolism includes both Chl anabolism (biosynthesis) and chl catabolism (degradation). It is estimated that billions of tonnes of chlorophyll are synthesized every year and equal amount of it is degraded leading to loss of green pigmentation during fruit

ripening and autumnal coloring in plants.The degradation of chlorophyll takes place in same cellular compartment as its biosynthesis. However, the mutual regulation of Chl biosynthesis and breakdown is still not known. Chl formation is mainly controlled by light and development, Chl catabolism initially occurs at a basal turnover level but is enhanced during senescence and fruit ripening.

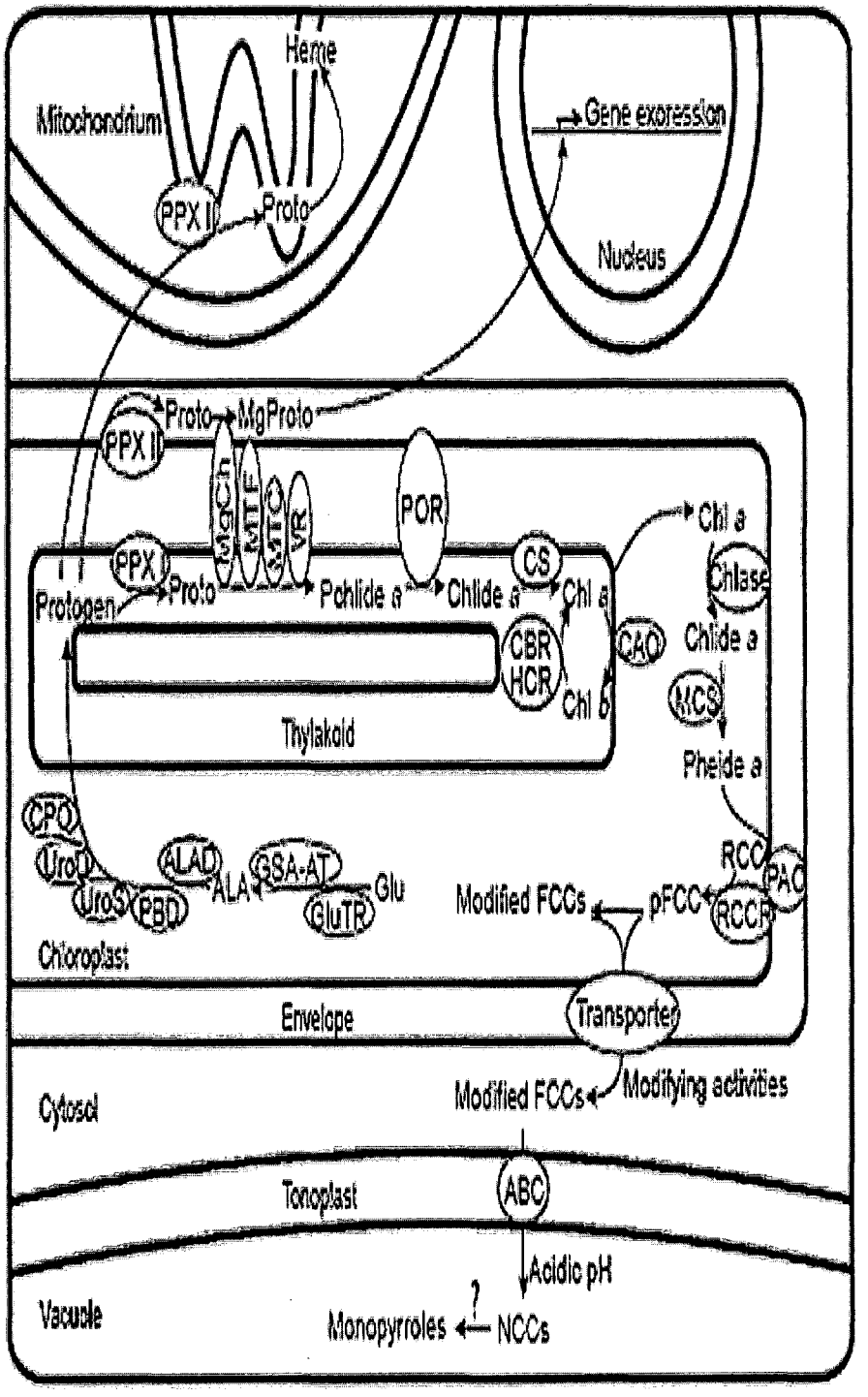


Figure:1 Chlorophyll metabolism pathway in Angiosperms

CHLOROPHYLL BIOSYNTHESIS

Chlorophyll plays a central role in photosynthesis by harvesting light energy and converting it to chemical energy. In higher plants Chl biosynthesis is a very conspicuous process and the metabolic flux is regulated by 17 enzymes, which are already cloned, sequenced and characterized (Figure I. & Table A). 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.

$tRNA^{Glu}$: A cofactor in ALA synthesis

Activation of glutamate by ligation to $tRNA^{Glu}$ is the first step in ALA synthesis. A stable complex is formed between glutamyl-tRNA synthetase (GluRS) and glutamyl-tRNA reductase (GluTR) in presence of ATP, glutamate, tRNA(Glu) and NADPH. So aminoacylated tRNA(Glu) is necessary for complex formation. Once complexed to the two enzymes tRNA(Glu) was found to be partially protected from ribonuclease digestion. The complexes provide the chloroplast with a potential channeling mechanism for Glu-tRNA(Glu) into chlorophyll synthesis in order to compete with the chloroplastic protein synthesis machinery (Jahn, 1992). RNAse A inhibits the ALA forming capacity in vitro in barley plastids (Kannangara *et al.*, 1984; Rieble *et al.*, 1989), whole cells of *Chlamydomonas reinhardtii* (Huang *et al.*, 1984) and in *Chlorella vulgaris* (Weinstein and Beale, 1985), which indicated that $tRNA^{glu}$ is required in ALA formation. Jahn *et al.*,

1992 have shown that aminoacylated $tRNA^{GLU}$ is required as a substrate for enzyme GluRS. Once the GluRS, GluTR and $tRNA^{GLU}$ forms the complex they become partially protected from ribonuclease digestion (Jahn *et al.*, 1992). tRNA(Glu) is a specific cofactor for the NADPH-dependent reduction by GluTR, an enzyme that recognizes the tRNA in a sequence-specific manner. This RNA is the normal tRNA(Glu), a dual-function molecule participating both in protein and in chlorophyll biosynthesis. A chlorophyll-deficient mutant of *E. gracilis* (Y9ZNalL) does not synthesize ALA from glutamate, although it contains GluTR and GSA-2, 1-aminomutase activities. The tRNA(Glu) isolated from the mutant can still be acylated with glutamate in vitro and in vivo. However, it is a poor substrate for GluTR. Sequence analysis of the tRNA and of its gene revealed a C56-->U mutation in the resulting gene product. C56 is therefore an important identity element for GluTR. Thus, a point mutation in the T loop of tRNA uncouples protein from chlorophyll biosynthesis (Stange-Thomann *et al.*, 1994)., The level of $tRNA^{GLU}$ remains unchanged after light treatment of etiolated seedlings (Schon *et al.*, 1992). It has been reported that a tRNA (Glu) uncouples protein and tetrapyrrole biosynthesis (Levican *et al.*, 2005). They have found that *Acidithiobacillus ferrooxidans*, a bacterium that synthesizes a vast amount of heme, contains three genes encoding tRNA (Glu). All tRNA (Glu) species are substrates in vitro of GluRS1 from *A. ferrooxidans*. Glu-tRNA (3)(Glu), that fulfills the requirements for protein synthesis, is not substrate of GluTR. Therefore, aminoacylation of tRNA (3) (Glu) might contribute to ensure protein synthesis upon high heme demand by an uncoupling of protein and heme biosynthesis (Levican *et al.*, 2005).

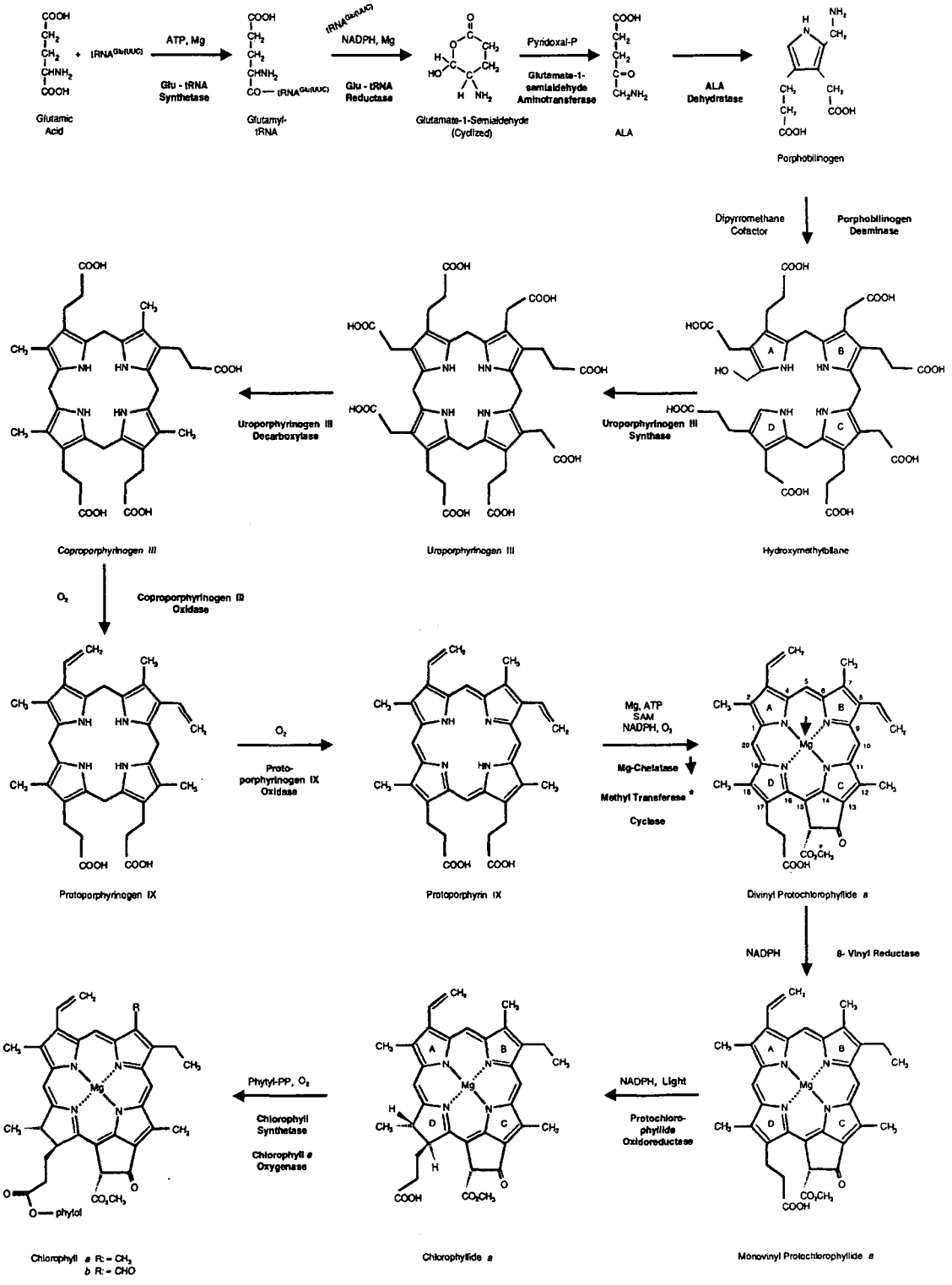


Figure2. Chlorophyll biosynthetic pathway in Angiosperms.

Table-A. List of enzymes involved in tetrapyrrole biosynthesis and their respective gene names and localization in the cell. **M:** Mitochondria, **P:** Plastid

Step	Enzyme name	Localization	Gene name(s)
1	Glutamyl-tRNA synthase	P	*GluRS*
2	Glutamyl-tRNA reductase	P	*HEMA1* *HEMA2* *HEMA3*
3	Glutamate-1-semialdehyde aminotransferase	P	*GSAT1* *GSAT2*
4	Porphobilinogen synthase		*AlaD/HEMB* *HEMB1* *HEMB2*
5	Porphobilinogen deaminase	P	*PbgD/HEMC*
6	Uroporphyrinogen III synthase	P	*UroS/HEMD*
7	Uroporphyrinogen III decarboxylase	P	*UroD/HEME* *HEME1* *HEME2*
8	Coproporphyrinogen oxidase	P	*CPO/HEMF* *HEMF1* *HEMF2*
9	Protoporphyrinogen oxidase	P/M	*PPX/HEMG* *HEMG1* *HEMG2*
10	Mg-chelatase	P	*CHL D* *CHLH* *CHLI 1* *CHLI 2*
11	Mg-protoporphyrin IX methyltransferase	P	*CHLM*
12	Mg-protoporphyrinogen IX monomethyl ester cyclase	P	*CRD1*
13	Divinyl reductase	P	*DVR*
14	NADPH-protochlorophyllide oxidoreductase	P	*PORA* *PORB* *PORC*
15	Geranylgeranyl reductase	P	*Chl P*
16	Chlorophyll synthase	P	*Chl G*
17	Chlorophyllide a oxygenase	P	*CAO*

Glutamate tRNA synthase

Glutamyl-tRNA synthetase (GluRS), the first enzyme of C_5 pathway and also known as aminoacyl-tRNA synthetase that catalyses ligation of glutamate to $tRNA^{GLU}$ (Huang *et al.*, 1984; Kannagara *et al.*, 1984, 1994). Glutamyl-tRNA synthetase (GluRS) belongs to the class I aminoacyl-tRNA synthetases and shows several similarities with glutaminyl-tRNA synthetase concerning structure and catalytic properties. Phylogenetic studies suggested that both diverged from an ancestral glutamyl-tRNA synthetase responsible for the gluta-mylation of tRNA (Glu) and tRNA (Gln), and whose Glu-tRNA (Gln) product is transformed into Gln-tRNA (Gln) by a specific amidotransferase (See Review, Freist *et al.*, 1997). Aminoacyl-tRNA synthetases catalyze the formation of an aminoacyl-AMP from an amino acid and ATP, prior to the aminoacyl transfer to tRNA.

Glutamate tRNA ligase has been purified from *Bacillus subtilis* (Breton *et al.*, 1990), *Chlamydomonas* (Wang *et al.*, 1984), *Chlorella* (Weinstein *et al.*, 1987), *Synechocystis* (Rieble and Beale, 1989), Barley (Bruyant and Kannagara, 1987), Wheat (Ratinaud *et al.* 1983), *Arabidopsis* (Siatecka *et al.*, 1995; Day *et al.*, 1998).

In eukaryotic cells two different kinds of glutamate synthesizing enzymes are present, one inside the chloroplast and other in the cytosol. Both the enzymes are encoded by nuclear DNA and one of the enzymes is imported into the chloroplasts where it ligates glutamate to tRNAs containing glutamate ($tRNA^{GLU}$) as well as glutamine $tRNA^{GLN}$ anticodons (Schon *et al.*, 1988).

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).

The enzyme purified from *Chlamydomonas* (Chen *et al.*, 1990b), barley (Pontoppidan and Kannangara, 1994), *Synechocystis* (Verkamp *et al.*, 1989; Rieble, 1991), *E. coli*

STEPS INVOLVED IN METABOLISM OF ALA TO PROTOIX

Steps involved in metabolism of ALA to protoIX are common to both heme and chlorophyll biosynthesis and in both plants and animals. The enzymes catalyzing these reactions are as follows:

5-Aminolevulinic acid dehydratase

This enzyme is also known as PBG synthase. It is a homooctameric metalloenzyme that catalyzes the condensation of two 5-aminolevulinic acid molecules to form the tetrapyrrole 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). Spinach enzyme is found to be a hexamer with molecular weight of 300 kDa (Liedgens *et al.*, 1980) while radish leaves found to have two isozymes of ALAD (Tchuinmogne *et al.*, 1992). Enzyme from radish cotyledons showed pH optimum 8.0 (Shibata and Ochiai, 1977) and requires Mg^{2+} and Mn^{2+} for its activity while K^{+} was less effective (Shibata and Ochiai, 1977). The enzyme from bacteria requires K^{+} for its activity (van Heningen and Shemin, 1971) while the enzyme from yeast require Zn^{2+} ions for its activity (Wilson *et al.*, 1972). It has shown that, a putative Mg^{2+} binding domain characteristic to plant ALADs is also present in *Chlamydomonas reinhardtii* (Matters and Beale, 1995b). Using an artificially synthesized PBG synthase in pea (*Pisum sativum* L.), Kervinen *et al.*, 2000 showed that this enzyme is most active at slightly alkaline pH and shows a maximal binding of three Mg(II) per subunit. Enzymes from tobacco leaves and radish cotyledons were found to be inhibited by Zn^{2+} and Fe^{2+} (Shetty and Miller, 1969; Shibata and Ochiai, 1977), arsenic inhibits ALAD activity in maize leaf (Jain and Gadre, 2004), $PbCl_2$ and $CdCl_2$ inhibit ALAD in amaranthus (Bhattacharjee and Mukherjee,

2003). The gene, *hemB* has isolated from *Bradyrhizobium japonicum* and *R. sphaeroides* (Chauhan and O'Brian, 1993; Delaunay *et al.*, 1991).

Porphobilinogen deaminase

The enzyme is a soluble chloroplast protein (Castelfracko *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 cosynthatase or non-enzymatically cyclized to uroporphyrinogen I (Frydman and Frydman, 1978 a, 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).

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). In yeast aerobic coprogen oxidase was stimulated in the presence of divalent ions, where as anaerobic enzyme activity had an absolute requirement for a metal ion (Poulson and Polgolasse, 1974). The enzyme from tobacco leaves was found to be activated by Fe^{2+},

Mn^{2+} and inhibited by EDTA and o-phenanthroline, suggesting that enzyme from plant sources requires some metal ions for the activity (Hsu and Miller, 1970). The gene from soybean encodes a polypeptide with a molecular mass of 43 kDa (Madsen *et al.*, 1993). It has shown that tobacco plants containing antisense RNA for coprogen oxidase are more resistant to tobacco mosaic virus (Mock *et al.*, 1999). 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.

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).

Uroporphyrinogen decarboxylase

This enzyme catalyzes stepwise decarboxylation uroporphyrinogen III to yield coproporphyrinogen III. The reaction is substrate concentration dependent (Luo and Lim, 1993) and oxidation products of the substrates inhibited the enzyme (Smith and Francis, 1981). Enzyme has been purified from *Euglena* (Jukant *et al.*, 1989), from Yeast (Felix and Brouillet, 1990) and from *R. sphareoides* (Jones and Jordan, 1993). The molecular weight of the enzymes ranges from 39-57 kDa. Martins *et al.*, 2001a, b reported the first crystal structure of a plant (tobacco) UROD.

Nucleotide sequences encoding *UroD* have been isolated from *Synechococcus* (Kiel *et al.*, 1992), *E. coli* (Nishimura *et al.*, 1993), tobacco and barley (Mock *et al.*, 1995). The

(Chen *et al.*, 1998). The *hemA* gene from barley was expressed in *E. coli* and its activity was shown (Vothknecht *et al.*, 1996). The catalytically essential amino acids are conserved throughout all GluTR (Schubert *et al.*, 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-diaminovalerate. The enzyme then releases amino group from position-4 of this intermediate, releasing δ-ALA. The enzyme is inhibited rapidly by gabaculine (Gough *et al.*, 1992). Tolerant GAB 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 NH_2 terminus but can also be affected by the point mutation alone. (Grimm *et al.*, 1991; Smith & Grimm, 1992).

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* (*gsa1*and *gsa2*) (Ilag *et al.*, 1994), *Glycine max* (Sangwan and O'Brian, 1993), *Nicotiana tabacum* (Hofgen *et al.*, 1994) and *Brasicca napus* (Tsang *et al.*, 2003). The amino acid sequences of these genes show extensive homology among themselves.

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 *GSA* gene was strongly expressed in leaves of etiolated soybean plantlets and to a lesser extent, in leaves of mature plants and is expressed in nodule (Sangwan and O'Brian, 1993) but not in roots (Frustaci *et al.*, 1995). It has shown that the *gsa1*, an isoform of GSAT in soybean is light induced and GAGA elements were found to be involved in the transcriptional control (Frustaci *et al.*, 1995). Two cDNA clones encoding two isomers of *GSAT* were found in tobacco with calculated Mr of 52 kDa and 53 kDa respectively (Hofgen *et al.*, 1994).

UroD was isolated from tobacco and barley and expressed in *E. coli*. The overexpressed protein was of 43 kDa and processed to 39 kDa after imported into the chloroplast.

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 is highly resistant to proteases (trypsin, endoproteinase Glu-C, or carboxypeptidases A, B, and Y), because the protein is folded into an extremely compact form (Arnould & Camadro, 1998Protox 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 (*PPX1 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).

STEPS INVOLVED IN METABOLISM OF PROTOIX TO CHLOROPHYLL:

The Mg-chelation step is branch point in chlorophyll and heme biosynthesis. Mg-chelatase catalyses the insertion of Mg-to proto IX leading to synthesis of chlorophyll, and Ferrochelatase catalyses the insertion of Fe-to proto IX leading to synthesis of heme..

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 BchI 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).

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 $MgCl_2$. It was 89 kDa protein expressed in soluble form and was active in a Mg-chelatse reconstitution assay (Luo *et al.*, 1999).

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 CsFeC1 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).

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 enzyme was partially purified from *Euglena* (Hinchigeri and Richards, 1982), wheat (Ellsworth *et al.*, 1974). The gene *bchM* encoding methyltransferase has been identified in *R. capsulatus*, which has an apparent molecular weight of 22kDa (Bollivar *et al.*, 1994). Co-expression of Mg-chelatase (all subunit) 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).

Block *et al.*, 2002 identified MgPixMT sequence from *Arbidopsis* genome owing to its similarity with the *Synechocystis* PCC 6803 *MgPixMT* gene. The ORF of *Arabidospis* consists of 939bp and encodes a mature protein of 33.8 kDa. The mature protein contains two functional regions. The C-terminal truncated region down stream of the cleaved transit peptide binds to Ado-met and shown to harbor the MgPix MT activity whereas the 40 N-terminal amoniacids are very hydrophobic and enhance the association of the potein with the membrane. In *Arabidopsis* and spinach, this protein has a dual localization in chloroplats membranes as well as thylakoids. Averina *et al.*, 2002 showed that SAM-MgPixMT was located not only in prothylakoids but also in prolamenar bodies of barley containing photoactive Pchlide. *ChlM* from tobacco encodes a 35-kDa protein.

Mg-protoporphyrin IX monomethylester cyclase

This enzyme catalyzes the formation of an isocyclic ring from MPE. Two pathways exist for the formation of the isocyclic ring, i.e., aerobic cyclization pathway found in plants, green algae, cyanobacteria where the ketone oxygen of divinyl protochlorphyllide is derived from molecular oxygen (Walker *et al.*, 1989) and a second anaerobic pathway found in purple photosynthetic bactria where the cyclase reaction is catalysed using a cobalamin cofactor (Gough *et al.*, 2000) and the oxygen atom of the isocyclic ring is derived from water (Porra *et al.*, 1995). The reaction is a three-step reaction (Wong *et al.*, 1985). First step is the steriospecific hydroxylation of the methyl-esterified ring C propionate resulting B-hydroxy-MME, followed by oxidation of the alcohol to the corresponding ketone that results B-keto-MME. The now activated methylene group reacts with the γ-meso carbon of the porphyrin nucleus to form divinyl

protochlorophyllide, which requires NADPH and O_2. The pH optimum of the cyclase activity is approximately 9.0, consistent with a location of the reaction in the chloroplast stroma or on the stromal side of the membrane (Walker *et al.*, 1989). Both pellet and soluble fractions are required for the activity of the enzyme (Walker *et al.*, 1991). In reconstituted system enzyme was found to be inhibited by CN- and N_3- (Whyte and Castelfranco, 1993), enzyme activity in extracts of *C. reinhardtii* and *Synechocystis* sp. PCC 6803 was inhibited by chelators of Fe suggesting that nonheme Fe is involved in the reaction (Bollivar and Beale, 1996). Lipid-soluble metal-complexing agents and Mg-protoporphyrin dimethyl ester also inhibit the enzyme activity. The involvement of *acsF* (encoding a precursor protein of 39 kDa) in mono-methyl-ester (MME) cyclization was demonstrated in *R. gelatinosus*, where disruption of the gene blocked the synthesis of bacteriochlorophyll when cells were grown under respiratory growth conditions with high aeration (Pinta *et al.*, 2002). *Crd1* and *Cth1*, two genes that code for MME hydroxylase in *Chlamydomonas* expressed in a reciprocal manner (Moseley *et al.*, 2002).

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. POR is a member of the 'RED' (Reductases, Epimerases, Dehydrogenases) superfamily of enzymes (Wilks and Timko, 1995) which generally catalyse NADP(H)- or NAD(H)-dependent reactions involving hydride and proton transfers. Sequence alignment of pea POR showed similarity with short-chain alcohol dehydrogenase (Wilk and Timko, 1995). It's a peripheral membrane protein that accumulates to high level in prolamellar bodies (PLBs) in wheat etioplasts, where it forms a ternary complex with Pchlide and NADPH (Oliver and Griffiths, 1982) and is present at low levels in the thylakoid membranes of developing and mature plastids. Upon illumination the protochlorophyllide is photoconverted to chlorophyllide and the level of the enzyme decreases in barley (Santel and Apel, 1981). POR converts Pchlide to Chlide, by adding two hydrogen atoms at C17 and C18 on ring D (Figure II). 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). 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.

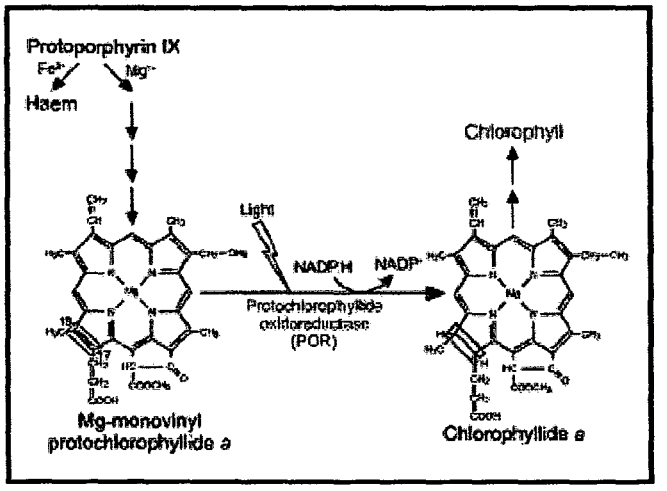


Figure3. The reduction of Pchlide in the biosynthesis of Chlorophyll

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 *Arbidopsis* (Benli *et al.*, 1991; Armstrong *et al.*, 1995; Oosawa *et al.*, 2000), cyanobacterium *Plectonema boryanum* and *Phormidium lamonosum* (Fujita *et al.*, 1998; Rowe and Griffiths, 1995), from Liverwort (Takio *et al.*, 1998), from Chlamydomonas (Li and Timko, 1996), Synecocystis (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.

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 arabidospis 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 was 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, 1996).

POR gene expression is also regulated by phytohormone, particularly by Cytokinins and Abscissic acid. Cytokinins are known to promote greening of etiolated seedlings (Fletcher and McCullagh, 1971). In Cucumber, Abscissic acid inhibits *POR* expression whereas, cytokinins upregulated the level of *POR* transcripts (Kuroda *et al.*, 1996) by transcriptional activation (Kuroda *et al.*, 2001).

Chlorophyllide a oxygenase

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 both present in thylakoid and envelop membrane (Eggink *et al.*, 2004), it could accept electrons from ferredoxin-NADPH oxidoreductase or from ferredoxin itself.

In higher plants light plays a major role in regulating the *CAO* gene and protein expression. 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 charectrised 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 geranyl-geranyl 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 chlorophll 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 stochiometry, composition and activity of both photosystems.

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). 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 (Rüdiger *et al.*, 1980), while in chloroplasts; the preferential substrate is phytyl diphosphate (PhPP) (Soll *et al.*, 1983). Chlorophyll synthetase in chloroplast thylakoid membranes incorporates phytol in presence of ATP and a stromal kinase (Benz and Rüdiger, 1981a). 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 O_2 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. 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. 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.

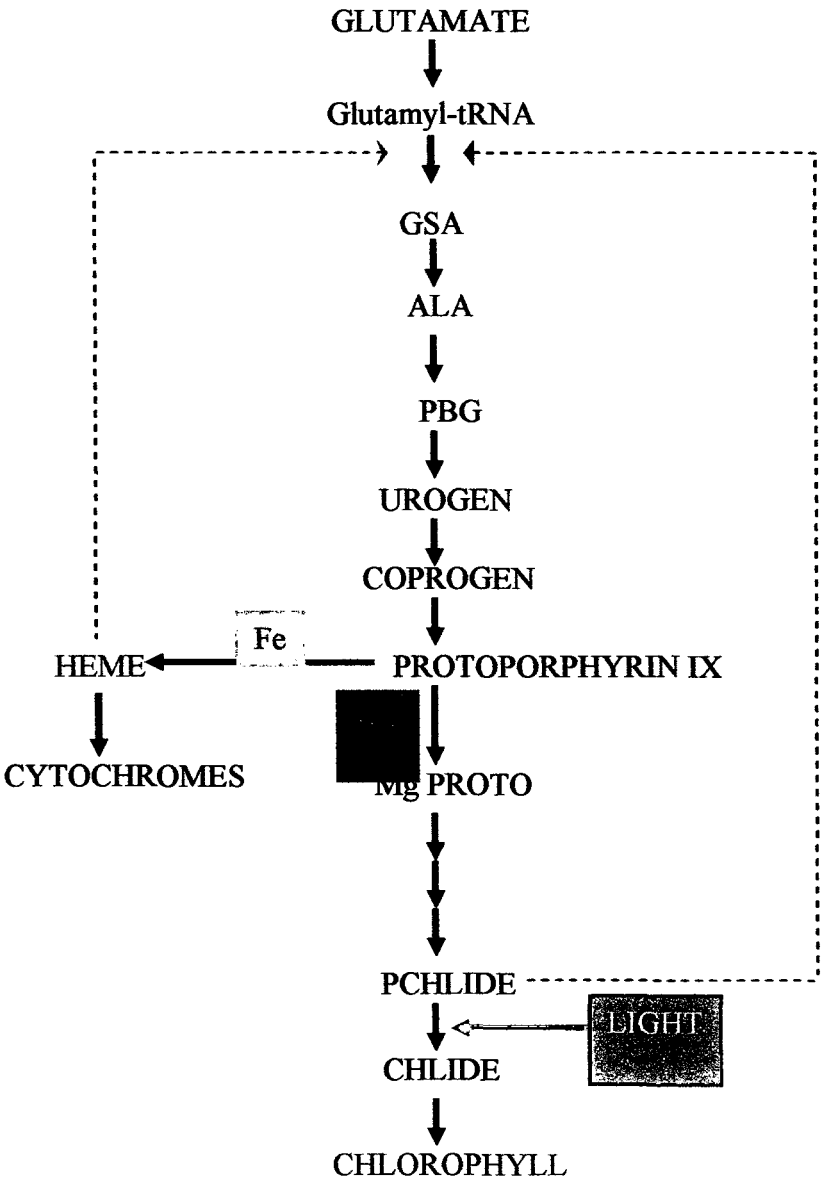


Figure4. Schematic representation of the regulatory loops of chlorophyll biosynthetic pathway. (Wang *et al.*, 1977)

2. 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 (Figure 4).

CHLOROPHYLL CATABOLISM DURING SENESCENCE:

Senescence in leaves is mainly characterized by the decrease in pigment, total protein and RNA, accompanying loss of structure in chloroplast and cytoplasm. The breakdown processes associated with it are indispensable for the internal recycling of nutrient elements such as N, P, K, S and Mg. Chlorophyll metabolism is probably the most visible manifestation of life, the seasonal appearance and disappearance of the green pigments are probably the most visual sign of life on Earth, observable from outer space(Krautler et al 1991 ,1999, 2002). The degradation of chlorophyll has been described as a biological enigma (Hendry et al., 1987). About 1 billion tons of Chl are seasonally synthesized and degraded per year, most of them in oceans (Rudiger, 1997).

The metabolic process responsible for autumnal yellowing of leaf and color changes in ripening fruits was poorly understood until recently .In year 1991 the structure of final product of Chl breakdown was elucidated (Krautler 1991). The catabolism reactions can be divided into early steps that are common to all plants followed by species – specific modifications of Chl breakdown products. The transfer of catabolites from senescent chloroplast (Gerontoplasts) to the vacuole is mediated by ATP – dependent transport system. Four enzymes are necessary to degrade Chl to a colorless, blue-fluorescing intermediate (pFCC). Chl is first dephytylated to chlorophyllide (Chlide) by chlorophyllase (CLH) and, subsequently, a metal chelating substance (MCS) removes Mg. The product, pheophorbide (Pheide) *a*, is then converted to pFCC in a two-step reaction by Pheide *a* oxygenase (PAO) and red Chl catabolite reductase(RCCR). pFCC undergoes several modifications before catabolites are finally stored inside the vacuole in the formof NCCs (figure 5).

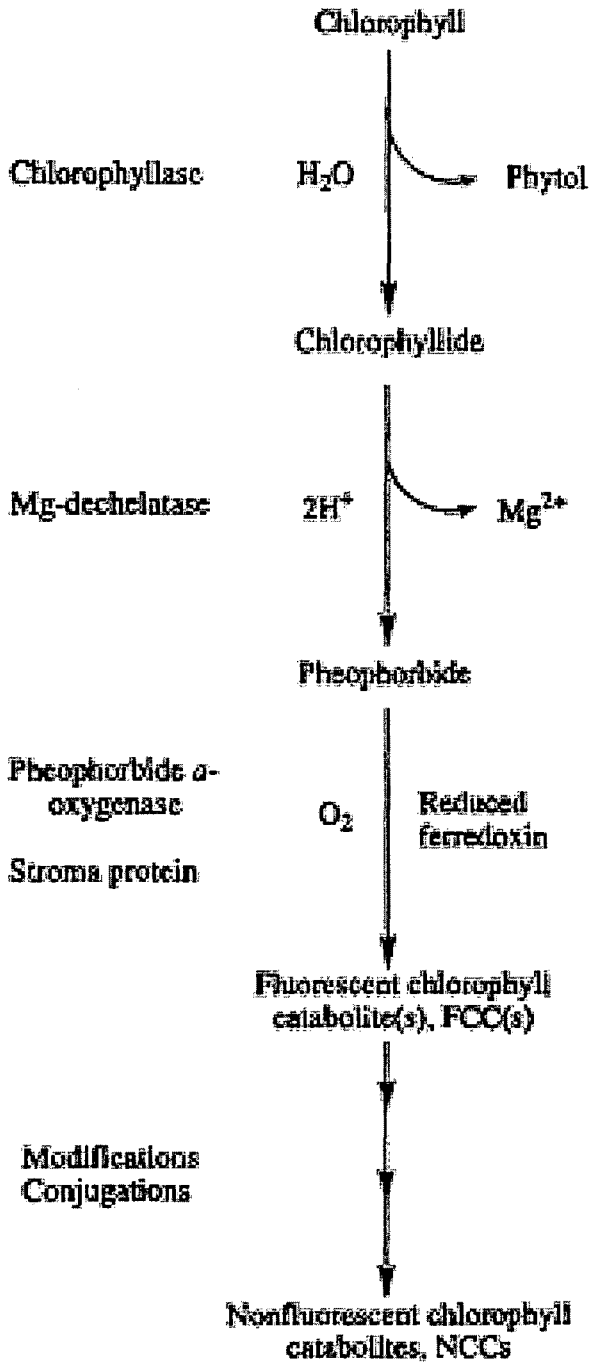


Figure: 5 Chlorophyll breakdown in senescent leaves: putative sequence of enzymic reactions.

ENZYMES INVOLVED IN CHLOROPHYLL CATABOLISM

CHLOROPHYLLASE (CHLOROPHYLL-CHLOROPHYLLIDO HYDROLASE)

CLH catalyzes the hydrolysis of the ester bond of Chl converting it into Chlide and phytol(Gossauer and Engel, 1996; Matile etal., 1996, 1999).This reaction is considered to be the first step during Chl catabolism , because the products of Chl breakdown in different plant species present mainly nonesterified structures(Amir-Shapira etal., 87; Engel etal., 1991; Shio etal., 1991). It acts preferentially on Chl a (Benedetti CE 2002), but also accepts Chl b and pheophytins as substrates (Hortensteiner S. 1999, 2004).

Chlase activity has been correlated to reduced Chl contents in senescing leaves (Jenkins etal., 1987; Kura-Hota etal., 1987; Rodriguez etal., 1987) and to respond to ethylene during fruit ripening (Trebish etal., 1993). However Chlase activity has also been found in presenescent leaves, in greened tissues, and during periods of increased Chl synthesis, suggesting a role in Chl turnover (Tanaka etal., 1982; Matile etal., 1996; Minguez-Mosquera and Gallardo-Guerrero, 1996). Chlase has been purified from a variety of plants and shown to be a glycosylated protein associated to plastid membranes (McFeeters, 1975; Terpstra, 1981; Schellenberg and Matile, 1995; Brandis etal., 1996; Matile etal., 1997; Tsuchiya etal., 1997).Takamiya etal., 2000 ; Tsuchiya etal., 1999 has recently identified the genes . The localization of CLH distinct from the thylakoid membrane is rationalized on the basis of a need for spatial separation of CLH from its substrates; therefore, entry of the substrates into the pathway requires a specific transport of Chl from the thylakoids to the envelope (Matile etal., 1999). Members of the family of water soluble Chl proteins (WSCPs), which can remove Chl from pigment-protein complexes(Satoh H etal., 1998), had been proposed to function in this delivery system, but recently a role for WSCPs as pigment carriers during Chl biosynthesis was suggested instead (Reinbothe C, etal., 2004). Alternatively, plastoglobules could be the hypothesized delivery vehicles. Their size and abundance increase during senescence, and plastoglobules contain a defined set of proteins (Kessler F etal., 1999), among them are Chl *a/b* binding proteins, and significant quantities of Chl (Grimm B. etal 1998). Plastoglobules are often tightly associated with the chloroplast envelope (Grimm B. etal 1998), indicating a role related to envelope function.

CLH has been purified repeatedly and obtained from different sources (Hortensteiner S etal., 1999). Yet N terminal and internal amino acid sequences were obtained and determined only for the enzymes purified from *Citrus sinensis* (CsCLH1)(Trebish T etal., 1993) and *C. album* (CaCLH) (Tsuchiya T, etal., 1997). *CLH* genes have been isolated from *Citrus* (Jakob-Wilk D, etal., 1999), *C. album* (Tsuchiya T, etal., 1999), *Arabidopsis* (*AtCLH1* and*At-CLH2*) (Tsuchiya T, etal., 1999), wheat (*TaCLH1*) (Arkus KAJ, etal 2005), *Gingko biloba,* and *Brassica oleracea* (Tang L, etal., 2004). Both phylogenetic analysis (Tang L, etal., 2004) and biochemical characterization (Tsuchiya T, etal., 1999) predict that CLHs cluster into two groups. Expression of members of one group (AtCLH1 and CsCLH1) is regulated by methyl jasmonate (MeJA) and ethylene, respectively, whereas AtCLH2 and CaCLH genes, in the second group, are constitutively expressed at a low level (Tsuchiya T, etal., 1999, Jakob-Wilk D, etal., 1999). The overall sequence identity between CLH sis rather low (31–42%), but they contain some common characteristics, including a conserved serine lipase motif (Takamiya K, etal., 2000, Tang L, etal., 2004), which was shown by site-directed mutagenesis to be indispensible for in vitro activity (Tsuchiya LA, etal. 1998). A charge-relay mechanism similar to other carboxylesterases was proposed (Arkus KAJ, etal 2005). Although CLHs were indicated to be membrane localized (Brandis A, etal. 1996, Matile P, etal. 1997), the absence of predicted transmembrane domains from cloned CLHs suggests that they are not intrinsic membrane proteins. Furthermore, the various sequences identified indicate different localization, either inside (e.g., AtCLH2) or outside (AtCLH1 and CaCLH) the plastid (Tsuchiya T, etal. 1999). It was hypothesized that an additional extra plastidic pathway for Chl breakdown might exist (Takamiya K, etal. 2000). The finding of a "mass exodus" from the chloroplast of Chl containing globules during senescence supported this idea (Guiamett JJ, etal 1999). These data implied a role for CLH, together with unknown oxidative activities, in Chl degradation in a pathway that is located inside the vacuole (Takamiya K, etal 200).Subcellular localization experiments and analysis of CLH mutants are required to elucidate the in vivo role(s) of CLHs. Notably, alteration of AtCLH1 levels by sense or antisense gene expression changed the Chl-to-Chlide ratio in leaves and flowers, but a senescence related Chl breakdown phenotype was not evident (Benedetti CE, etal 2002,Kariola T,

etal 2005). Furthermore, not all of the predicted CLHs might hydrolyze Chl in vivo: ɛsides Chl, recombinant TaCLH1, but notAtCLH1, also efficiently cleaved the ester ıds of other synthetic hydrophobic esters, such as *p*-nitrophenyl decanoate (Arkus KAJ ı05, Benedetti CE, etal 2002).

g-dechelation.

eports on activities that catalyze a release of Mg2+ from Chlide were conflicting in the ıst (Matile P etal 1999). Two types of activities were distinguished that either were sociated with a heat-stable low-molecular weight compound or were catalyzed by a otein. The low-molecular weight compound was tentatively named MCS (formerly Mg ɛchelating substance) (Shio Y, etal 1996). In contrast, a constitutive Mg-dechelatase ızyme was described, which was associated with chloroplast membranes (Vicentini F, al. 1995). The discrepancy between these two activities was explained by the possibility at MCS could merely represent a cofactor (chelator) of Mg-dechelatase, which is active ı its own. Recent attempts from the Shioi lab (Kunieda T, etal. 2005, Suzuki T, etal. ı05, 2002) readdressed the issue: Mg dechelating proteins only act on the frequently ɛed artificial substrate chlorophyllin, but not on Chlide, whereas MCS removes Mg2+ om both substrates. Properties of the two activities differ significantly, particularly with ɛspect to inhibition by chelators, and MCS could not be extracted from dechelatase ɔntaining fractions. The sizes of MCS compounds are different in *Chenopodium album* <400 Da) and strawberry (2180 + 20 Da), and they differ in their sensitivity to treatment ith proteinase K (Costa ML, etal. 2002, Suzuki T, etal. 2002). We can conclude that lg2+-removal from Chlide is most probably catalyzed by different kinds of low- ıolecular weight compounds whose structure is not known. Elucidation of their ıolecular nature is an important prerequisite to understand the mechanism of MCS ɛducing their origin.

hlorophyll b reduction:

ı the photosystems, Chl *b* is a component of antenna complexes and occurs at variable ıtios to Chl *a*. Despite this, all but one of the NCCs identified so far from vascular plants ɛe derivatives of Chl *a*. An explanation for this is found in the exclusive specificity of AO for Pheide *a*, and consequently conversion to the *a*-form is a prerequisite of Chl *b*

breakdown via PAO. Furthermore, senescence of barley leaves in the presence of D2O leads to the formation of Chl catabolites that are specifically labeled in their 7-methyl group, indicating that they are derived from Chl *b* (Folley P, etal. 1999,). A cycle of reactions that is able to interconvert ` Chl(ide) *a* and *b* was recently described (Rudiger W. etal, 2002). The oxidative half of the cycle acts mainly on dephytylated pigments (Rudiger W. etal, 2002), and is catalyzed by Chlide *a* oxygenase, a Rieske-type iron sulfur oxygenase(Oster U, etal 2000; Tanaka A, etal, 1998). By its action, the C7 methyl group of Chlide *a* is oxidized to formyl by two successive hydroxylations, followed by the spontaneous loss of water (Oster U, etal 2000). In contrast, *b* to *a* conversion occurs on both Chlide or Chl with C7-hydroxy Chl(ide) *a* as a stable intermediate. The two consecutive reductions are catalyzed by different enzymes, an reductase. (Rudiger W, etal 2002). Neither the enzymes nor the genes of these reductases have been molecularly identified so far. Although an alternative role for Chl(ide) *b* reductase in protochlorophyllide *b* to protochlorophyllide *a* reduction during de-etiolation was suggested recently (Reinbothe S, etal 2003) the requirement of the reductive reactions of the Chl(ide) cycle for Chl *b* degradation is substantiated by a marked increase of chl(ide) *b* reductase activity during dark-induced senescence of barley leaves (Ssheumann V, etal, 1999). Chl(ide) *b* reductase is the only enzyme of Chl breakdown that localizes to the thylakoid membrane (**Figure 6**), qualifying it as the first enzyme of Chl *b* degradation .

PHEOPHORBIDE a OXYGENASE:

PAO is a Fe-dependent monooxygenase located at the envelope membrane of gerontoplasts. Electrons required to drive the redox cycle of PAO are supplied from reduced ferredoxin. PAO activity was originally shown to be strictly limited to senescence, although recent investigations using improved assay conditions show that low PAO activity is present before the onset of senescence (Pruzinska A, etal 2003, 2005; Roca M, etal 2004). PAO has an intriguing specificity for Pheide *a,* with Pheide *b* inhibiting in a competitive manner. The recent identification of *At*-NCC-3, which is hydroxylated at C71 (Pruzinska A, 2005) may disprove this specificity.

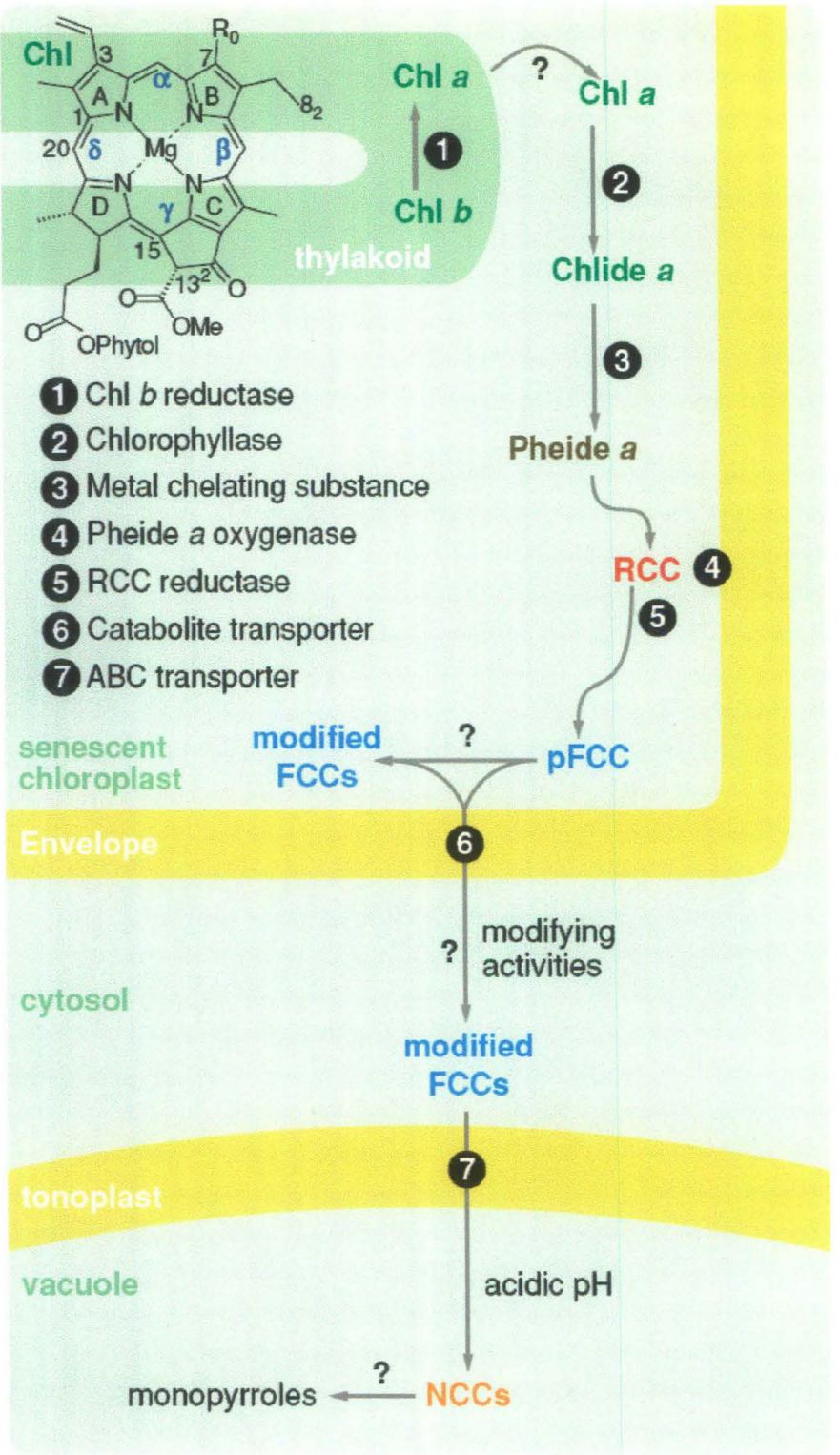


Figure 6: Conversion of Chl b into Chl a and further downstream Chl catabolism.

Although the mechanism of its formation has not been elucidated, *At*-NCC-3 could be assumed to derive from Chl *b* that entered the catabolic pathway through PAO/RCCR before complete reduction to Chl(ide) *a* in the Chl cycle. Consequently, we could hypothesize that PAO is able to accept C7-hydroxy Pheide *a* as substrate. In *Chlorella protothecoides*,

a PAO-like monooxygenase has been identified (Curty C, etal. 1995) that is more unspecific, causing the occurrence of both Chl *a*- and Chl *b*-derived degradation products (Engel N, etal 1996; Iturraspe J , etal 1994).

The biochemical properties of PAO have been investigated intensively, but attempts to purify the protein by means of classical protein purification have largely failed (Horteneister S, etal 1998; Wuthrich K, 1999). Based on the characteristics of PAO, candidate genes of *Arabidopsis* were identified in a functional genomics approach. *Accelerated cell death* (*ACD*) *1* (At3g44880), which after expression in *E. coli* exhibited PAO activity with properties similar to native PAO (PruzinskaA,etal2003).ACD1/AtPAO is the ortholog of lethal leaf spot 1 in maize (Gray J etal 1997; Yang M, etal. 2004). The in vivo role of these proteins as PAO was demonstrated by the analysis of respective mutants. Maize *lls1* and an *Arabidopsis* knockout line in At3g44880, *pao1*, retain Chl during dark induced senescence (Pruzinska A etal, 2003; 2005). Furthermore, they do not accumulate colorless catabolites and are devoid of PAO activity. Both mutants show a light-dependent cell death phenotype in leaves, which is due to the accumulation of Pheide *a* (Pruzinska A etal, 2003; 2005). In *Arabidopsis*, *PAO* is a single copy gene and AtPAO belongs to a small family of Rieske-type iron-sulfur oxygenases (Gray J etal, 2002). Besides PAO, two other Rieske-type oxygenases are involved in Chl metabolism, i.e., Chlide *a* oxygenase (Oster U, etal 2000) and a proposed protochlorophyllide *a* oxygenase (Reinbothe S, etal 2004). Like other Riesketype oxygenases, the plant proteins require ferredoxin as the source of electrons (Tanaka A, etal 1998; Horteniester S, etal 1995). PAO-like proteins are highly conserved in plants and have been found in the genomes of oxygenic, but not anoxygenic, photosynthetic prokaryotes (Gray J 2004)

This indicates that the capacity to degrade Chl to colorless—and hence nonphototoxic—catabolites coevolved with oxygenic photosynthesis.

Red Chlorophyll Catabolite Reductase:

RCCR is a soluble protein of chloroplasts, but in young *Arabidopsis* seedlings is also associated with mitochondria (Mach JM, etal 2001). Reduction of the C20/C1 double bond of RCC is catalyzed by RCCR in an intriguing stereospecific manner: Depending on the source of RCCR, one of two C1 isomers of pFCC (Muhlecker W, etal 2000; 1997) is formed in the PAO/RCCR assay. Thus, with *Arabidopsis* RCCR, for example, pFCC-1 is formed, whereas pFCC-2 occurs with the tomato enzyme (Horteinsteiner S, etal 2000). Screening the RCCR activities of more than 60 plant species indicated that, within a family, all genera and species produce the same isomer (Horteinsteiner S, etal 2000). Identification of genes encoding RCCR allowed a search for domains that define RCCR stereospecificity. For this, chimeric RCCRs composed of portion of the *Arabidopsis* and the tomato proteins were expressed in *E. coli*. It turned out that an exchange in the *Arabidopsis* RCCR of Phe218 to Val was sufficient to change the specificity of the protein from pFCC-1 to pFCC-2 production (I. Anders, S. Aubry, and S. H¨ ortensteiner, unpublished). In planta, the stereospecificity of RCCR defines the C1- configuration of respective NCCs. Thus, *Hv*- NCC-1 and *So*-NCC-2 are C1 isomers (Oberhuber M , etal 2001), at the same time, barley and spinach RCCR produce pFCC-1 and pFCC-2, respectively (Horteinsteiner S, etal 2000). Like PAO, RCCR is ferredoxin dependent, but appears to lack a metal or flavin cofactor, indicating that electrons are directly transferred from ferredoxin to RCC (Krautler B, etal 2003). This was interpreted to ascribe a role to RCCR as a "chaperone" rather than a catalytic reductase (Krautler B etal 2003, Iturraspe J etal 1994). Despite the absence of RCCR in the *Arabidopsis acd2-2* mutant (Mach JM etal 2001), (FCCs and NCCs accumulate during dark-induced senescence in the mutant; some catabolites occur simultaneously in both C1 isomeric forms (A. Pruˇzinsk´a & S.H¨ ortensteiner, unpublished). This indicates loss of stereospecificity in the mutant, and hence the presence of an unknown, nonselective reducing activity. RCC is reduced to pFCC by electrochemical means (Oberhuber M 2001, 2002). Nevertheless, after complementation of *acd2-2* with chimeric RCCR constructs that exhibit different stereospecificity, NCC formation follows the C1 specificity of the respective RCCR (A. Pruˇzinsk´a, I. Anders, and S. H¨ ortensteiner, unpublished). This indicates a true involvement for RCCR. RCCR could be required to mediate an efficient interaction

between RCC (still bound to PAO) and ferredoxin, thereby enabling a fast, regio-, and stereoselective reduction to pFCC.

RCCR has been purified by conventional protein purification methods (Rodoni S 2002; Wuthrich KL 2000), and has been cloned from barley and *Arabidopsis* using amino acid sequence information obtained from the purified barley protein (Wuthrich KL 2000). Functional identity with native RCCR was confirmed after expression of *AtRCCR* in *E. coli*, and a recombinant 35-kDa precursor protein was, after import into isolated chloroplast, cleaved to mature AtRCCR with a size of 31 kDa (Wuthrich KL 2000).

In *Arabidopsis*, the enzyme is encoded by a single gene (At4g37000) and is identical to ACD2 (Mach JM 2001). RCCR is a novel protein, but is distantly related to a family of bilin reductases. These include phytochromobilin synthase, catalyzing the last step in phytochrome chromophore biosynthesis from biliverdin in higher plants, and different bilin reductases of photosynthetic bacteria required for phycobilin biosynthesis (Kohchi T 2001; Frankenberg N 2001). These enzymes are stereospecific, exclusively producing the product Z-isomers. Although the overall sequence identity is rather low, several amino acid residues are highly conserved, indicating a role in protein structure or catalysis (Frankenberg N 2001). Among these is a Phe residue, which in AtRCCR corresponds to the Phe218 that is required for maintaining the stereospecificity of pFCC production. RCCR is widely distributed within higher plants (Wuthrich KL 2000), but *RCCR* genes are not readily identifiable in the genomes of *C. reinhardtii* or of photosynthetic prokaryotes (Frankenberg N 2001). This explains the identification in green algae of RCC-like compounds, which as final products of Chl breakdown are excreted into the medium (Engel N 1996; Hortensteiner S 2000). It is possible that the evolution of *RCCR* genes was a prerequisite for land colonization by terrestrial plants: Disposal had to be developed to render red Chl catabolites into colorless ones that could be accumulated safely inside the vacuole. Mutants in RCCR were originally isolated as *acd2* mutants that exhibit a light-dependent cell death phenotype with spontaneous spreading lesions (Greenberg JT 1994) RCC was suggested to be responsible for the observed phenotype (Mach JM 2001) and, indeed, RCC accumulates during dark-induced senescence in *acd2-2* leaves

CHLOROPHYLL CATABOLITES:

Chl breakdown starts with the removal of the hydrophobic phytol chain, followed by the release of the central Mg atom. These reactions are catalyzed by two early enzymes CLH, and Mg- dechelatase, leading to the formation of chlide and **Pheide a.** In some species, pyro forms of Chl, in particular pyropheophorbide, have been described as Chl breakdown product. In higher plants, pyropheophorbide formation has been attributed to the activity of pheophorbidase which catalyzes the conversion of Pheide a to an intermediate, $C13^2$-carboxyl-pyropheophorbide a, followed by a nonenzymatic decarboxylation(Suzuki etal 2002). But its cytosolic localization questions its action upstream of PaO\RCCR, as an unlikely transport of catabolites out and back into the plastid has to be postulated.

Pheide a is converted into **pFCC** in a two step reaction, in the first step the macro ring is oxygenolytically opened between pyrroles A and B(Figure7) to produce a red colored intermediate, **RCC** catalyzed by PaO and the second part is catalyzed by RCCR, which depends on Fd as a source of electrons to stereospecifically reduce the C20/C1 double bond of RCC. In addition two of the methine bridges are reduced .**RCC** is bound strongly to PaO so has not been observed in degreening plants, only trace amounts found in vitro catabolic reactions. **pFCC** rapidly tautomerizes to the respective **NCC,** the mechanism of tautomerization has been proposed to be a two fold protonation/ deprotonationreaction leading to an intermediary **NCC** in which the methoxycarbonyl function at $C13^2$and pyrrole D are cis **to** each other, with a half life of 2h, this instable **NCC** isomerizes to the final product, thereby establishing a trans arrangement of the bulky groups at $C13^2$and C15.

All **NCCs** from plants exhibit the same basic structure of a 19-formyl-1-oxo-bilane,they differ from each other by modifications of peripheral side chains, which are restricted to the three positions (R1-R3)and which can occur separately or simultaneously in a NCC, complete deconjugation of the four pyrrolic unit is characteristic of the chromophore. Six reactions can be distinguished in conversion of **FCC** to **NCC**; dihydroxylation of the vinyl group of pyrrole A, hydroxylation atC8, followed by glucosylation and/ or malonylation , $C13^2$ demethylation, and finally tautomerization.

Name[a]	R1[b]	R2[b]	R3[b]	CE-chemistry[c]	Identity with
At-NCC-1	vinyl	O-glucosyl	H	1	Bn-NCC-2
At-NCC-2	vinyl	OH	H	1	Bn-NCC-3
At-NCC-3	vinyl	OH[d]	H	1	—
At-NCC-4	vinyl	O-glucosyl[d]	CH_3	1	—
At-NCC-5	vinyl	H	H	1	Bn-NCC-4
Bn-NCC-1	vinyl	O-malonyl	H	1	—
Bn-NCC-2	vinyl	O-glucosyl	H	1	At-NCC-1
Bn-NCC-3	vinyl	OH	H	1	At-NCC-2
Bn-NCC-4	vinyl	H	H	1	At-NCC-5
Cj-NCC-1	vinyl	OH	CH_3	2	So-NCC-4
Cj-NCC-2	vinyl	H	CH_3	2	
Hv-NCC-1	dihydroxyethyl	OH	CH_3	1	
Lo-NCC-1	vinyl	OH	CH_3	nd	
Ls-NCC-1	vinyl	OH	CH_3	nd	
Nr-NCC-1	vinyl	O-glucosyl-malonyl	CH_3	2	
Nr-NCC-2	vinyl	O-glucosyl	CH_3	2	Zm-NCC-2
So-NCC-1	dihydroxyethyl	OH	H	2	
So-NCC-2	dihydroxyethyl	OH	CH_3	2	
So-NCC-3	vinyl	OH	H	2	
So-NCC-4	vinyl	OH	CH_3	2	Cj-NCC-1
So-NCC-5	vinyl	H	CH_3	2	
Zm-NCC-1	dihydroxyethyl	O-glucosyl	CH_3	2	
Zm-NCC-2	vinyl	O-glucosyl	CH_3	2	Nr-NCC-2

At; Arabidopsis thaliana

Bn: Brassica napus

Cj: Cercidiphyllum japonicum

Hv: Hordeum vulgare

Lo: Liquidambar orientalis

Ls: Liquidambar styraciflua

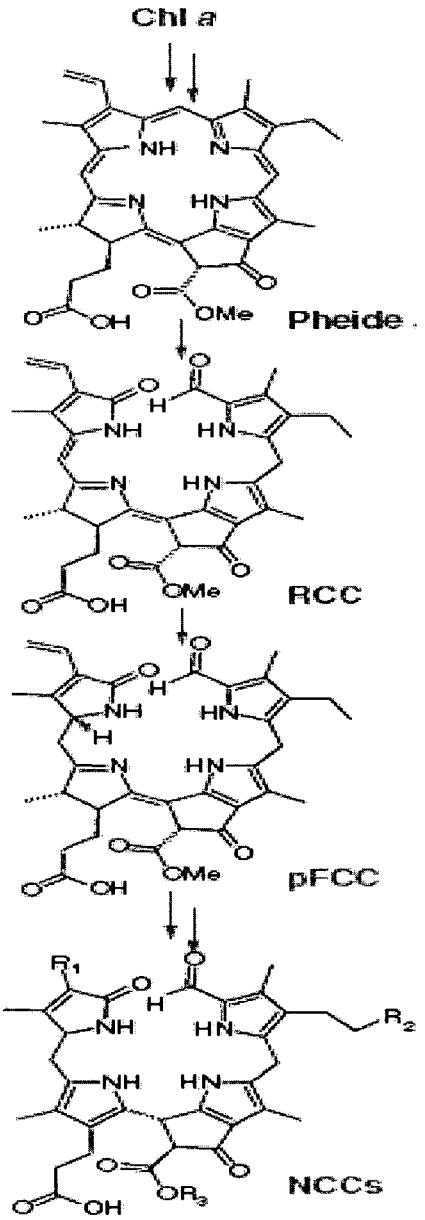
Nr: Nicotiana rustica

So: Spinacia olearacea

Zm: Zea mays

Table B : Structure of NCC in higher plants

.



RCC –RED CHLOROPHYLL CATABOLITE

pFCC –PRIMARY FLUORESCENT CHLOROPHYLL CATABOLITE

NCC-NON FLUORESCENT CHLOROPHYLL CATABOLITE

Figure 7: Structure of Chlorophyll Catabolites

COMPARTMENTATION OF BREAKDOWN IN THE CELL

The spatial organization within senescent mesophyll cells and gerontoplasts is crucial for understanding Chl breakdown. (Figure8). To start with the end of the process, the central vacuole of senescent mesophyll cells has been identified as the dumping ground of Chl catabolites (Matile et al., 1988; Diiggelin et al., 1988b; Bortlik et al., 1990; Hinder et al., 1996). This is not particularly surprising, since structures of NCCs demonstrate that catabolites are modified and conjugated in the same manner as many water-soluble secondary compounds that are also known to be deposited in cell saps. Disposal of catabolites in vacuoles is achieved by a specific ATP-dependent carrier in the tonoplast that has been demonstrated to function in the primary active mode (Hinder et al., 1996).

When isolated, intact gerontoplasts are incubated in the presence of ATP or Glc-6-P, FCCs are produced in organelle (Schellenberg et al., 1990; Ginsburg et al., 1994). The most abundant FCC of barley gerontoplasts, Hv-FCC-2, is released into the medium if ATP is provided at the cytosolic face of the envelope (Matile et al., 1992). Thus, gerontoplasts appear to be equipped with a carrier that is responsible for the export of newly produced catabolites into the cytosol. Chlorophyllase activity is latent. Although the enzyme is associated with chloro-plast membranes apparently close to its substrate, hydrolysis of the endogenous Chl does not take place unless the membranes are solubilized in the presence of an appropriate detergent (Amir-Shapira et al., 1986) or acetone (e.g. Garcia and Galindo, 1991). Recently, an unexpected explanation for latency in the intact organelle has emerged; chlorophyllase appears to be localized in the chloroplast envelope (Matile et al., 1996). This spatial separation of chlorophyllase from the thylakoidal pigment-protein complexes is unchanged when Chl breakdown takes place in developing gerontoplasts. Therefore, breakdown of Chl is likely to require, in addition to the enzymes mentioned so far, an additional tool that mediates between the site of Chl in the thylakoids and the site of catabolic enzymes such as chlorophyllase (Matile et al., 1996) and Pheide a oxygenase (Matile and Schellenberg, 1996) in the envelope. The newly synthesized, senescence-specific protein is required for the establishment of contact between Chl and chlorophyllase. Thus, the oxygenase-deficient stay-green lesion in *F.* pratensis causes progressive accumulation of dephytylated species of Chl during senescence, indicating that chlorophyllase and dechelatase are served with

substrate, and this phenomenon is abolished when cytoplasmic protein synthesis is inhibited in the presence of cycloheximide (Thomas et al., 1989). The protein(s) in question (marked X in Fig. 8) function by luring Chl molecules out of complexes in the thylakoids and carrying them to the catabolic machinery in the envelope. It may well turn out that such a putative Chl carrier provides an answer to the enigma of thylakoid deconstruction in developing gerontoplasts.

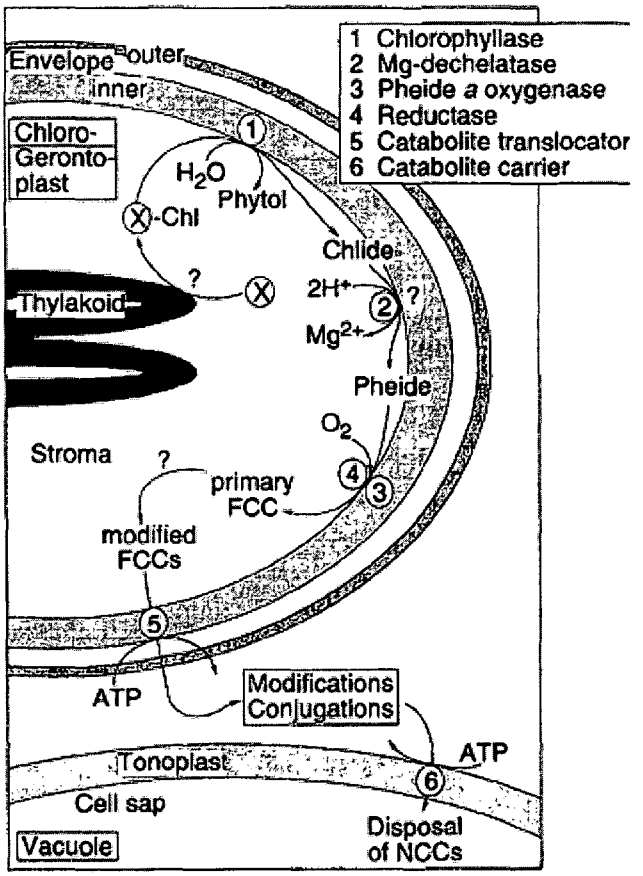


Figure 8: Compartmentalization of Chl catabolism

SIGNIFICANCE OF CHLOROPHYLL BREAKDOWN:

In general it can be stated that Chl is not catabolized beyond the level of linear tetrapyrroles and that these catabolites are stored in the mesophyll cells down to the end of the senescence period. In other words, the four N atoms contained in each molecule of Chl are unlikely to be recycled from senescent leaves to other parts of the plant. They

may contribute to the diet of soil microorganisms when leaves are finally shed. And yet, the question of why plants catabolize Chl at all and in such a complicated and energy-consuming fashion remains to be answered. **A** look at stay-green mutants that are characterized by high retention of Chl in senescent leaves or in ripening fruits (Thomas and Smart, 1993) is enlightening. Chl stability in such plants is associated with the persistence of Chl-binding proteins in the thylakoids (Thomas and Hilditch, 1987; Guiamet et al., 1991; Cheung et al., 1993; Bachmann et al., 1994). Apoproteins seem to be protected from proteolysis as long as they are properly complexed with pigments. Since the proteins of pigment complexes in the thylakoids account for over 30% of the total protein of chloroplasts, their recycling contributes substantially to the overall N budget. When *F.* pratensis plants are cultivated under nutritional conditions that promote internal N recycling, the stay-green line pays a significant penalty compared with the wild type in terms of rates of growth and development, largely because thylakoid protein N is immobile in senescent leaves. It would be expected, therefore, that stay green genotypes might be less competitive under natural conditions of N limitation than are wild types with a more efficient internal N economy. It is no wonder then that stay-greens are found above all in cultivated plant species, particularly in legumes such as soybean, French bean, and pea (Thomas and Smart, 1993). Another important reason why the cellular machinery for breakdown in senescent leaves needs to be so elaborate concerns the photodynamic nature of Chl. Disassembly of pigment-protein complexes is associated with the separation of Chl from the various mechanisms that, in the intact thylakoid, prevent photooxidative damage by activated oxygen. Therefore, it is crucial for the viability of senescent mesophyll cells that dismantling of the photosynthetic membranes remains tightly coupled with the photodynamic inactivation of Chl. In this sense, Chl breakdown may be regarded as a process of detoxification. It is significant that the metabolic sequence that transports Chl catabolites to the vacuole and sequesters them as conjugates is also reminiscent of the fate of xenobiotics and toxic secondary compounds in plant cells

FATE OF CAROTENOIDS DURING SENESCENCE:

Carotenoids, together with chlorophylls, are located in the thylakoid membrane where they are non covalently bound to apoproteins in pigment –protein complexes. The

reaction centre core complexes are enriched in *B*-carotene and the light harvesting complexes contain xanthophylls such as lutein, violaxanthin and neoxanthin. Carotenoids have two major functions (Siefermann-Harms, 1985, 1987). First they act as accessory light harvesting pigments, absorbing light and transferring excitation energy to Chl molecules. In addition, they will photo-protect the pigment-protein complexes by quenching triplet state Chl molecules and singlet oxygen. Singlet oxygen is a particularly reactive species which if quenched, will initiate membrane destruction, leading to the death of plant.

During senescence, Chls are destroyed more rapidly than the carotenoid, resulting in yellowing of tissue. Gut and co workers (1987) reported that of carotenoids, *b*-carotene was the least sensitive to the destructive events associated with senescence. Lutein, the main xanthophylls is generally least affected by the conditions leading to photooxidation.

EFFECT OF KINETIN ON SENESCENCE:

Kinetin controls the degradation of Chl by suppressing the action of Chl catabolizing enzymes (Richmond and Lang, 1957; Mothes 1964; Kulayeva 1962; Osborne, 1962; Srivastava, 1967). Almost nothing is known about the action of kinetin on Chl biosynthetic enzymes.

MATERIALS AND METHODS

PLANT MATERIAL:

Rice (*Oryza sativa CSR10)* seeds were used as experimental materials. Seeds of CSR10 were obtained from Central Soil Salinity Research Institute (C.S.S.R.I.), Karnal

CHEMICALS:

Chemicals like MOPS, Kinetin, and Nitrocellulose membrane were purchased from Amersham chemical company and other chemicals were purchased from Sigma, Qualigens, BDH, S.d.fine etc.

PLANT GROWTH CONDITIONS:

Rice seeds were soaked overnight, and were grown in vermiculite using half strength Murashige and Skoog (MS) liquid media having no agar and vitamins as nutrient solution. Seeds were grown in continuous light for 7 days in plant growth chamber under white fluorescent light of constant intensity of 100 μmols m^{-2} s^{-1}, 28^0C temperature and 75% humidity. Fully expanded first leaves were excised and incubated in Petri dishes containing incubation medium (set A with 2mM MOPS and set B with 2mM MOPS and 200μM kinetin) and placed in continuous dark for 7 days.

Nutrient Sol. used for Growth of Rice Seedlings:

For the growth of rice seedlings Murashige and Skoog medium was prepared.

Major Salt Solution:

g/500ml (20x)

NH_4NO_3:	16.5g
KNO_3:	19g
$CaCl_2.2H_2O$:	4.4g
$MgSO_4.7H_2O$:	3.7g
KH_2PO_4:	1.7g

Minor Salt Solution::

g/500ml (200x)

KI:	0.083g
H_3BO_3:	0.62g
$MnSO_4.4H_2O$:	1.69g
$ZnSO_4.7H_2O$:	0.86g
$CuSO_4.5H_2O$:	0.0025g
$CoCl_2.6H_2O$:	0.0025g
$Na_2MoO_4.2H_2O$:	0.0025g

IRON SOURCE

250ml

$FeSO_4.7H_2O$:	0.695g
$Na_2EDTA.2H_2O$:	0.93g

Dissolve both Ferrous sulphate and Sodium EDTA separetely in minimum quantity of distilled water and than mixed, and final volume made 250ml with distilled water.

For 1 litre of M.S. media 50 ml of major salt solution is mixed with 5ml of minor salt solution and 10 ml of iron source and rest of the volume is made up with double distilled water. Media was without any vitamins and agar.

SPECTROPHOTOMETRY:

Spectrophotometric studies were done on UV-160A (Shimadzu Corporation, Kyoto, Japan) double-beam spectrophotometer.

Chlorophyll and carotenoid estimation:

The extraction of Chlorophylls & carotenoids from tissues and thylakoid membranes was done under a dim, green safe light. Leaf tissues were homogenized in 90% chilled ammoniacal acetone (10 ml) in a pre-chilled mortar and pestle. For preparing 90% ammonical acetone 1 N ammonia solution (7.48 ml in 100 ml distilled water) was prepared and then diluted ten times. This 0.1N ammonia solution was taken and acetone

was added so as to obtain 90% ammonical solution. Three replicates were taken for each batch. Homogenate was centrifuged at 10,000 rpm for 10 min at 4^0C. Supernatant was taken for estimating Chl and carotenoids. Absorbance was taken at 663 nm, 645 nm and 470 nm. Reference cuvette contained 90% ammonical acetone. Chl was calculated as described by Porra *et al.*, 1989 and carotenoids were calculated as described by Welburn and Lichenthaler, 1984.

Chl a = $(14.21 \times OD_{663} - 3.01 \times OD_{645})$ V/W

Chl b = $(25.23 \times OD_{645} - 5.16 \times OD_{663})$ V/W

Chl (a+b) = $(9.05 \times OD_{663} + 22.2 \times OD_{645})$ V/W

Carotenoids = $(1000 \times OD_{470} - \{3.27 \times$ Chl a $- 1.04 \times$ Chl b$\}/5)$ V/227 x W

PROTEIN ESTIMATION:

For estimating protein, leaves were cut into pieces and hand homogenized in prechilled mortar and pestle in 10 ml of ice cold 90% ammonical acetone at 4°C and in green safe light. Homogenate was passed through 8 layers of cheesecloth and centrifuged in sorvall centifuge in SS-34 rotor at 10,000 rpm for 10 min at 4°C. The pellet was taken and dissolved in 0.1N KOH and kept at 4°C for overnight. Standard curve was prepared as described by Lowry et al (1951) using stock solution of BSA (1 mg/ml). Different dilutions ranging from 5µg/ml to 200 µg/ml of BSA were prepared with distilled water. To this 5ml of alkaline copper sulphate was added, vortexed and incubated for 10 min. To this 0.5 ml Folin reagent was added, vortexed and incubated for 20-30 min in dark at room temperature. The absorbance was recorded at 750 nm. To prepare alkaline copper sulphate, stock solutions of 2% sodium potassium tartrate and 1% copper sulphate were prepared. These were then mixed in the ratio of 1:1. To this 2% sodium carbonate in 0.1N NaOH was added in ratio of 1:50.

SPECTROFLUOREMETRY:

The fluorescence spectra were recorded in ratio mode in SLM-8000 (SLM Instruments, Inc.Urbana IL, USA) spectrofluoremeter having a photon counting device. The instrument was interfaced with an IBM microcomputer. Rhodamine B was used in the reference channel as a quantum counter. A tetraphenylbutadiene (TPD) block was used

to adjust the voltage in both samples as well as reference channel to 10,000 counts per second at excitation and emission wavelengths of 348 nm and 422 nm, respectively. The excitation and emission bandwidths were adjusted to 4 nm. The photon count was integrated for 1sec.

HEAR PREPARATION:

For preparing HEAR, acetone extract of sample was taken in a separating funnel and equal volume of ice cold hexane was added to it. This was mixed properly and the two layers were allowed to separate. Lower layer was taken. To this layer 1/3rd volume of hexane was added. Again the two layers were mixed and allowed to separate. Lower layer was taken. This layer was hexane-extracted acetone-residue solvent mixture (HEAR).

δ-AMINO LEVULINIC ACID DEHYDRATASE (ALAD) ASSAY:

ALA is the precursor of chlorophyll biosynthesis in plants. ALAD utilizes ALA and converts two molecules of ALA to one molecule of Porphobilinogen (PBG). ALAD Assay was done as described by Sassa (1982). Three replicates of 250 mg leaves were homogenized at 4°C in 5 ml of 0.1 M tris pH 7.6, 0.01M 2-mercaptoethanol in mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C in sorvall SS-34 rotor. Supernatant was taken for enzyme assay. Reaction mixture contained tris 300 µmol pH 8.5, ALA 12 µmol, 2-mercaptoethanol 15 µmol, KCl 150 µmol. To this 0.5 ml of protein was added. Final volume was made to 3.0 ml with distilled water. Reaction mixture was incubated at 37°C for 2 hrs. 1.0 ml of 20% TCA with 0.1 M mercuric chloride was added to stop the reaction. This was centrifuged at 5000 rpm for 10 min in sorvall SS-34 rotor to settle down precipitated protein. Supernatant was taken. 1.0 ml aliquot was taken and 1.0 ml of Ehlrich reagent was added to it. Absorbance was taken at 555 nm. For calculations absorption coefficient was 6.2 $x10^4M^{-1}$ was used (Sassa, 1982). Reference cuvette contained water and Ehlrich in ratio of 1:1.

PREPARATION OF EHLRICH REAGENT:

Ehlrich reagent was prepared as described by Mauzerall and Granick (1956). For preparing Ehlrich reagent 2 g of dimethyl amino benzaldehyde (DMAB) was dissolved in 30 ml of glacial acetic acid and 16 ml of 70% perchloric acid (4 N) was added. Final volume was made to 50.0 ml with glacial acetic acid.

PORPHOBILINOGEN DEAMINASE (PBGD):

Leaves (250 mg) were taken and Enzyme was assayed as described by Williams et al., (1981). Leaves were hand homogenized in mortar and pestle in 5.0 ml of phosphate buffer (pH 8.0) and 0.6 mM EDTA at 4°C and passed through 4 layers of cheesecloth. Homogenate was centrifuged at 10,000 rpm for 10 min in sorvall SS-34 rotor at 4°C. Supernatant was taken for assay. Enzyme activity was assayed as amount of porphyrin synthesized in 3.0 ml of reaction mixture having following composition as final concentrations: -

Freshly prepared 0.6 ml PBG (550 µM), 90µL EDTA solution (0.6mM), 428µL phosphate buffer (pH8.0), 1.8ml of enzyme extract and 82µL distilled water. In blank distilled water was added instead of enzyme. The reaction mixture was incubated at 37°C for 1 h. 750 µl of this reaction mixture was taken to which 1.7 ml of TCA (7.1%) was added to stop the reaction. Centrifuge at 10,000 rpm for 10 minutes in sorvall SS-34 at 4°C. Then 10µl of iodine solution (1%) was added and incubated for 5 min at 37°C. Finally 20µl of freshly prepared Sodium thiosulphate (2 %) was added to reduce iodine. Absorbance was measured at406 nm. Extinction coefficient used at 406 nm was 5.48 $x10^5$ M^{-1} cm^{-1}. Absorbance of all samples was taken from 400-700 nm to check for a peak at 405.5 or 406 nm.

ISOLATION OF BROKEN PLASTIDS AND PLASTIDIC MEMBRANES:

Isolation was done as described by Tripathy and Mohanty (1980). Around 5 g of leaves were homogenized in 40 ml of isolation buffer containing sorbitol 0.4 M, hepes 0.05 M pH 7.3, $MgCl_2$ 1mM, ethylene diamine tetraacetic acid (EDTA) 1mM at 4°C and in green safe light. Homogenate was passed through 8 layers of cheesecloth and 1 layer of miracloth and centrifuged in sorvall centrifuge in SS-34 rotor at 4000 rpm for 3 minutes

at 4°C. Pellet was obtained. Pellet containing broken plastids was suspended in suspension buffer containing sorbitol 0.4 M, tris 0.05 M pH 7.5, $MgCl_2$ 1 mM and EDTA 1 mM. For isolating plastidic membranes pellet was taken and suspended in 1 ml of TE buffer containing Tris 0.01M pH 7.5 and EDTA 1 mM and kept on ice for 10 min and centrifuged at 8000 rpm for 5 min. Pellet containing plastid membrane was suspended in suspension buffer containing sorbitol 0.4 M, tris 0.05 M pH 7.5, $MgCl_2$ 1 mM and EDTA 1 mM.

PREPARATION OF SAMPLES FOR RUNNING SDS-PAGE AND FOR WESTERN BLOTTING:

Broken plastids or plastid membranes were isolated as described. Chlorophyll and protein contents were measured and calculated. Samples were loaded on equal protein basis. To the samples suspended in suspension buffer and 4X treatment buffer. Bromophenol blue was added to the treatment buffer. Samples were then incubated at 45°C for 1 h, centrifuged in Beckmann microfuge at maximum speed for 2 min and then loaded on the gel.

Polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli, 1970. The chemicals and solutions used for SDS-PAGE:

Acrylamide (30%):	Acrylamide	58.4g
	Bis-acrylamide	1.6g
	Distilled water added to make vol. 200ml	
	This stock solution was filtered and stored at 4^0C in a amber bottle.	
Separating gel buffer (4X):	Tris (1.5M)	36.3g
	Final vol. made to 200ml with distilled water after adjusting pH to 8.8 with HCl. Stored at 4^0C.	

Stacking gel buffer (4X):	Tris (0.5M)	6.0g
	Final vol. made to 100ml with distilled water after adjusting pH to 6.8 with HCl. Stored at 4^0C.	
SDS (10%):	SDS	10g
	Vol. made to 100ml with distilled water and stored at room temperature.	
Sample buffer (2X):	Tris Cl (0.125M, pH 6.8)	2.5ml of stacking gel buffer
	SDS (4%)	4ml of 10% SDS
	β-mercaptoethanol (10%)	1ml
	Glycerol (20%)	2ml
	Distilled water added to make up the vol. to 10ml.	
Tank buffer (4X):	Tris (0.025M)	15g
	Glycine (0.192M)	72g
SDS (0.1%)		1g
	Distilled water added to make up the vol. to 1L.	
	APS 10%:	Always prepared fresh.

Recipes	**12.5% Separating gel**	**5% Stacking gel**
Monomer stock solution	10.0 ml	1.7 ml
Buffer	6.0 ml (pH 8.8)	2.5 ml (pH 6.8)
SDS (10%)	0.25 ml	1.0 ml
Water	8.3 ml	4.7 ml
TEMED	15 µl	5 µl
APS (10%)	400 µl	150 µl
Total volume	**25 ml**	**10 ml**

SDS-PAGE was carried out in a vertical gel electrophoresis apparatus (regular size) (ATTO Corp., Japan) according to Laemmli, 1970. Gels were prepared and electrophoresed under reducing and denaturing conditions in presence of β-ME and SDS. Protein samples (50 μg) were prepared by mixing with ½ volumes of 2X sample buffer (constituents of sample buffer is mentioned above). The samples were boiled for 3 min in a water bath and centrifuged at 13000 rpm for 2 min at room temperature. The supernatant was loaded on the stacking gel. Gels were run either at a constant voltage (100V) or a constant current (20 mA). After electrophoresis, the gels were stained with Coomassie blue R 250 (CBB R 250) for visualization of the proteins.

Staining with coomassie brilliant blue R 250 (CBB R 250):

Fixing solution:	40% methanol, 10% acetic acid in double distilled water
Staining solution:	CBB (0.05%) in 50% methanol and 5% acetic acid CBB was dissolved in methanol. Acetic acid was added, followed by Distilled water to make the volume 1L.
Destaining solution:	5% methanol, 7.5% acetic acid in double distilled water

The gel, after electrophoresis, was incubated in 10 volumes of fixing solution for 20 min on a shaker. The gel was stained in 5 vol of CBB 2 h on the shaker. After staining, stain was removed; gel was rinsed with double distilled water and was then left in 20 vol destaining solution for 2-3 h. The destaining solution was replaced 2-3 times at 1h interval, until the background was clear. The gel was preserved in 7% acetic acid in distilled water.

Western blot:

Western blot analysis was done according to_Towbin *et al.*, 1979. The chemicals and solutions used for western analysis:

TBS (Tris buffered saline):	Tris (25mM)	3.03g (pH 7.4)
	NaCl (136mM)	7.95g

	KCl	0.2g
	Distilled water added to make up the vol. to 1L.	
TBST buffer:	TBS + 0.05% Tween 20	
Blocking solution:	TBST + 4% BSA	
Transfer buffer:	Tris (0.1M)	6.05g
	Glycine (0.192M)	7.2g
	5% Methanol (v/v)	25ml
	Distilled water added to make up the vol to 500ml.	
Alkaline phosphatase (AP) buffer:	Tris HCl (100mM, pH 9.5)	1.211g
	NaCl (100mM)	0.5844g
	$MgCl_2$ (5mM)	0.10165g
	Distilled water added to make up the vol. to 100ml.	
AP colour development solution:	AP buffer	10ml
	NBT sol	66µl
	BCIP	33µl
Ponceau S:	0.1% (w/v) in 1% acetic acid (v/v)	

Transfer of proteins from polyacrylamide gels to nitrocellulose (NC) membranes was carried out in a semi-dry Transblot apparatus (ATTO Corp., Japan), as per the manufacturer's instructions. Protein (20 µg) loaded on SDS-PAGE was run in a ATTO gel electrophoresis apparatus as described above. After the run, gel was first equilibrated in transfer buffer for 15 min. NC membrane and Whatman papers (3mm) were also soaked in the transfer buffer. For transfer, 4-6 pieces of 3mm Whatman paper were placed on the platform of the apparatus, on the top of which membrane was placed followed by gel and 4-6 layers of Whatman paper. Air bubbles trapped were removed. Constant current equal to twice the area of gel was applied (e.g. if the gel area was 50 sq.

cm, 100mA current was applied) during transfer. Handling of membrane was done wearing gloves. After the transfer was over, gel-facing side of the membrane was marked and the membrane was stained in Ponceau S (0.1% Ponceau S in 1% acetic acid). Markers were marked with a ballpoint pen and the membrane was destained in water. Membrane was then kept in blocking solution containing 4% BSA in TBST, at room temperature for 2 h. After this the membrane was washed in TBST thrice (5 min each) with constant shaking. The membrane was incubated for 1 h at room temperature with primary antibody at the appropriate dilution. Dilution was made in TBST containing 0.1% BSA. After the incubation, membrane was washed in TBST thrice (5 min each) with constant shaking and then incubated with alkaline phosphatase-conjugated secondary antibody (1:7500 dilutions) for 1 h at room temperature. Membrane was again washed in TBST thrice (5 min each) with constant shaking. The NC membrane was then stained using substrate for alkaline phosphatase. 16µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 33µl of nitroblue tetrazolium (NBT) were added to 5 ml of AP buffer. Stock solution of BCIP was prepared by dissolving 0.5 g BCIP disodium salt in 10 ml of 100% dimethyl formamide and stored at -20°C and of NBT was prepared by dissolving 0.5 g in 10 ml of 70% dimethylformamide and stored at -20°C. The blot was developed till purple-blue bands appeared at the site of antibody binding (Towbin *et al.*, 1979).

Table: Antibodies used in this study

Antibodies	Abbreviation	Dilution
Glutamate 1-semialdehyde Aminotransferase (46 kDa)	GSAT	1:1000
Uroporphyrinogen Decarboxylase (39 kDa)	UROD	1:500
Coproporphyrinogen oxidase (39 kDa)	CPO	1:500
Mg-chelatase Subunit I (42 kDa)	CHLI	1:1000
Protochlorophyllide oxidoreductase B (36 kDa)	POR B	1:200
Geranyl-geranyl reductase (37 kDa)	CHLP	1:1000
PSII Reaction Center subunit (32 kDa)	D1	1:3500
PSII Reaction Center subunit (34 kDa)	D2	1:3000
Reiske (20 kDa)	Pet C	1:2000
PSI Subunit III (16 kDa)	PS I F	1:1000
PSI Subunit V (17 kDa)	PS I L	1:1000
PS1 Subunit 1V (17 kDa)	PS I E	1:2500
OEC (33 kDa)	OEC	1:1000
Cyt f (34 kDa)	Pet A	1:2000

RESULTS

The Chlorophyll contents

Rice seedlings (CSR10) were grown under continuous light (100 μmole photons m^{-2} s^{-1}) for 7 days in Conviron plant growth chamber as described in materials and methods. The leaves were excised above leaf sheath, weighed, divided into two sets and incubated in dark for 6 days to induce senescence. One set was incubated in the absence of kinetin i.e., in 2 mM MOPS solution (pH 4.0) and the other one in the presence of kinetin i.e., 2 mM MOPS + 200 µM kinetin (pH 4.0) solutions. Total chlorophyll contents of the incubated leaves were measured after different days of dark incubation. As shown in figure 1, the Chl contents continuously decreased upto 6^{th} day in both MOPS and kinetin incubated samples. However the decrease was severe in the absence of kinetin (control) as compared to kinetin samples. The reduction in Chl contents was most prominent on 4^{th} day i.e., 82% in MOPS incubated samples whereas 51% in kinetin incubated samples.

Carotenoid contents

During dark induced senescence carotenoid contents of both MOPS and MOPS + kinetin incubated samples also decreased continuously (Figure 2). However the decrease was not as prominent as observed in case of Chl amounts. After 6 days of dark incubation the decline in the carotenoid content was 69.3% in control whereas the same 38.6% in kinetin samples.

Chlorophyll a/b ratio

As compared to control the Chl a/b ratio increased during dark induced senescence in both MOPS and kinetin samples (Figure 3). On 4^{th} day the Chl a/b ratio was 3.95 in MOPS incubated samples whereas in Kinetin treated samples it was 3.11. This demonstrates more loss of Chl b as compared to that of Chl a.

Protein contents

There was substantial decrease in protein contents in both dark incubated MOPS and MOPS+ kinetin samples (Figure 4). However decline in protein content was much higher

in MOPS treated samples. Senescence accounted for 75% decrease in MOPS samples and 57% in MOPS + kinetin samples on 4th day.

Protochlorophyllide contents

The control sample was taken from the excised rice leaves in light where most of the phototransformable Pchlide were converted to Chlide. Upon dark treatment the Pchlide level of rice leaves increased upto the 4th day and then declined over the next 2 days (Figure 5). Since the leaves were incubated in dark, the decline in Pchlide content could not be due to its phototransformation to Chlide but must be caused by its net degradation. Kinetin treatment enhanced the Pchlide content of leaves to 18% over MOPS on 4th day of dark treatment.

Enzyme activities

ALA Dehydratase (ALAD)

This enzyme converts ALA to porphobilinogen. As shown in Figure 6, the enzymatic activity of ALA dehydratase to synthesize PBG from exogenously added ALA declined in both MOPS and kinetin incubated excised leaves. After 1 d of dark incubation the activity showed 18% decline in MOPS incubated samples and 8% in kinetin incubated samples. It was further declined to 54% in MOPS and 40% in kinetin on 3rd day. Finally on 5th day it was reduced to 80% in MOPS and 60% in kinetin as compared to the original activity.

Porphobilinogen deaminase (PBGD)

The PBG deaminase in concert with uroporphyrinogen (Urogen) co-synthase converts four PBG molecules to one urogen III molecule. Its enzymatic activity to synthesize uroporphyrin from the exogenously added PBG declined comparably to that of ALAD. In kinetin treated leaves the enzymatic activity was higher than that of MOPS (Figure 7). After 1 d of dark incubation its activity declined by 33% in control and 24% in kinetin incubated samples. Its activity further declined to 50% in MOPS and 34% in kinetin on 3rd day and finally on 5th day it was reduced to 63% in MOPS and 54% in kinetin as compared to the original activity recorded on the first day.

Protein profile of broken plastid

Protein profile in broken plastids isolated from control and dark incubated leaves (MOPS and MOPS+Kinetin) was studied by SDS-PAGE (Figure 8). SDS-PAGE revealed that most of the high molecular weight proteins (~60 kDa) and low molecular weight protein (~14-20 kDa) were present till 3rd samples and were substantially reduced in 4th day and 5th day samples. As the dark induced senescence period increased the amount of RUBISCO large subunit (53 kDa) was substantially reduced. However few new proteins were observed in between 20-43 kDa ranges after 2nd day. These induced proteins were prominent in MOPS incubated samples as compared to kinetin treated samples.

Western blot analysis

To have a better insight of the regulation of enzymes involved in Chl biosynthesis, western blot analysis of a few Chl biosynthetic enzymes was performed (Figure 9).

GSA-AT

This enzyme converts glutamatesemialdehyde to ALA. Its protein abundance (46 kDa) remained unchanged in both MOPS and kinetin samples on the 1st day of senescence. On the 2nd and 3rd day its protein expression decreased in MOPS treated samples whereas remained unaltered in Kinetin treated samples. However, it was undetected on 4th and 5th day in both MOPS and Kinetin incubated samples.

UROD

This enzyme converts Uroporphyrinogen III to Coproporphyrinogen III. Its protein abundance remained unchanged in both MOPS and Kinetin samples on the 1st day of senescence. Thereafter its abundance constantly decreased till the 5th day in MOPS samples and remained unchanged in presence of kinetin.

CPO

This enzyme catalyzes the oxidative decarboxylation of propionate acid side chains on ring A and B of coproporphyrinogen III to yield Protoporphyringen IX. It was undetectable after 4th and 5th day of dark incubation in MOPS. However, in the presence of kinetin its protein abundance remained unchanged.

CHLI

This is a subunit of the enzyme Mg-chelatase, which catalyzes insertion of Mg^{2+} to protoporphyrin IX. Its protein abundance (42 kDa) increased in MOPS treated samples from 2nd day to 5th day of dark incubation in both control and kinetin-treated samples.

PORB

This is one of the isoforms of protochlorophyllide oxidoreductase enzyme that converts Pchlide to Chlide. Its protein amount (36 kDa) remained unaltered in senescence-induced samples.

CHLP

This enzyme catalyzes reduction of geranyl-geranyl diphosphate to phytol diphosphate. Its protein abundance (47 kDa) increased in MOPS treated samples after 2nd day of dark incubation.

Thylakoid protein profile

Thylakoids were isolated from control along with different treated samples as described in materials and methods. Their protein amount was measured and checked by SDS-PAGE for equal loading (Figure 10).

Western blot analysis of photosynthetic proteins

To check the effect of senescence on photosynthetic thylakoid proteins Western blot analysis of certain proteins was done (Figure 11).

D1 and D2

The amount of PSII reaction center protein D1 (32 kDa) substantially decreased after 3rd day of dark-induced senescence. Kinetin treatment could not protect D1 degradation after 3rd of senescence. Abundance of D2 (34 kDa), the other protein of PSII reaction center continuously decreased and completely disappeared on 4th day of senescence in control. It was present in kinetin-treated samples. However, on 5th day of dark incubation it disappeared from control and kinetin-treated samplesOEC 33

The abundance of 33 kDa protein of oxygen evolving complex continuously declined in control samples till 5th day of senescence. Upon kinetin treatment the protein abundance was higher than control till 5th day of senescence.

Cytochrome f

Cyt f is an electron carrier protein present in cytochrome b/f complex of thylakoid membrane. Its abundance continuously declined in control samples till 5th day. In kinetin-treated samples the Cyt f protein abundance was higher than that of control on all days of senescence.

Reiske protein

The abundances of hydrophobic Rieske protein present in cytochrome b/f complex of thylakoid membrane continuously declined in control samples till 5th day of senescence. Kinetin was able to halt the degradation of Rieske protein.

Subunit IV

The abundances of subunit IV protein of cytochrome b/f complex continuously declined in control samples till 5th day of senescence. Kinetin was able to block the degradation of subunit IV protein.

PSI subunit III & V

The photosystem I subunit III & V protein contents remained unchanged till 3rd of senescence. However on 4th and 5th day, their protein abundance decreased in MOPS incubated samples as compared to kinetin samples.

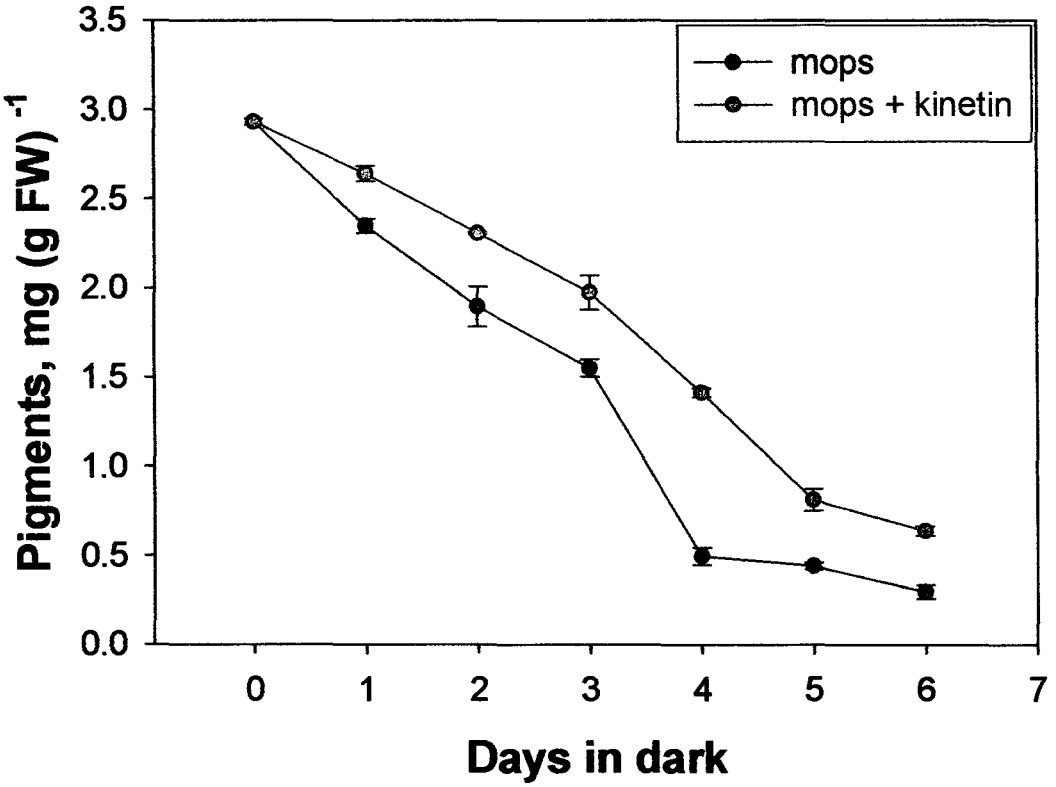


Figure 1. Senescence induced changes in chlorophyll contents.
Leaves were excised from 7-d-old rice seedlings grown under continuous light (100 μmole photons m^{-2} s^{-1}) at 28°C. The excised leaves were incubated in dark with MOPS (pH 4) or MOPS solution containing 200 μM kinetin for 1-6 days. Their chlorophyll contents were measured from control and kinetin-treated samples as described in materials and methods. Each data point is the average value of 4 replicates. The error bar represents standard deviation (SD).

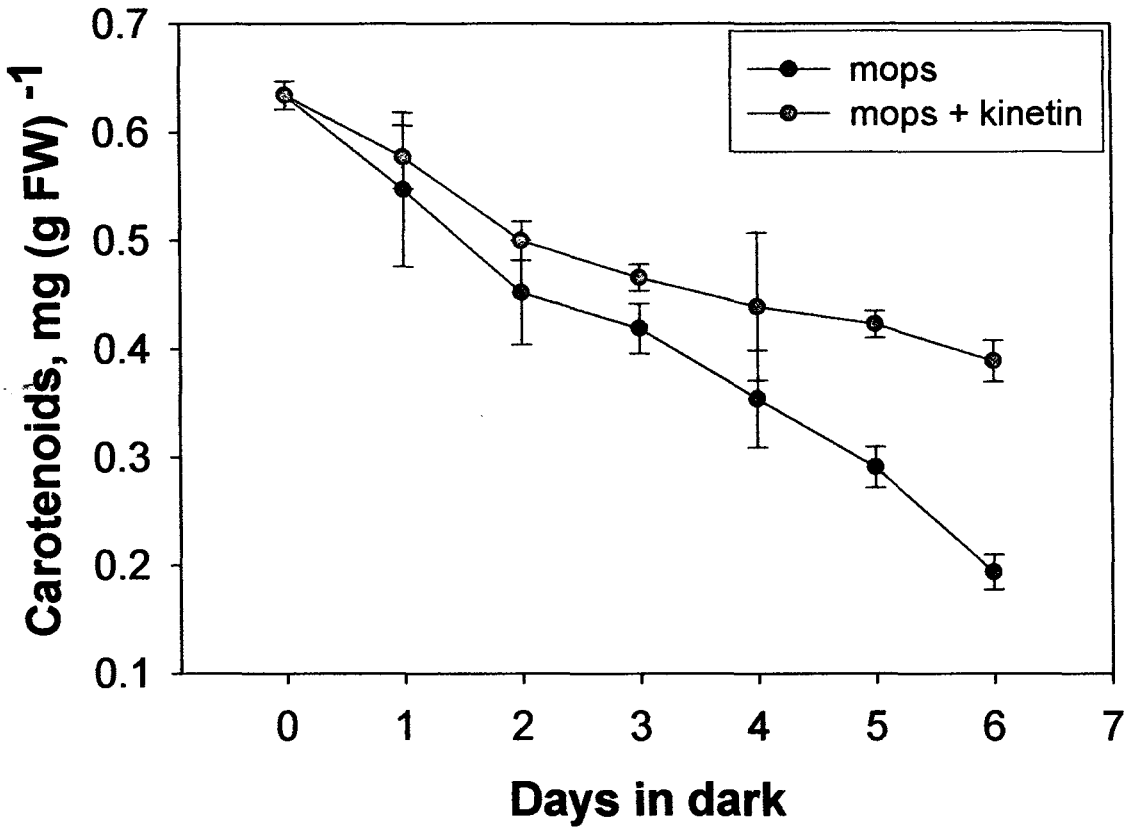


Figure 2. Senescence induced changes in carotenoid contents.
Leaves were excised from 7-d-old grown rice seedlings grown in continuous light (100 μmole photons m^{-2} s^{-1}) at 28°C. The excised leaves were incubated in dark with MOPS (pH 4) or MOPS solution containing 200 μM kinetin for 1-6 days. Their carotenoid contents were measured from control and kinetin-treated samples as described in materials and methods. Each data point is the average value of 4 replicates. The error bar represents standard deviation (SD).

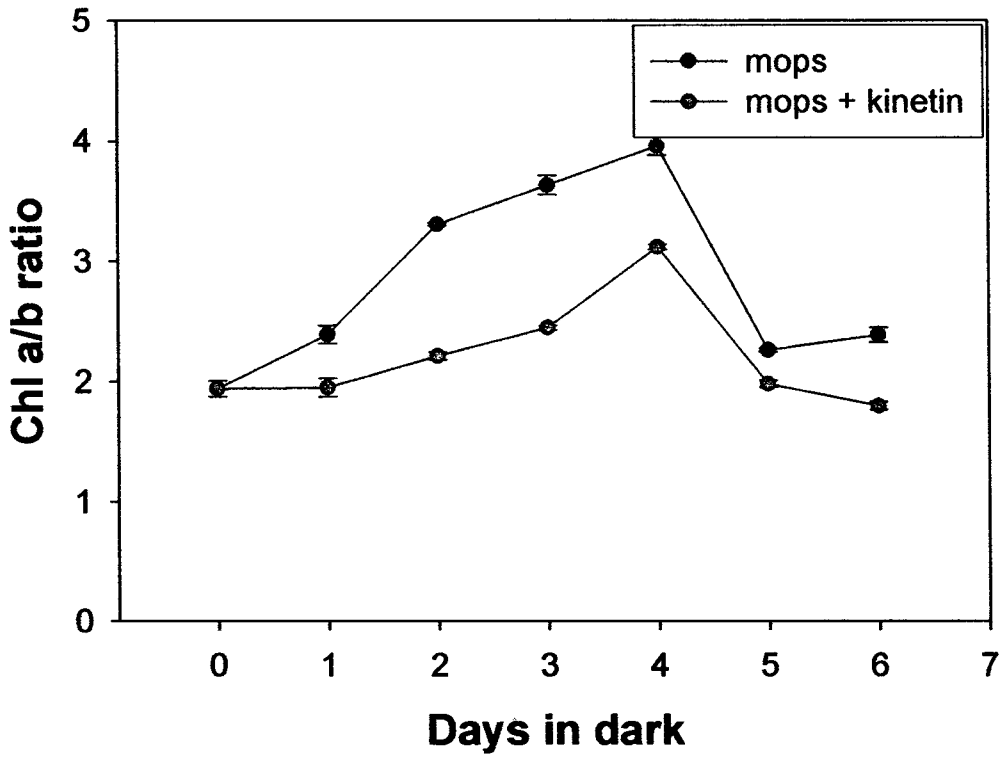


Figure 3. Senescence induced changes in Chl a/b ratio.
Leaves were excised from 7-d-old rice seedlings grown in continuous light (100 μmole photons m^{-2} s^{-1}) at 28°C. The excised leaves were incubated in dark with MOPS (pH 4) and MOPS solution containing 200 μM kinetin for 1-6 days. Their Chl a/b ratio was measured from control and kinetin-treated samples as described in materials and methods. Each data point is the average value of 4 replicates. The error bar represents standard deviation (SD).

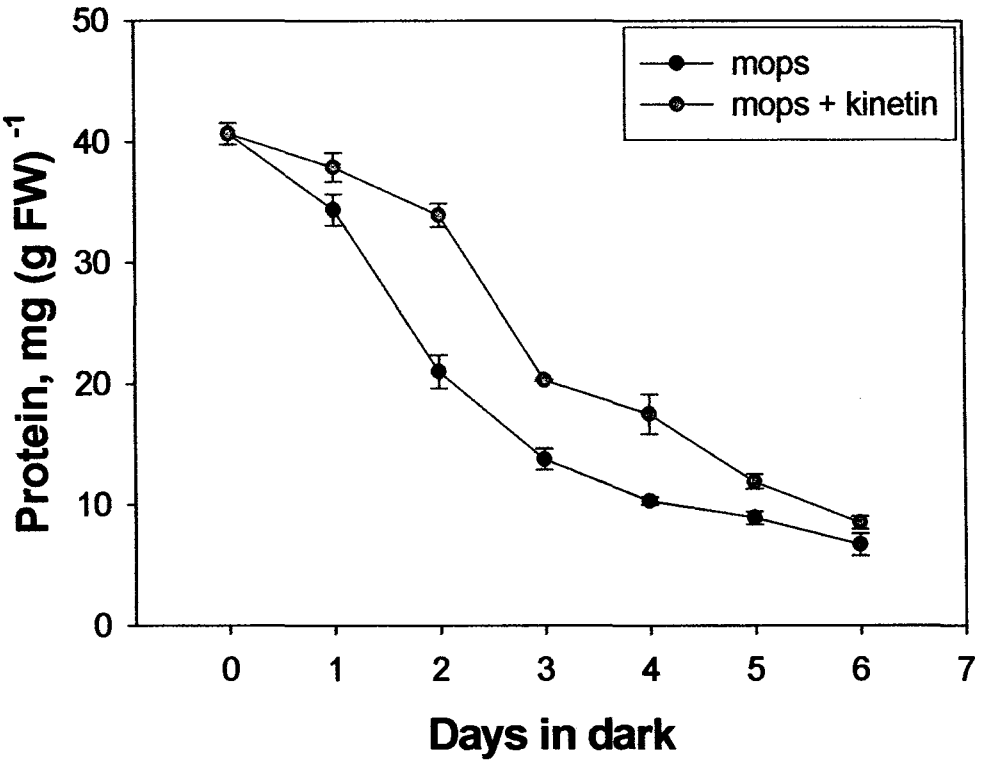


Figure 4. Senescence induced changes in protein contents.
Leaves were excised from 7-d-old rice seedlings grown under continuous light (100 μmole photons m^{-2} s^{-1}) at 28°C. The excised leaves were incubated in dark with MOPS (pH 4) and MOPS solution containing 200 μM kinetin for 1-6 days. Their protein contents were measured from control and kinetin-treated samples as described in materials and methods. Each data point is the average value of 4 replicates. The error bar represents standard deviation (SD).

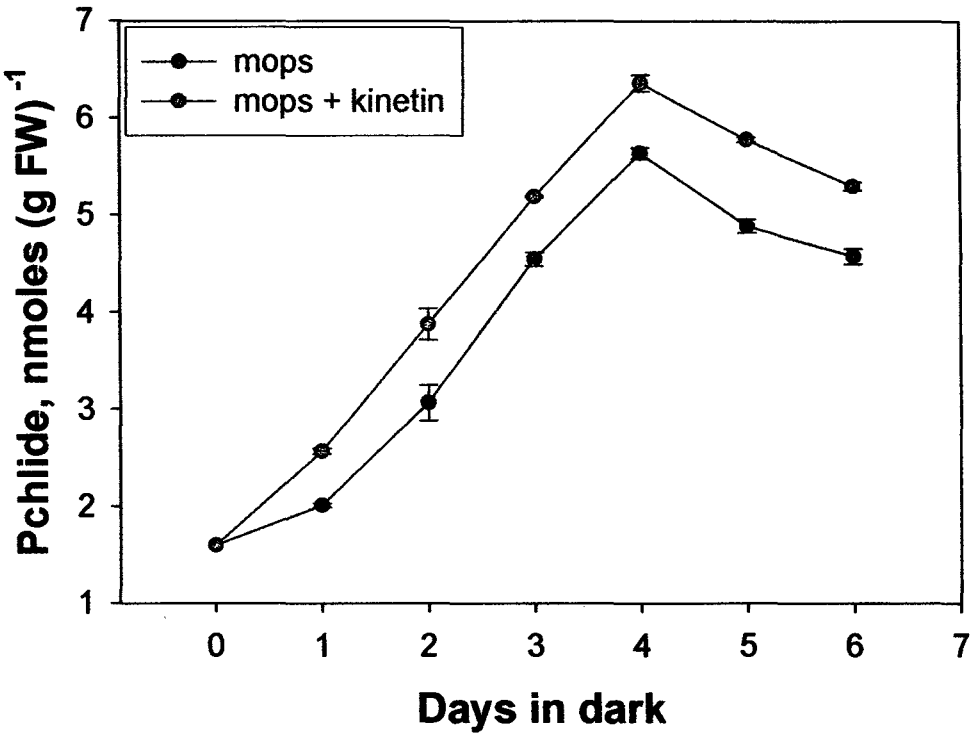


Figure 5. Senescence induced changes in protochlorophyllide contents. Leaves were excised from 7-d-old rice seedlings grown under continuous light (100 µmole photons m^{-2} s^{-1}) at 28^{0}C. The excised leaves were incubated in dark with MOPS (pH 4) and MOPS solution containing 200 µM kinetin for 1-6 days. Their protochlorophyllide contents were measured in dark from control as well kinetin-treated samples as described in materials and methods. Each data point is the average value of 4 replicates. The error bar represents standard deviation (SD).

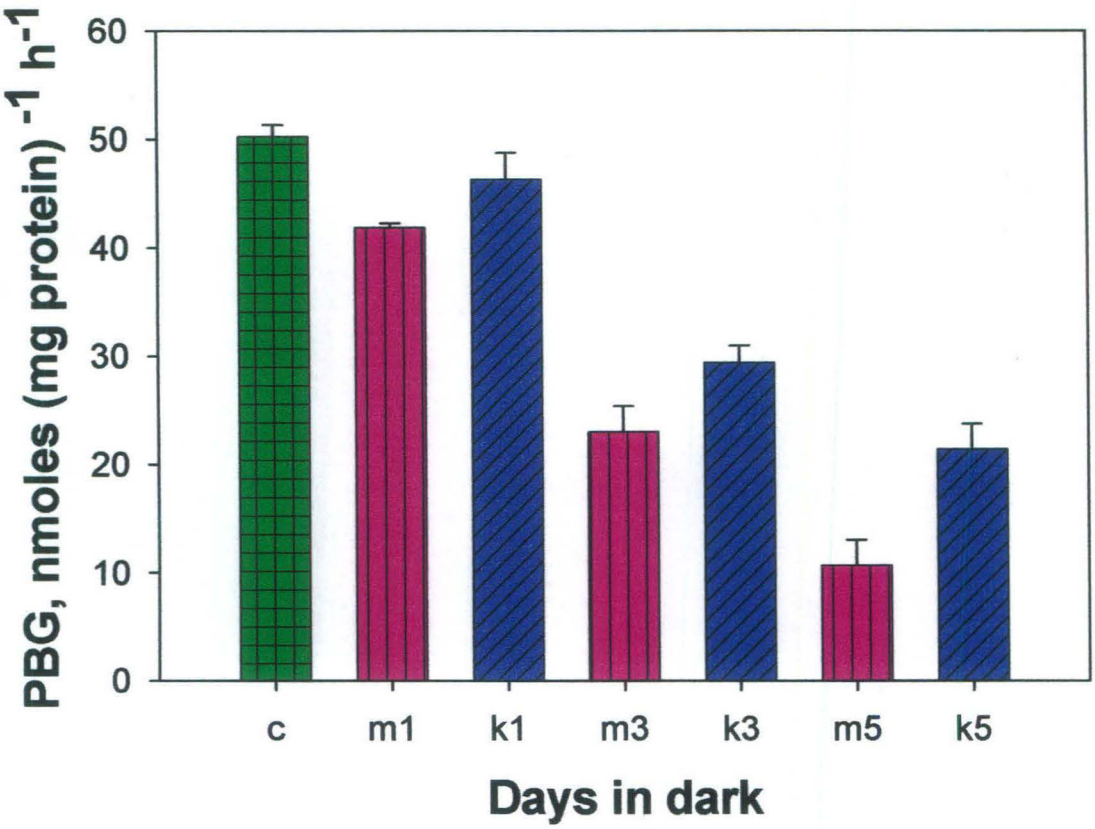


Figure 6. Senescence induced changes in activity of 5-aminolevulinicacid dehydratase.

Leaves were excised from 7-d-old rice seedlings grown in continuous light (100 μmole photons m^{-2} s^{-1}) at 28^0C. The excised leaves were incubated in dark with MOPS (pH 4) and MOPS solution containing 200 μM kinetin for 1-5 days. Their ALA dehydratase activity was measured from control as well as dark incubated samples as described in materials and methods. 'C' represents control sample measured on 0 day, 'm' represents mops and 'k' represents kinetin. 1, 2, 3, 4, 5 are for different days of dark incubation. Each data point is the average value of 4 replicates. The error bar represents standard deviation (SD).

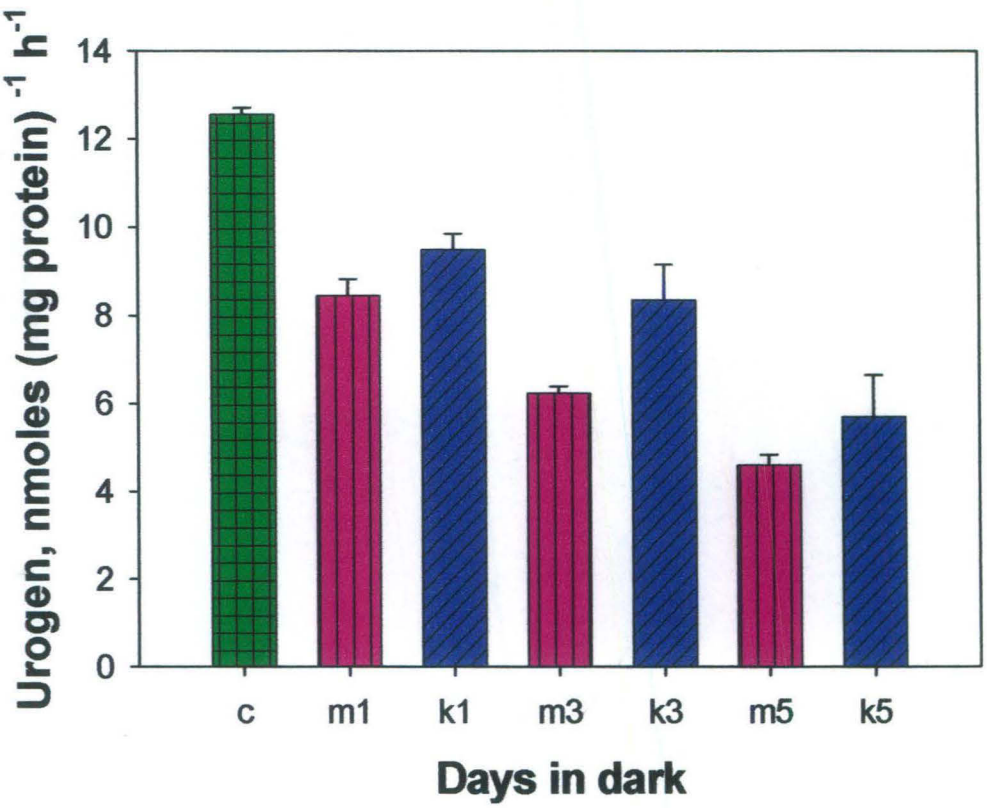


Figure 7. Senescence induced changes in activity of porphobilinogen deaminase.

Leaves were excised from 7-d-old rice seedlings grown in continuous light (100 µmole photons m^{-2} s^{-1}) at 28^0C. The excised leaves were incubated in dark with MOPS (pH 4) and MOPS solution containing 200 µM kinetin for 1-5 days. Their porphobilinogen deaminase activity was measured from control as well as dark incubated samples as described in materials and methods. 'C' represents control sample measured on 0 day, 'm' represents mops and 'k' represents kinetin. 1, 2, 3, 4, 5 are for different days of dark incubation. Each data point is the average value of 4 replicates. The error bar represents standard deviation (SD).

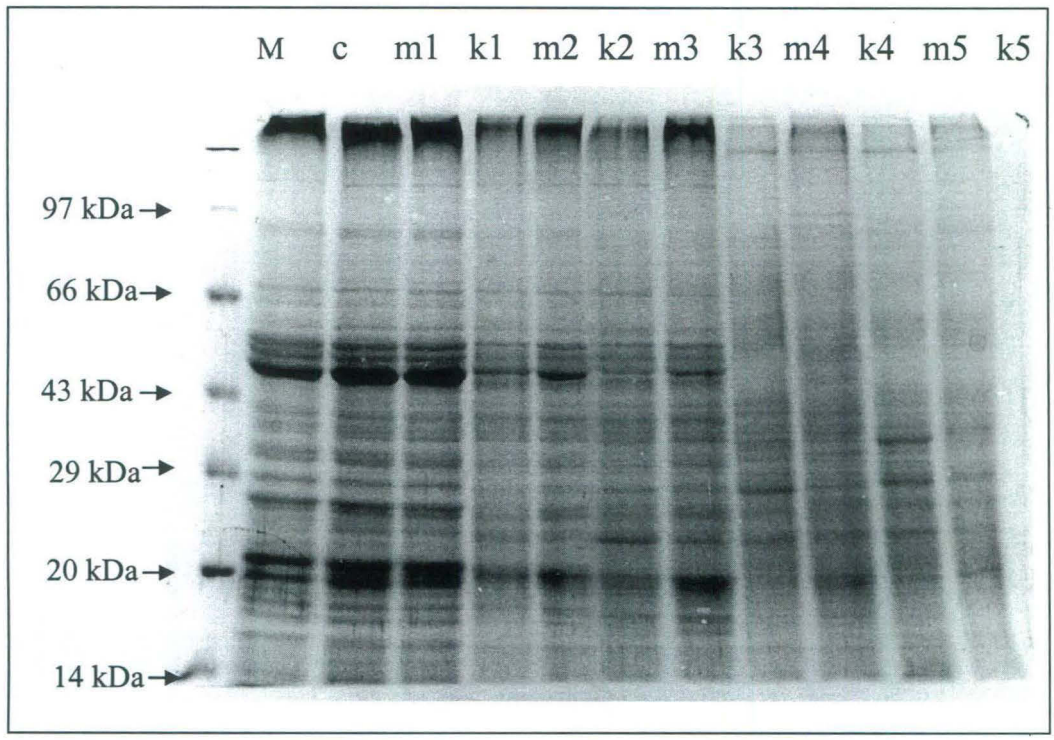


Figure 8. Senescence induced changes in the protein profile of broken plastids.

Leaves were excised from 7-d-old rice seedlings grown in continuous light (100 μmole photons m^{-2} s^{-1}) at 28^0C. The excised leaves were incubated in dark with MOPS (pH 4) and MOPS solution containing 200 μM kinetin for 1-5 days. Plastids were isolated from leaf samples and their protein profile was checked by running 12.5% SDS-PAGE as described in materials and methods. 'M' represents the molecular weight marker. 'C' represents control sample on 0 day, 'm' represents mops and 'k' represents kinetin. 1, 2, 3, 4, 5 are for different days of dark incubation.

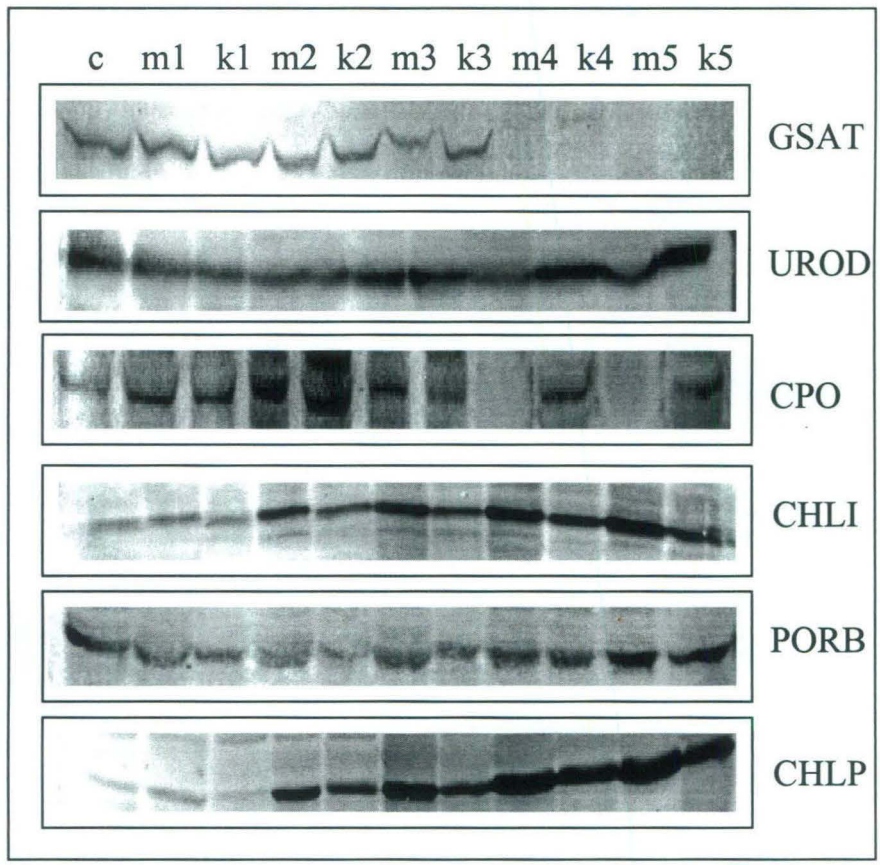


Figure 9. Western blot analysis of chlorophyll biosynthetic proteins.
Leaves were excised from 7-d-old rice seedlings grown under continuous light (100 μmole photons m^{-2} s^{-1}) at 28^0C. The excised leaves were incubated in dark with MOPS (pH 4) and MOPS solution containing 200 μM kinetin for 1-5 days. Plastids were isolated from leaf samples and their protein profile was checked by running 12.5% SDS-PAGE as described in materials and methods. The proteins from the SDS-PAGE were transferred to nitrocellulose membrane and the membranes were probed with different antisera (GSA-AT, UROD, CPO, CHLI, PORB and CHLP). 'C' represents control sample on 0 day, 'm' represents mops and 'k' represents kinetin. 1, 2, 3, 4, 5 are for different days of dark incubation.

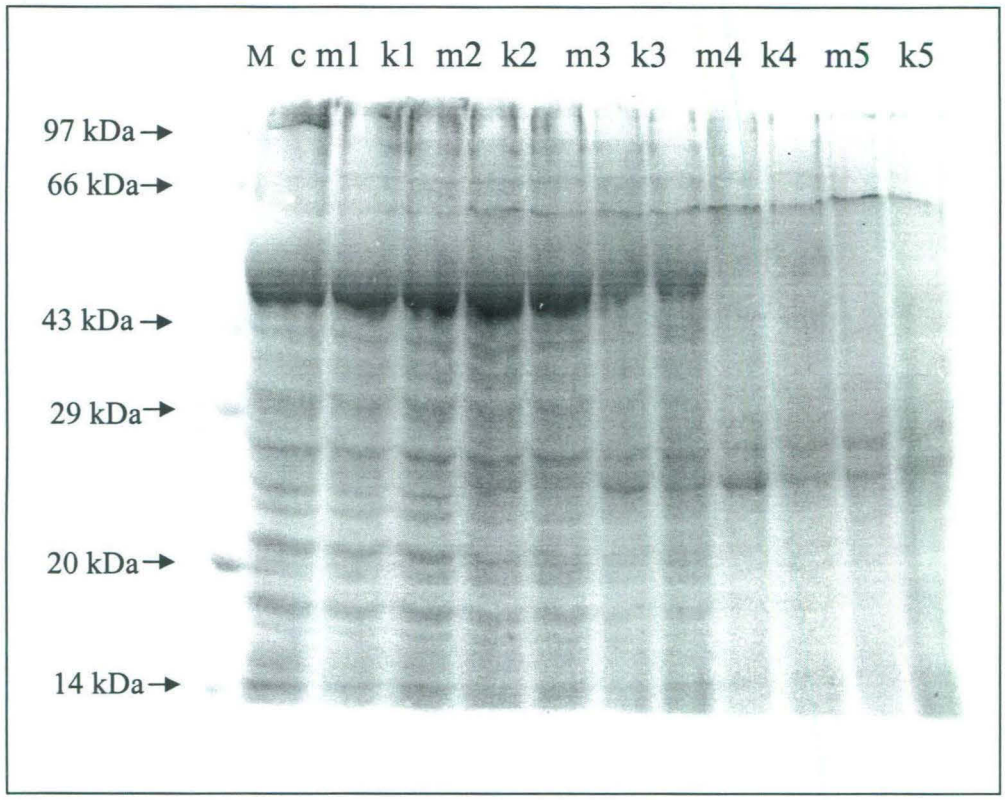


Figure 8. Senescence induced changes in Broken plastids.
Leaves were excised from 7-d grown rice plants under continuous light (100 μmole photons m^{-2} s^{-1}) at 28^0C. The excised leaves were incubated in dark with MOPS (pH 4) and MOPS solution containing 200 μM kinetin for 1-5 days. Thylakoid membranes were isolated from different treated samples and their protein profile was checked by running 12.5% SDS-PAGE as described in materials and methods. 'M' represents the molecular weight marker. 'C' represents control sample, 'm' represents mops and 'k' represents kinetin. 1, 2, 3, 4, 5 are for different days of dark incubation

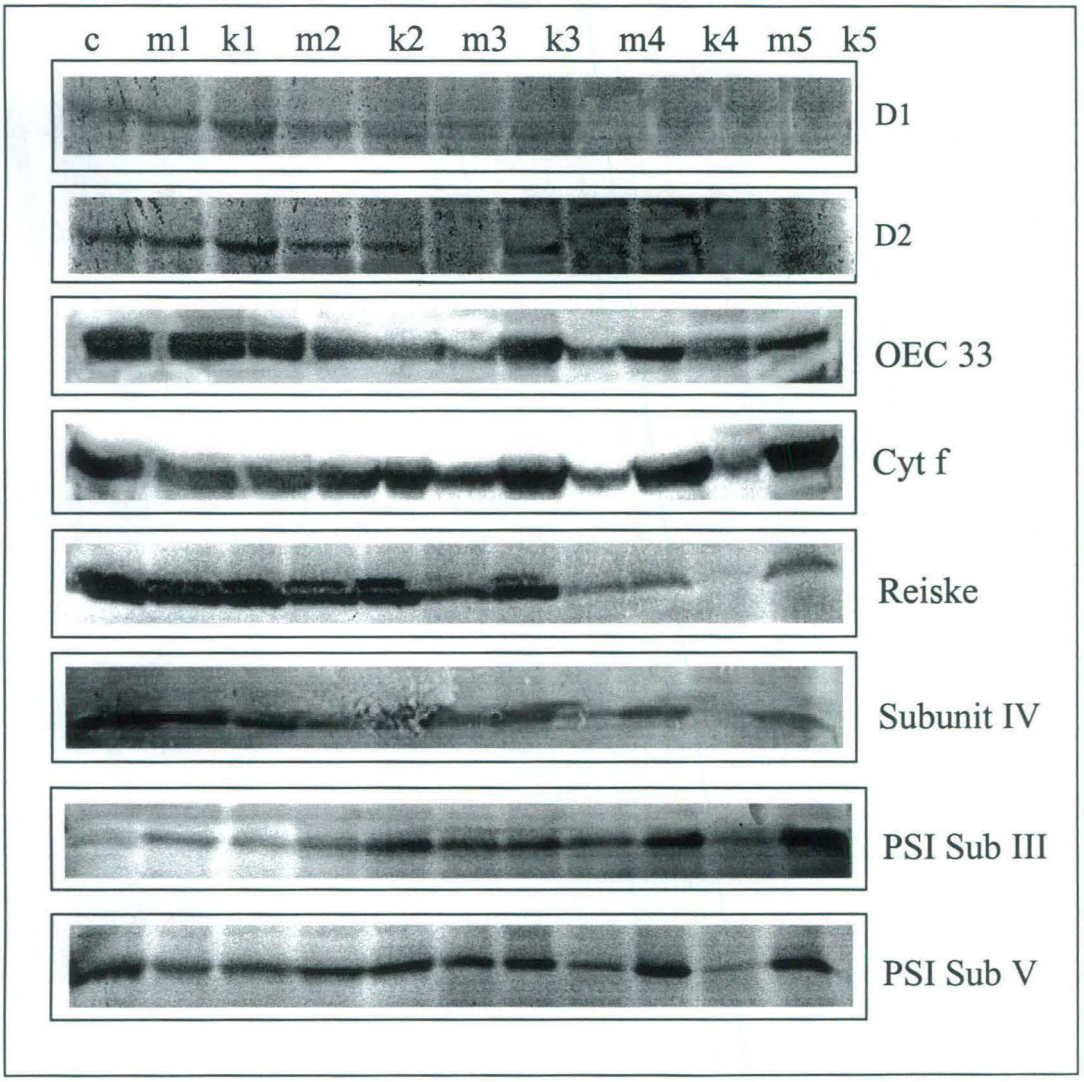


Figure 11. Western blot analysis of electron transport chain proteins.
Leaves were excised from 7-d grown rice plants under continuous light (100 μmole photons m^{-2} s^{-1}) at 28^0C. The excised leaves were incubated in dark with MOPS (pH 4) and MOPS solution containing 200 μM kinetin for 1-6 days. Thylakoid membranes were isolated from different treated samples and their protein profile was checked by running 12.5% SDS-PAGE as described in materials and methods. The proteins from the SDS-PAGE were transferred to nitrocellulose membrane and the membranes were probed with different antisera (D1, D2, OEC33, Cyt f, Reiske and Subunit IV) of the electron transport chain proteins. 'C' represents control sample, 'm' represents mops and 'k' represents kinetin. 1, 2, 3, 4, 5 are for different days of dark incubation

DISCUSSION

af senescence represents an endogenously controlled degenerative process that imately leads to organ death. It involves massive programmed cell death (*Gan and iasino, 1997 Yuehui and Susheng, 2002*). It progresses in an age-dependent manner, t it is also affected by a complex interaction of developmental age with other internal external factors (Biswal and Biswal, 1984). During leaf senescence they lose their otosynthetic and other anabolic capacity, and catabolism of chlorophyll predominates *uchanan and Wolleston, 1997 Congming etal 2001).* Shading and darkness are two portant external factors that enhance leaf senescence in higher plants. Most of the work dark- induced senescence has been done on either detached leaves or those attached to tyledons or primary leaves of the seedlings. Leaf senescence is regulated by the ordinated expression of specific genes and many senescence-associated genes/proteins.

Gradual disappearance of Chl is a universal indicator of leaf senescence. When tached rice leaves of 7-d-old light-grown seedlings were incubated with 2 mM MOPS lution in dark for different days it is shown that the Chl contents declined in a linear shion (Fig. 1). However incubation with Kinetin retarded senescence and as a result the crease in Chl content was less. This work confirms the previous observations lukmani and Tripathy, 1994; Richmond and Lang, 1957; Mothes 1964; Kulayeva 1962; sborne, 1962; Srivastava, 1967). As compared to control, the decrease in carotenoid ntents was more in MOPS incubated samples (Fig. 2). This observation confirms that Gut et al., 1987. Degradation of Chl is accompanied by change in Chl a/b ratio (Fig. . In control there is an increase of Chl a/b ratio than that of kinetin-treated samples. owever, on the 4th day of dark incubation the increase in Chl a/b ratio is prominent. nis indicates that during senescence Chl b degrades faster than Chl a. It was previously own that there is accelerated Chl b reduction in senescent barley leaves (Scheumann et ., 1999). This may be due to loss of light-harvesting chlorophyll-protein complex II that rich in Chl b. In fact, as shown in Fig. 8 and Fig. 10, the decline in 26 kDa LHCPII was pid in senescing leaves. This was also accompanied by loss of 53 kDa Rubisco large

subunit and other proteins in senescent leaves. It resulted in loss of protein contents (Fig. 4) suggesting a co-regulated degradation of Chl and proteins.

The present study confirms the previous observation of Hukmani and Tripathy, 1994 that Pchlide, an intermediate of Chl biosynthetic pathway accumulates in dark till 4^{th} day and then degrades and kinetin failed to stop the degradation of Pchlide (Fig. 5). Further investigations are required to ascertain the pathway of Pchlide degradation and to identify the Pchlide degrading enzymes. The decline in Pchlide content in senescing leaves was also due to its reduced synthesis. It is supported by the present observation that two early enzymes of Chl biosynthetic pathway leading to Pchlide synthesis i.e., ALA dehydratase and PBG deaminase decline during senescence (Fig. 6-7). Kinetin retarded the degradation of ALA dehydratase and PBG deaminase (Fig. 6, 7) that resulted in increased accumulation of Pchlide in kinetin-treated leaves.

The decline in protein expression of glutamate semialdehyde aminotransferase (GSA-AT) (Fig. 9) clearly suggests that ALA biosynthesis itself is affected in senescing leaves leading to reduced substrate (ALA) availability for ALA dehydratase enzyme. However, uroporphyrinogen decarboxylase (UroD) that converts uroporphyrinogen III to coproporphyrinogen III, expression was not severely affected in senescing leaves. The next enzyme in Chl biosynthetic pathway coproporphyrinogen oxidase (CPO), that converts coproporphyrinogen III to protoporphyrinogen IX, was downregulated during senescence and kinetin protected the CPO protein expression in senescing leaves. Interestingly, the protein expression of ChlI a component of Mg-chelatase, that incorporates Mg to protoporphyrin IX moiety to form Mg-protoporphyrin IX and that of ChlP involved in the reduction of geranyl-geraniol to phytol increased during senescence. Further investigations are needed to understand the increase in protein abundance of these two enzymes. It is also possible that plants overexpress these enzymes to synthesize Chl in the face of massive Chl catabolism. In the same vein the protochlorophyllide oxidoreductase (POR) expression did not decline during senescence. This also further confirms the report of Hukmani and Tripathy (1994) where efficient photoconversion of Pchlide to Chlide was observed in senescing barley leaves. Further studies of gene expression of all these Chl biosynthetic enzymes are needed to understand the co-regulation of transcriptional translational events of Chl synthesis during senescence.

It is well known that during senescence photosynthetic activities decline and kinetin partially protects the photosynthetic activities. To understand the mechanism of downregulation of photosynthetic light reaction during senescence, Western blot analysis of several proteins of PSII, Cytochrome bf complex and PSI involved in photosynthetic electron transport was studied. Several components i.e., D1 and D2 proteins present in the PSII reaction center degraded very fast and kinetin failed to prevent its degradation (Fig. 11). The oxygen evolving complex protein OEC 33 was partially degraded during senescence and was effectively protected by kinetin. Components of cytochrome bf complex i.e., Rieske Fe-S protein, cytochrome f and subunit IV declined in senescence. Their degradation was prevented by kinetin. However, the PSI proteins subunit III and V were only partially affected during senescence. These explain the previous observation that during senescence PSII activity declines very fast whereas the PSI reaction is only partially affected.

SUMMARY

Leaf senescence, encompassing a period from maturation to attrition, is a unique developmental process in the life history of plants. During leaf senescence there is disassembly of the photosynthetic apparatus within chloroplasts and the concomitant decrease in photosynthetic activity. Gradual disappearance of Chl is a universal indicator of leaf senescence.

To study the senescence induced changes in chlorophyll metabolism, detached rice leaves of 7-d-old light-grown seedlings were incubated with 2 mM MOPS or 2 mM MOPS + 200 μM kinetin solution. The Chl contents declined in a linear fashion during dark-induced senescence. However incubation with kinetin retarded senescence and as a result the decrease in Chl content was less. As compared to control, the decrease in carotenoid contents was more in MOPS incubated samples (Fig. 2). Degradation of Chl was accompanied by change in Chl a/b ratio indicating that during senescence Chl b degrades faster than Chl a.

Pchlide, an intermediate of Chl biosynthetic pathway accumulated in dark till 4^{th} day and then degraded and kinetin failed to stop the degradation of Pchlide. The decline in Pchlide content in senescing leaves was also due to its reduced synthesis. It is supported by the present observation that two early enzymes of Chl biosynthetic pathway leading to Pchlide synthesis i.e., ALA dehydratase and PBG deaminase declined during senescence. Kinetin retarded the degradation of ALA dehydratase and PBG deaminase that resulted in increased accumulation of Pchlide in kinetin-treated leaves.

The decline in protein expression of glutamate semialdehyde aminotransferase (GSA-AT) suggested that ALA biosynthesis itself was affected in senescing leaves leading to reduced substrate (ALA) availability for ALA dehydratase enzyme. The protein expression of Uroporphyrinogen decarboxylase (UroD), that converts uroporphyrinogen III to coproporphyrinogen III, was not severely affected in senescing leaves. The next enzyme in Chl biosynthetic pathway coproporphyrinogen oxidase (CPO), that converts coproporphyrinogen III to protoporphyrinogen IX, was

downregulated during senescence and kinetin protected the CPO protein expression in senescing leaves. Interestingly, the protein expression of ChlI a component of Mg-chelatase, that incorporates Mg to protoporphyrin IX moiety to form Mg-protoporphyrin IX and that of ChlP involved in the reduction of geranyl-geraniol to phytol increased during senescence. The protochlorophyllide oxidoreductase (POR) expression did not decline during senescence.

To understand the mechanism of downregulation of photosynthetic light reaction during senescence, Western blot analysis of several proteins of PSII, Cytochrome bf complex and PSI involved in photosynthetic electron transport was studied. Several components i.e., D1 and D2 proteins present in the PSII reaction center degraded very fast and kinetin failed to prevent its degradation. The oxygen evolving complex protein OEC 33 was partially degraded during senescence and was effectively protected by kinetin. Components of cytochrome bf complex i.e., Rieske Fe-S protein, cytochrome f and subunit IV declined in senescence. Their degradation was prevented by kinetin. However, the PSI proteins subunit III and V were only partially affected during senescence.

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