Chlorophyll metabolism in higher plants: A brief overview

Chlorophyll (Chl) is one of the most abundant organic molecules on Earth. As a component of the photosynthetic machinery, it absorbs light energy and is involved in energy transfer in the course of photosynthesis (von Wettstein et al., 1995 and Blankenship et al. 2002). Chlorophyll turnover is very important for proper functioning of photosynthetic apparatus under normal as well as in different environmental conditions. During the past few decades, most of the genes that encode the enzymes involved in Chl metabolism have been identified. Chlorophyll metabolism is a highly regulated phenomenon in plants. Chlorophyll metabolism can be subdivided into four distinct parts (Fig. I).

I. The synthesis of protoporphyrin IX: This is also called “common pathway” because protoporphyrin IX is the common precursor for Chl and protoheme. Protoporphyrin IX is synthesized from the first committed precursor, 5-aminolevulinic acid (ALA). 5-aminolevulinic acid (ALA) is the universal precursor for all biologically synthesized tetrapyrroles, including hemes, chlorophylls, cobalamin (vitamin B12), siroheme, and coenzyme F430 (Battersby et al., 2000 and Fukuda et al., 2005). ALA is synthesized from glutamyl-tRNAglu, by the activities of glutamyl-tRNA reductase (GluTR) and glutamate 1-semialdehyde aminotransferase (GSA-AT) in higher plants, algae and many bacteria. Glutamyl-tRNAglu, is also involved in protein synthesis (Kannangara et al., 1984). Two molecules of ALA are condensed to form the porphobilinogen. Four molecules of porphobilinogen are then sequentially polymerized linearly and subsequently to form the uroporphyrinogen III. After formation of uroporphyrinogen III, this pathway is branched for the formation of siroheme called as “siroheme branch”. Siroheme is the cofactors of nitrite and sulfite reductases that function in nitrogen and sulfur assimilation, respectively. Uroporphyrinogen III is converted to Coproporphyrinogen III by a decarboxilation step by Uroporphyrinogen III decarboxylase. Protoporphyrin IX is formed from Coproporphyrinogen III by further oxidation by, Coproporphyrinogen III oxidase and Protoporphyrinogen IX oxidase (PPOX1).

II. The insertion of Mg$^{2+}$ into protoporphyrin IX for Chl a biosynthesis, called “the Mg branch”. Protoporphyrin is the branching point for heme biosynthesis (“Heme branch”) and chlorophyll biosynthesis (Mg branch). Mg-chelatase and ferrochelatase
insert Mg\(^{2+}\) and Fe\(^{2+}\), respectively, into protoporphyrin IX, thereby directing the substrate into the Chl \(a\) or heme (“heme branch”) biosynthetic pathway. In the Mg branch, Mg-protoporphyrin IX is sequentially modified by methylation, formation of the fifth isocyclic ring and reduction of a side chain of the tetapyrrole ring. The D-ring of Pchlide is reduced stereospecifically to form chlorophyllide \(a\) by Protochlorophyllide oxidoreductase (POR) enzyme. It is the only light-requiring enzyme of the Chlorophyll biosynthetic pathway. The resulting movovinyl chlorophyllide (Chlide) \(a\) is esterified with a long chain polyisoprenol (geranylgeraniol or phytol) to synthesize Chl \(a\). Phytol is provided from geranylgeranyl pyrophosphate (GGPP), which is produced via isopentenyl pyrophosphate (IPP) in the nonmevalonate 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in plastids.

III. **Chlorophyll cycle:** The interconversion system between chlorophyll \(a\) and chlorophyll \(b\) refers to the chlorophyll cycle. A portion of Chlide \(a\) is converted to Chlide \(b\) by the activity of Chlide \(a\) oxygenase (CAO). Chl \(b\) can be reversibly converted to Chl \(a\) through two step reduction process. First Chl \(b\) is converted to 7-hydroxymethyl Chl \(a\) by Chl \(b\) reductase followed by again reduction by 7-hydroxymethyl Chl \(a\) reductase in to Chl \(a\) (Rudiger et al. 2002).

IV. **The degradation of Chl \(a\):** Amount of chlorophyll in leaf depends on the light condition, stress and leaf age. Its constant turnover is important to maintain proper photosynthetic complexes. The chlorophyll (Chl) breakdown takes place not only in the normal turnover of Chl, but also at specific stages of plant development, such as leaf senescence and fruit ripening. During senescence and abiotic stress, a large fraction of chlorophyll is broken down by pheophtinase (Pruz’inska’ et al., 2005) and Chlorophyllase (Jakob et al., 1999) in to chlorophyllide and giving rise to the accumulation of high amounts of free phytol (Soll et al., 1980). Chlorophyllide is converted in to fluorescent and non-fluorescent chlorophyll catabolites (H’ortensteiner et al., 2006 and Takamiya et al., 2000). Accumulated phytol could be recycled for Chl synthesis or could be used for the synthesis of tocopherol, phyloquinone and phytyl esters. Figure II represents different steps involved in chlorophyll catabolism (Ischebeck et al., 2006 and Valentine et al., 2006).
FIGURE I. The Chl metabolic pathway in higher plants. (Adopted from Masuda et al., 2008)
**FIGURE II.** Representative structural outline of major catabolites delineating the main paths of chlorophyll breakdown in higher plants (Kräutler et al., 2006 and 1991): Chls are degraded in the chloroplast by enzyme-catalyzed processes via pheophorbide (Pheide) a and the red chlorophyll catabolite (RCC) to give primary fluorescent chlorophyll catabolites (pFCC, or its C1-epimer, epipFCC). The relevant enzymes involved in this part are: a), Chl b reductase; b), 7-hydroxymethyl Chl reductase; c), pheophytinase, chlorophyllase (CLH); d), magnesium dechelatase; e), pheophytinase (PPH); f), Pheide a oxygenase (PAO); g), RCC reductase (RCCR). pFCCs are modified further by unidentified hydroxylating enzymes (h, i). When carrying a free propionic acid group, FCCs are transported into the vacuole, where they are suggested to isomerize by a spontaneous, acid catalyzed reaction (j) to the corresponding nonfluorescent chlorophyll catabolites (NCCs), such as Hv-NCC-1 (the main tetrapyrrolic catabolite found in senescent leaves of barley, *Hordeum vulgare*). Else, they are esterified by unknown enzymes at the propionic acid group (k) to give 'persistent' hypermodified FCCs, such as Me-FCC-56 (the main FCC in peels of ripe bananas, *Musa acuminata*, cavendish cultivar). Relevant atom numbering is specified. (Adopted from Hörtensteiner et al., 2011)
Table-A. List of enzymes involved in tetrapyrrole biosynthesis and their respective gene names and localization in the cell. M: Mitochondria, P: Plastid

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme name</th>
<th>Localization</th>
<th>Gene name(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glutamyl-tRNA synthase</td>
<td>P</td>
<td>GluRS</td>
</tr>
<tr>
<td>2</td>
<td>Glutamyl-tRNA reductase</td>
<td>P</td>
<td>HEMA1, HEMA2, HEMA3</td>
</tr>
<tr>
<td>3</td>
<td>Glutamate-1-semialdehyde aminotransferase</td>
<td>P</td>
<td>GSAT1, GSAT2</td>
</tr>
<tr>
<td>4</td>
<td>Porphobilinogen synthase</td>
<td>P</td>
<td>AlaD, HEMB, HEMB1, HEMB2</td>
</tr>
<tr>
<td>5</td>
<td>Porphobilinogen deaminase</td>
<td>P</td>
<td>PhgD, HEMC</td>
</tr>
<tr>
<td>6</td>
<td>Uroporphyrinogen III synthase</td>
<td>P</td>
<td>UroS, HEMD</td>
</tr>
<tr>
<td>7</td>
<td>Uroporphyrinogen III decarboxylase</td>
<td>P</td>
<td>UroD, HEME, HEME1, HEME2</td>
</tr>
<tr>
<td>8</td>
<td>Coproporphyrinogen oxidase</td>
<td>P</td>
<td>CPO, HEMF, HEMF1, HEMF2</td>
</tr>
<tr>
<td>9</td>
<td>Protoporphyrinogen oxidase</td>
<td>P/M</td>
<td>PPX, HEMG, HEMG1, HEMG2</td>
</tr>
<tr>
<td>10</td>
<td>Mg-chelatase</td>
<td>P</td>
<td>CHL D, CHL H, CHL1, CHL2</td>
</tr>
<tr>
<td>11</td>
<td>Mg-protoporphyrin IX methyltransferase</td>
<td>P</td>
<td>CHLM</td>
</tr>
<tr>
<td>12</td>
<td>Mg-protoporphyrinogen IX monomethyl ester cyclase</td>
<td>P</td>
<td>CRD1</td>
</tr>
<tr>
<td>13</td>
<td>Divinyl reductase</td>
<td>P</td>
<td>DVR</td>
</tr>
<tr>
<td>14</td>
<td>NADPH-protochlorophyllide oxidoreductase</td>
<td>P</td>
<td>PORA, PORB, PORC</td>
</tr>
<tr>
<td>15</td>
<td>Geranylgeranyl reductase</td>
<td>P</td>
<td>Chl P</td>
</tr>
<tr>
<td>16</td>
<td>Chlorophyll synthase</td>
<td>P</td>
<td>Chl G</td>
</tr>
<tr>
<td>17</td>
<td>Chlorophyllide a oxygenase</td>
<td>P</td>
<td>CAO</td>
</tr>
</tbody>
</table>
Protochlorophyllide oxidoreductase

It is the only light-requiring enzyme of the Chlorophyll biosynthetic pathway. Protochlorophyllide oxidoreductase (POR) catalyzes the light-dependent trans-addition of hydrogen across the C17–C18 double bond of the D-ring of protochlorophyllide (Pchlide) to produce chlorophyllide (Fig. 3) (For reviews see Reyes et al., 2005 and Masuda et al., 2004, Tripathy and Pattanayak, 2011). The reaction involves a highly endergonic (ground state to excited state) light-driven hydride transfer from the pro-S face of the nicotinamide ring of NADPH to C17 of the Pchlide molecule (Heyes et al., 2006 and 09), followed by an exergonic (ground state) proton transfer from a conserved Tyr residue to C18 (Fig. III) (Menon et al., 2009).

![Diagram of light-activated reduction of Pchlide catalyzed by POR](image)

**FIGURE III.** Scheme for the light-activated reduction of Pchlide catalyzed by POR. Upon illumination, a hydride is transferred from NADPH (blue) to C17 of Pchlide (green), followed by proton transfer from Tyr-193 (red) to C18. (Adopted from Heyes et al., 2011)

Light energy absorbed by the Pchlide in the complex may produce torsional strain in the molecule that provides a favorable condition for hydride/ hydrogen transfer from NADPH (Begley et al., 1989). POR is a member of a large family of enzymes known as short chain dehydrogenases/reductases (SDR) (Wilks et al., 1995) which generally catalyze NADP(H)- or NAD(H)-dependent reactions involving hydride and proton transfers. A tyrosine (Tyr) and a lysine (Lys) residue are both conserved throughout all members of the SDR family. In POR, it was also seen that Tyr and Lys residues are important for its activity (Lebedev et al., 2001; Wilks et al., 1995). The Tyr may be deprotonated, acting as a general acid to facilitate hydride transfer to or from NAD (P)+/H (Ehrig et al., 1994). The proton at the C-18 position of Pchlide is derived from Tyr and the hydride transferred to the C-17 position is derived from the pro-S face of NADPH. The close proximity of the Lys residue is thought to allow the deprotonation step to occur at physiological pH by lowering the apparent pKₐ of the phenolic...
group of the Tyr (Wilks et al., 1995). The mutation of either Tyr 275 or Lys-279 did not completely abolish the catalytic activity of POR. However, mutation of either residue impairs formation of the ground state ternary enzyme-substrate complex, indicating their key role in substrate binding. Both residues have multiple roles in catalysis, involving formation of the ground state ternary enzyme-substrate complex, stabilization of a Pchlide excited state species and proton transfer to the reaction intermediate formed after the light reaction (Menon et al., 2009). Recently it has been demonstrated that a light-activated conformational change of the protein is necessary to activate catalysis (Heyes et al., 2008; Sytina et al., 2008). The fact that POR is light activated means the enzyme–substrate complex can be formed in the dark. This has recently been exploited by studying Pchlide reduction at low temperatures to trap intermediates in the reaction pathway (Heyes et al., 2002, 2003; Heyes and Hunter, 2004). As a result, the reaction has been shown to consist of at least three distinct steps: an initial light-driven step, followed by a series of ‘dark’ reactions. An initial photochemical step can occur below 200 K (Heyes et al., 2002), whereas two ‘dark’ steps were identified for Synechocystis POR, which can only occur close to or above the ‘glass transition’ temperature of proteins (Heyes et al., 2003). 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 et al., 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 secondary structure analysis of POR reveals that it has 33% alpha helix, 19% beta-sheets, 20% turn and 28% random coil. A hydrophobic loop-region was supposed to be involved in membrane anchoring (Birve et al., 1996). Mutation studies by Dahlin et al., 1999 showed that, mutation in predicted α-helical regions of the protein showed the least effect on enzyme activity, whereas mutations in the predicted β-sheet regions showed an adverse effect on enzyme function. The replacement of charged amino acids by alanine in the N- and C-terminal regions of the mature protein did not affect POR assembly, whereas mutation within central core of the protein were incapable of proper attachment to the thylakoid.

POR is nuclear encoded, translated as a precursor protein in the cytosol and ultimately transported into plastids (Apel, 1981). It’s a peripheral membrane protein that accumulates to high level in PLBs, 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. It is observed that the Cys residues of POR are crucial for its membrane
association (Aronsson et al., 2001a), for the NADPH and pigment binding (Reinbothe et al.,
2006; Townley et al., 2001).

The association of POR with Pchlide results in three different spectral forms of Pchlide
based on their fluorescence emission maximum (in nm): Pchlide F631 (due to the pigment
structural arrangements), Pchlide F644 (due to association of POR), and Pchlide F655 (due to
localization in PLBs and/or prothylakoids) (Böddi et al., 1992, 1993). Spectroscopic studies
of the dark-grown bean seedlings gave the idea about two forms of Pchlide, a main
component with a red absorption band at 650 nm and a minor component absorbing at 636 nm
(Shibata 1957). On the basis of flash illumination, two kinds of Pchlide can be categorized;
one is transformed into Chlide and is called photoactive Pchlide, whereas the other remains
unchanged and is called nonphotoactive Pchlide. The latter is assembled into various
complexes with different molecular structure and spectral properties (Masuda and Takamiya
2004; Schoefs and Franck 2003). Plastids isolated from dark-grown wheat seedlings exhibit a
smaller fluorescence emission (77K) peak at 632 nm due to non-phototransformable Pchlide
and a larger peak at 657 nm due to phototransformable Pchlide. The non-phototransformable
Pchlide emitting at 632 nm is due to monomeric Pchlide complex or esterified Pchlide i.e.
Protochlorophyll (Lindsten et al. 1988), which spontaneously dimerizes to form (POR-Pchlide-
NADPH)2. The short-wavelength, monomeric Pchlide is not flash-photoactive; instead it
regenerates the long wavelength Pchlide forms (He et al. 1994; Schoefs and Franck 2003;
Schoefs et al. 1994, 2000a, 2000b). The dimer has the absorption maximum at 638 nm and
emission maximum at 645 nm (Lebedev and Timko 1999). The dimeric POR-Pchlide-
NADPH complex further polymerizes to form 16-mer or larger aggregates of POR-Pchlide-
NADPH complex i.e., (POR-Pchlide-NADPH)n having absorption maximum at 650 nm and
emission maximum at 657 nm (Böddi et al. 1989; Wiktorsson et al. 1993) and is flash
photoactive (Böddi et al. 1991). However long-term illumination i.e., more than a minute
usually converts non-active Pchlide to photo-active Pchlide. The photo-transformable Pchlide
(F657) rapidly decreases when 5-d-old etiolated seedlings are transferred to 42°C in dark for
24 h (Mohanty and Tripathy, 2011). In heat-stressed seedlings the Shibata shift is
substantially arrested while in chill-stress conditions, the same is partially affected.

Full-length cDNA clones of POR were isolated from barley (Holtorf et al., 1995;
Schulz et al., 1989), oat (Darrah et al., 1990), pea (Spano et al., 1992), wheat (Teakle and
Griffiths, 1993), cucumber (Kuroda et al., 1995), tobacco (Masuda et al., 2002),
Arabidopsis (Armstrong et al., 1995; Benli et al., 1991; Oosawa et al., 2000), and banana (Coemans et al.,
2005). The high degree of sequence similarity among PORs from different taxonomic group
implies a common mechanism of the enzyme action. A characteristic feature of POR accumulating in dark is its sensitivity to illumination. The POR mRNA expression was also decreased (Santel and Apel, 1981). Red and far-red light treatment also inhibits POR mRNA expression indicating that POR expression was controlled by phytochromes (Apel, 1981; Batschauer and Apel 1984; Mosinger et al., 1985). The negative effect of light on POR enzyme and its mRNA was observed in different dicotyledons like bean, pea, tomato, Arabidopsis (Armstrong et al., 1995; Forreiter et al., 1991; Spano et al., 1992) and in monocotyledonous plants maize and barley (Forreiter et al., 1991; Holtorf et al., 1995). However, some flowering plants have isoforms of POR. In Arabidopsis, (Armstrong et al., 1995; Oosawa et al., 2000; Pattanayak and Tripathy, 2002; Su et al., 2001), barley (Holtorf et al., 1995; Holtorf and Apel, 1996a, b) and tobacco (Masuda et al., 2002) there are different PORs present. The N-terminus of PORA and PORB of barley etioplast has recently been characterized (Ploscher et al., 2009). In Arabidopsis there are three isoforms of POR, namely PORA, PORB and PORC. These three isoforms are differentially regulated by light. The level of porA mRNA and protein decreases on illumination of etiolated plants (Holtroff and Apel, 1996a) 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, 1996a). Both porB and porC of Arabidopsis exhibit diurnal fluctuation but only the porB mRNA of Arabidopsis exhibits circadian regulation (Su et al., 2001). porC mRNA and protein expression also increased under high light intensity (Masuda et al., 2003; Su et al., 2001). In cucumber the levels of the por mRNA increased in etiolated cotyledons when they were illuminated with continuous light (Fusada et al., 2000; Kuroda et al., 1995). The plant hormone cytokinin regulates cucumber por gene expression by binding to the cis-elements present at 5’ region of the POR promoter (Fusada et al., 2005). In tobacco, two POR isoforms have been isolated, the expression of which was not negatively regulated by light, persisted in mature green tissue and showed diurnal fluctuations with a similar oscillation phase (Masuda et al., 2002).

A plant specific downstream element in the 3’ untranslated region of the porA transcript confers porA mRNA instability, where as it was not responsible for porB mRNA degradation (Holtorf and Apel, 1996a). POR gene expression in cucumber is regulated by phytohormone, particularly by cytokinins and abscissic acid (Kuroda et al., 2001). In lipI mutant of pea, cytokinins restored the formation of PLB and photoactive Pchlide in dark (Seyedi et al., 2001a), but in Arabidopsis its application results in loss of PLBs (Chory et al., 1994).
lupine, *por* expression is also regulated by cytokinins and abscissic acid (Kusnetsov et al., 1998).

*Por* gene expression is also organ specific. *Arabidopsis porB* and *porC* are expressed in all photosynthetic tissues of the mature plants but not in root (Armstrong et al., 1995; Oosawa et al., 2000). *Por* expression in photosynthetic tissue is also observed in Cucumber plant (Kuroda et al., 1995). *Por* gene expression is also developmentally regulated and depends on the age of the plant. In *Arabidopsis* and barley the *porA* expression is only observed in young seedlings whereas *porB* is expressed both in young and matured green tissue (Armstrong et al., 1995; Schunmann and Ougham, 1996). In *Arabidopsis* both *porB* and *porC* expression is observed in green tissue (Oosawa et al., 2000; Su et al., 2001). In the leaves of dark-grown seedlings, highest level of expression is observed in 8-10 days of post germination of seedlings (Spano et al., 1992). The transcript level of pea *por* did not decrease after 48 h of light exposure whereas immunoblot analysis showed there was no POR protein after 48 h of light exposure. These results suggested that in pea, Pchlide reductase activity is primarily regulated post-transcriptionally, most likely at the level of translation initiation/elongation or protein turnover (Spano et al., 1992).

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

In vivo functions of each POR isoform have been extensively studies in *Arabidopsis* seedlings. In det340 (de-etiolated) mutant of *Arabidopsis*, *porA* is constitutively down regulated, resulting in a lack of PORA and photoactive Pchlide-F655 in dark-grown seedlings and susceptible to photooxidative damage at extremely low light intensities (Lebedev et al., 1995). The photoprotective mechanism of PORA is also described by Buhr et al., 2008.
Arabidopsis etiolated seedlings grown under continuous far-red light are unable to green when subsequently transferred to white light, which is called far-red blocking of greening process. This process involves depletion of PORA, partial depletion of PORB and the concomitant loss of PLBs resulting in photo-oxidative damage (Barnes et al., 1996; Runge et al., 1996). From these studies, PORA has been proposed to play a special role in the formation of POR ternary complexes containing photoactive Pchlide-F655, PLB assembly, and protection against photo-oxidative damage caused by non-photoactive Pchlide (Reinbothe et al., 1999). However, overexpressed lines of porA and porB overcome the photooxidative damage (Sperling et al., 1997, 1998). Franck et al., 2000 examined in detail redundant roles of PORA and PORB in etioplast differentiation by manipulating the total POR content and the PORA-to- PORB ratio of Arabidopsis seedlings using antisense and overexpression approaches.

It was recently observed that the cyanobactrial POR overexpression in Arabidopsis porA mutant could restore the prolamellar body formation. However the amount of photoactive pchlide in the etioplasts of the complementing lines was retained at a low level as in the parent PORA knockdown mutant (Masuda et al., 2009). The lip1 mutant of pea lacked PLBs and can store PLBs if treated with cytokinin (Seyedi et al., 2001a). But unlike Arabidopsis mutant it did not undergo photooxidative damage (Seyedi et al., 2001b). The physiological function of specific POR isoforms in vivo has been well characterized in knockout mutants of Arabidopsis (Frick et al., 2003; Masuda et al., 2003). Single por mutants display no obvious phenotypes at the whole plant or chloroplast ultrastructural levels, except that porB mutants have less extensive etioplast inner membranes. However the porB porC double mutant, which displayed a seedling-lethal xantha phenotype at the cotyledon stage, contained only a small amount of Chl a, and possessed chloroplasts with mostly unstacked thylakoid membranes (Frick et al., 2003). Masuda et al., 2003 focused on the greening process of por mutants, and showed that the etiolated porB mutant seedling was able to green to a similar extent as the wild type, and the greening of porC was repressed under high light conditions.

From a molecular evolutionary perspective, the LPOR enzymes are extraordinarily highly conserved. Comparative analysis of complete plastid genome sequences indicate that LPOR genes were lost from the plastid at some point during the early evolution (Martin et al., 1998, 2002), and analysis of LPOR proteins in species of conifer show evidence for loss of enzyme activity (Kusumi et al., 2006). The discovery of genes for LPOR in the plastid
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genomes of diverse cryptophytes algae suggests that these genes have been lost relatively recently.

**Intraplastidic Route of Chlorophyll Biosynthesis**

Chloroplast has two limiting envelope membranes, the outer membrane in contact with the cytoplasm of the cell and the inner membrane surrounding the stroma. The inner envelope membrane is essential for the biosynthesis of plastid components such as glycolipids and prenylquinones (Douce and Joyard 1990). In addition, the envelope membrane plays a key role in the sorting of plastid proteins that are coded by the nuclear genome. The envelope also plays a significant role in chlorophyll (Chl) degradation. Chlorophyllase and Mg-dechelatase are present in the inner envelope membrane (Matile et al. 1996, 1999).

Chl is bound to pigment-protein complexes of thylakoid membranes. Chl and its precursors are essential for chloroplast development and nuclear gene expression (Eichacker et al. 1990; Jilani et al. 1996; Kropat et al. 1997, 2000). Interplay of envelope, stroma and thylakoids is shown for Proto IX synthesis and enzymes responsible for conversion of ALA to Proto IX i.e., ALA dehydratase, porphobilinogen deaminase, uroporphyrinogen decarboxylase and coproporphyrinogen oxidase are mostly located in the stromal phase (Manohara and Tripathy 2000). All subsequent steps of Chl biosynthesis are catalyzed by membrane-bound or membrane-associated enzymes (Joyard et al. 1990; Matringe et al. 1992a, Manohara and Tripathy, 2000). Pchlide (Pineau et al. 1986) and Proto IX (Mohapatra and Tripathy, 2002, 2003, 2007) are present both in the thylakoid and envelope membranes. Out of total plastidic Pchlide, envelope membranes contained 1.5%, thylakoids have the maximum 98.48% and stroma has a trace fraction of 0.02%. Distribution of the Mg-proto IX and its monoester was 89.0% in thylakoids, 10.0% in stroma and 1.0% in envelope. A substantial fraction (33.77%) of plastidic proto IX was partitioned into stroma. Envelope contained 0.66% and thylakoids had 65.57% of the total plastidic proto IX pool. Had there been trafficking of Pchlide from the envelope to thylakoids or vice versa, significant amounts of Pchlide should have been present in the stroma. Therefore, in the developed chloroplast, Pchlide present in the envelope may be synthesized *de novo* independent of thylakoids. During greening Pchlide content (mg protein)\(^{-1}\) decreased in thylakoids and increased in envelope membranes (Barthelemy et al. 2000).
ALA biosynthetic enzymes are located in the stroma (Kannangara et al. 1994). Enzymes responsible for conversion of ALA to protogen IX i.e., ALA dehydratase, porphobilinogen deaminase, and uroporphyrinogen decarboxylase are mostly located in the stromal phase (Manohara and Tripathy 2000). Coproporphyrinogen oxidase activity is predominantly observed in the stroma and a small fraction in the envelope (Manohara and Tripathy, unpublished). Once synthesized in the stroma, protoporphyrinogen IX may associate either to the envelope or thylakoid membranes where it is oxidized to Proto IX. The presence of protoporphyrinogen oxidase is shown both in the envelope and thylakoid membranes (Matringe et al. 1992a). Part of plastidic protoporphyrinogen IX migrates to mitochondria where it is oxidised by protoporphyrinogen oxidase II to Proto IX, the substrate of heme synthesis (Lermontova et al. 1997). Substantial amount of Proto IX present in the stroma may be due to its own diffusion from the site of its synthesis i.e., envelope and thylakoids. The next step in Chl biosynthesis is the conversion of Proto IX to Mg-proto IX by Mg-chelatase. The association of MP(E) with envelope membranes suggests that Mg-chelatase is functional in envelope membranes. It is often argued that Chl biosynthetic enzymes present in envelope membranes are protein translocation intermediates. As there is an obligate requirement of three subunits of Mg-chelatase i.e., Chl D, Chl H and Chl I to assemble in a definite proportion to form the functional enzyme (Kannangara et al. 1997; Papenbrock et al. 1997; von Wettstein et al. 1995), it is unlikely that translocation intermediates of Mg-chelatase enzyme could mediate the synthesis of Mg-porphyrin. Proto IX is converted to Mg-Proto IX in the stroma in close association with inner envelope membrane or thylakoid. This explains the presence of MP(E) in the envelope membrane, stroma and thylakoids.

It is likely that the amphiphilic tetrapyrrole Mg-Proto IX subsequently migrates to both envelope and thylakoids where it is independently esterified to MPE and subsequently metabolized to Pchlide. The POR protein is present both in the envelope and thylakoid membranes and phototransform Pchlide to Chlide. As Chl synthetase is absent from envelope membrane, Chl synthesis is not advanced in the envelope membrane. Chlide present in the envelope membrane may play important role for stabilization of light-harvesting proteins during their post-translational protein import. Although envelope membranes participate in tetrapyrrole biosynthesis leading to the synthesis of Chlide, the major role is played by the thylakoid membranes during late steps of Chl biosynthesis. The detailed intraplastidic Chl biosynthesis pathway is shown in Fig. IV.
Regulation of chlorophyll metabolism

Regulation of chlorophyll metabolism is very important for proper functioning of different metabolic activities. Requirement of different tetrapyrroles in different cell types and their supply is changed with the development of the plant. For example, chlorophyll level dramatically increase during the initial stage of greening process of etiolated seedlings (Castelfranco et al., 1975 and Papenbrock et al., 2000b). Thus, there is a need for much greater allocation of intermediates to the Mg branch for Chl biosynthesis at this stage and after the development of matured chloroplast, the Chl supply should be distinctly controlled to maintain a functional photosynthetic apparatus. To achieve distinct control, plants must coordinately regulate the enzyme activities of the branch points as well as those of the common pathway.

The Mg and Fe (Heme) branches of tetrapyrrole biosynthesis are also regulated. Ferrochelatase, enzyme act on the Heme branch point is inhibited by ATP (Cornah et al., 2002). During the day time when ATP level is higher, the magnesium branch of the pathway
would be favored as Mg-chelatase needs ATP for Mg-proto IX synthesis. Conversely, in the night time the steady-state level of total heme increases in tobacco plants during the dark period and corresponds to the Fe-chelatase activity (Papenbrock et al., 1999). In Arabidopsis, the magnesium chelatase subunit ChlH reaches a peak at the beginning of the light phase and ferrochelatase reaches a peak at the end of the light phase, indicating a diurnal (Harmer et al., 2000) regulation of Mg and Fe branches of tetrapyrrole biosynthesis.

The most important external modulator of the tetrapyrrole pathway in plants is light. In angiosperms, it plays a direct role in the chlorophyll branch as light-dependent POR phototransforms most of Pchlide to Chlide that removes Pchlide-mediated feedback inhibition of ALA biosynthesis leading to its increased availability of proto IX for augmented ATP-dependent (light-dependent) Mg-chelation reaction. In spite of this many tetrapyrroles and chlorophyll intermediates are photodynamic molecules. These molecules absorb light and leads to the formation of reactive oxygen species resulting cell death. One important aspect of this control is the coordination of tetrapyrroles and cognate apoprotein synthesis, which is mainly attributed to transcriptional control (Masuda et al. 2008).

Some chlorophyll biosynthetic intermediates and catabolites act as signalling molecules for nuclear gene expression and plant cell death because of this generation and transduction of such signalling molecules must be tightly regulated (Masuda et al. 2008). Under different light environment plants change their chlorophyll antenna size of photosystems by changing the expression of light harvesting chlorophyll proteins (LHC) subsequently changing the interconversion of Chl a and b. Such qualitative control of chlorophyll is also important for efficient photosynthesis (Masuda et al. 2008).

The initial part of the chlorophyll biosynthesis pathway i.e. ALA formation is crucial for metabolic flow through the pathway and is generally accepted as the rate limiting in Chl synthesis. In maize, a lag-phase of about 3h for ALA synthesis exists prior to the formation of porphyrins suggesting an inhibition of ALA synthesis by Pchlide (Stobart and Ameen-Bukhari, 1984) or heme (Fig. 7). GluTR, one of the enzymes of ALA production from glutamyl-tRNA is the major regulatory point in the pathway. The gene encoding for GluTR, i.e., HEMA is regulated by hormones, the circadian clock (Kruse et al., 1997), by light, through the action of phytochrome and cryptochrome (McCormac and Terry MJ, 2002a, b; McCormac et al., 2001), and sugars (Ujwal et al., 2002). Thus multiple external factors can influence the flux through the tetrapyrrole pathway, by the synthesis of the initial precursor.

There is also evidence that heme, whose synthesis may be enhanced in the dark by the accumulation of pathway intermediates behind the block at POR, acts to repress ALA
synthesis, since GluTR activity is inhibited by heme (Vothknecht et al., 1996) (Fig. V). This is supported by the observation that phytochrome chromophore-deficient mutants, such as hy1 and hy2, have reduced protochlorophyllide and ALA synthesis in the dark (Papenbrock et al., 2001). The reduced ability to turnover heme may cause an increase in its levels. Tobacco plants transformed with antisense ferrochelatase RNA exhibited necrotic lesions induced by an accumulation of protoporphyrin IX (Papenbrock et al., 1999), whereas tobacco plants antisense for subunits of magnesium chelatase had a decrease in chlorophyll content in leaves, but did not show necrotic lesions, suggesting that porphyrin accumulation was prevented by heme feedback (Papenbrock et al., 2000a, 2000b).

The Arabidopsis flu mutant accumulates very high levels of Pchlide in the dark, and if the plants are exposed to white light, photobleaching is observed due to the phototoxicity of Pchlide (Goslings et al., 2004; Danon et al., 2005). Subsequently, FLU was shown to interact directly with GluTR in the yeast two-hybrid system (Goslings et al., 2004) and was specific for the GluTR. ulf3, a suppressor of flu gene was isolated which reduced ALA synthesis and Pchlide accumulation. It was found to be allelic to hy1; supporting the model that heme antagonizes the effect of the flu mutation by inhibiting GluTR independently (Goslings et al., 2004). The branch point for proto IX represents another regulatory step at which the quantitative distribution of intermediate is controlled in the direction of Chl and heme. In plastids, the insertion of Mg\(^{2+}\) or Fe\(^{2+}\) into the porphyrin ring determines the flux to either Chl or heme. Magnesium chelatase has a higher affinity for proto IX than Fe-chelatase (Guo et al., 1998). It suggested that while both the chelatases contribute to a coordinated allocation of proto IX, the excessive flow of proto IX is towards Chl synthesis in irradiated plants. Ferrochelatase is inhibited by ATP (Cornah et al., 2002), so in the light, when ATP levels are higher, the magnesium branch of the pathway would be favored; conversely, in the dark, magnesium chelation would be reduced. The steady-state level of total heme increases in tobacco plants during the dark period and corresponds to the Fe-chelatase activity (Papenbrock et al., 1999). In Arabidopsis, the magnesium chelatase subunit ChlH reaches a peak at the beginning of the light phase and ferrochelatase reaches a peak at the end of the light phase, indicating a diurnal rhythm (Harmer et al., 2000).

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

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

Regulation of tetrapyrrole biosynthesis in higher plants has been attributed to negative feedback control of steps prior to delta-aminolevulinic acid (ALA) formation. One of the first mutants with a defect in this control had been identified in barley. The tigrina (tig) d mutant accumulates 10-15-fold higher amounts of Pchlide than wild type, when grown in the dark. The identity of the TIGRINA d protein and its mode of action are not known yet. Initially this protein had been proposed to act as a repressor of genes that encode enzymes involved in early steps of ALA formation. The TIGRINA d gene of barley is an ortholog of the FLU gene of Arabidopsis thaliana (Lee et al., 2003). Phytochrome-interacting factor1 (PIF1) negatively regulates Chl biosynthesis in dark and its activity is negatively regulated by light (Huq et al., 2004). pif1 mutant seedlings accumulate excess free protoclorophyllide when grown in the dark, with consequent lethal bleaching upon exposure to light.

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

Nuclear genes control plastid differentiation in response to developmental and environmental signals. Chlorophyll biosynthesis pathway intermediates plays crucial role in plastid development and signaling between chloroplast to nucleus. Susek and Chory, 1992 isolated mutants that do not repress Lhcb transcription completely in Arabidopsis seedlings, in which chloroplast development is prevented due to photooxidative damage by norflurazon treatment. This group of mutants is referred to as gun for genomes uncoupled (Susek et al., 1993; Mochizuki et al., 2001). The lhcb1 expression is not repressed in norflurazon treated GUN5 mutant, as compared to wild type (Mochizuki et al., 2001). Interestingly transgenic Arabidopsis lines overexpressing porA or porB restored the loss of nuclear gene expression
due to the norflurazon treatment, indicating that the transgenic lines phenotypically resemble gun mutants (McCormac and Terry 2002, 2004).

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

**Vitamin E pathway**

The nutritional value of vitamin E in the human diet was recognized >75 years ago (Evans and Bishop, 1922). Vitamin E is made up of a group of structurally related compounds (vitamers), eight major forms of which are known to occur in nature: α-, β-, γ-, and δ-tocopherol and four corresponding unsaturated derivatives, α-, β-, γ-, and δ-tocotrienol (Fig. VI). Tocotrienols are distinguished from tocopherols by the presence of three double bonds in the isoprenoid side chain, and Greek letters that refer to the grade of methylation on the aromatic head group, with α-tocopherol or α-tocotrienol being the highest methylated isoforms (Fig. VI). Of these, α-tocopherol has the highest vitamin E activity in animals and humans (Sheppard et al. 1993; Bramley et al. 2000), presumably due to its preferred retention and distribution throughout the mammalian body (Traber and Sies 1996).

![FIGURE VI. Tocopherols and tocotrienols with major isoforms](image-url)
tocopherols are derived from their radical scavenging activity in lipophilic environments, resulting in the stabilization of polyunsaturated fatty acids in membrane lipids and oils. Recent studies using *Arabidopsis thaliana* mutants have provided experimental evidence supporting the protective role of tocopherols against oxidative stress (Kanwischer et al., 2005) as well as beneficial effects on seed longevity and germination as a result of lipid stabilization by tocopherols in plants (Sattler et al., 2004a).

Tocopherols and tocotrienols function as the principle lipid-soluble oxidation chain-breaking antioxidants in biological membranes and lipoproteins (Liebler 1998; Brigelius-Flohé et al. 2002). Tocopherols and tocotrienols are thought to have a number of vital functions in plants, including the protection of chloroplasts from photo oxidative damage (Munne-Bosch and Alegre, 2002). In contrast to cytoplasmic systems, such as the glutathione redox cycle, or the super oxide dismutase, which depend on enzymatic inactivation of oxygen radicals, the antioxidant reactions of tocochromanols do not require involvement of enzymes. The physiological role of tocopherols and tocotrienols is thought to be the protection of polyunsaturated fatty acids (PUFA) from lipid oxidation by quenching free radicals in cell membranes and other lipophilic environments (Kamal-Eldin and Appelqvist 1996).

Tocopherols are synthesized in photosynthetic microorganisms and plants, with the highest concentrations being found in seeds. The seeds of most plants have significant amounts of γ-tocopherol, whereas leaves have predominately α-tocopherol. Unfortunately, γ-tocopherol has only one-tenth the vitamin E activity of α-tocopherol. The major tocopherols are the highly abundant γ-tocopherol (60 to 65% of the total) and δ-tocopherol (20 to 26% of the total) (Tan, 1989). Manipulating the seed tocopherol biosynthetic pathway in soybean to convert the less active tocopherols to α-tocopherol could have significant human health benefits and make this crop an attractive target for the improvement of tocopherol composition.

All tocopherols are amphipathic molecules, with the hydrophobic tail associated with membrane lipids and the polar head groups remaining at the membrane surface. In plants, tocopherols are synthesized and localized in plastids (Soll et al., 1980, 1985; Lichtenthaler et al., 1981; Fryer, 1992; Kruk and Strzalka, 1995; Arango and Heise, 1998) and accumulate to varying degrees in all tissues, with seed generally containing the highest levels (Sheppard et al., 1993). ROS generated as byproducts of photosynthesis and metabolism are potential sources of lipid per oxidation in plant cells. All enzymes of the tocopherol biosynthetic pathway have been cloned and characterized in the past several years and exhibit a remarkable degree of evolutionary conservation between plants and cyanobacteria (Shintani
and DellaPenna, 1998; Collakova and DellaPenna, 2001; Porfirova et al., 2002; Shintani et al., 2002; Cahoon et al., 2003; Cheng et al., 2003; Sattler et al., 2003). The only exception is 2-methyl-6-phytyl-1,4-benzoquinol/2-methyl-6-solanyl-1,4-benzoquinol methyl transferase, which is encoded by evolutionarily unrelated protein families in cyanobacteria and plants (Shintani et al., 2002; Cheng et al., 2003). These cloned pathway enzymes have been used in various transgenic approaches to successfully modify the amount and types of tocopherols that accumulate in leaves and seeds (Shintani and DellaPenna, 1998; Savidge et al., 2002; Tsegaye et al., 2002; Cahoon et al., 2003; Collakova and DellaPenna, 2003a).

Although tocopherols are only synthesized by photosynthetic organisms, little is known concerning their functions in plants. Instead, because of their essential role in human nutrition as vitamin E, our understanding of tocopherol chemistry and function has been derived primarily from studies in artificial membranes and animal systems (Liebler, 1998; Azzi et al., 2000). Although the biological activities of tocopherols will likely differ in plants and animals because of fundamental differences in their biology, the chemical properties of tocopherols are likely to be the same between the two kingdoms. In vitro studies in animals and artificial membranes have shown that tocopherols interact with the polyunsaturated acyl groups of lipids, stabilize membranes, and scavenge and quench various reactive oxygen species (ROS) and lipid soluble byproducts of oxidative stress (Brigelius-Flohe and Traber, 1999; Wang and Quinn, 2000).

Singlet oxygen quenching by tocopherols is highly efficient, and it is estimated that a single α-tocopherol molecule can neutralize up to 120 singlet oxygen molecules in vitro before being degraded (Fukuzawa et al., 1982). Because of their chromanol ring structure, tocopherols are capable of donating a single electron to form the resonance-stabilized tocopheroxyl radical (Liebler, 1993; KamalEldin and Appelqvist, 1996), which contrasts with other phenolic antioxidants, such as hydroxyquinones, that must donate two electrons to attain a stable structure (Liebler and Burr, 2000). Tocopheroxyl radicals can also donate a second electron, resulting in opening of the chromanol ring to form the corresponding tocopherol quinone and other oxidized derivatives, which can also participate in electron transfer reactions (Liebler, 1998; Wang and Quinn, 2000).

Tocopherols also function as recyclable chain reaction terminators of polyunsaturated fatty acid (PUFA) radicals generated by lipid oxidation (Girotti, 1998). Tocopherols scavenge lipid peroxyl radicals and yield a tocopheroxyl radical that can be recycled back to the corresponding tocopherol by reacting with ascorbate or other antioxidants (Liebler, 1993). This property allows each tocopherol molecule to participate in many lipid peroxidation...
chain-breaking events before being degraded. Finally, recent studies in animal systems have also demonstrated other nonantioxidant functions of tocopherols related to the modulation of signal transduction pathways and transcription (Brigelius-Flohe and Traber, 1999; Sen et al., 2000; Chan et al., 2001; Ricciarelli et al., 2001; Yamauchi et al., 2001; Clement et al., 2002; Nobata et al., 2002). Whether some or all of the functions identified for tocopherols in animal systems also occur in plants remains to be determined.

Tocopherol levels increase in photosynthetic plant tissues in response to a variety of abiotic stresses (Munne-Bosch and Alegre, 2002), and this is often cited as circumstantial evidence for a protective role. However, the engineering of several-fold increase in Arabidopsis thaliana leaf tocopherol levels did not alter chlorophyll and carotenoid losses during high light stress in comparison with the wild type (Collakova and DellaPenna, 2003b). Mutations disrupting the tocopherol cyclase enzyme in plants (the VITAMIN E 1 [VTE1] locus in Arabidopsis result in tocopherol-deficient plants that accumulate the pathway intermediate 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) (Porfirova et al., 2002; Sattler et al., 2003). Despite this tocopherol deficiency, mature Arabidopsis vte1 plants are phenotypically indistinguishable from the wild type and exhibit only a minor decrease in photosynthetic efficiency after 5 d of high light treatment (Porfirova et al., 2002), whereas sxd1 plants have defective plasmodesmata between the bundle sheath cells and the vascular parenchyma that results in a defect in sucrose export from leaves (Provencher et al., 2001). These data suggest that tocopherol functions in photosynthetic tissues may be varied and species dependent. Alternatively, the presence of DMPBQ in tocopherol cyclase mutants may interfere with normal cellular processes in a species-specific manner.

In addition to photosynthetic tissues, seeds also accumulate tocopherols, often to the highest level of any plant tissue. The occurrence of high levels of tocopherols and PUFAs in seeds suggests that tocopherols may protect storage lipids from oxidation. However, like the tocopherols in photosynthetic tissues, functions for the high seed tocopherol levels are unclear, though attempts have been made to correlate seed tocopherol content with either seed PUFA levels or seed viability during storage. In some studies, a positive correlation has been reported, whereas other studies have failed to observe such a relationship (Wilson and McDonald, 1986; KamalEldin and Andersons, 1997).
FIGURE VII. Tocopherol biosynthetic pathway

TYRA- Bifunctional chorismate mutase-prephenate dehydrogenase, IPP-Isopentenylidiphosphate, DMAPP-Dimethylallyldiphosphate, GGH- Geranylgeranylidiphosphate hydratase, HPPD- p-hydroxyphenylpyruvate dioxygenase, MEP- 2-methyl-D-erythritol 4-phosphate, VTE1- Tocopherol cyclase, VTE2- Homogentisate phytoltransferase, VTE3- 2-methyl-6-phytylbenzoquinol methyltransferase, VTE4- \( \gamma \)-tocopherol methyltransferase, VTE5- Phytol Kinase

**Tocopherol Biosynthesis**

Tocopherols, synthesized by photosynthetic organisms, are micronutrients with antioxidant properties that play important roles in animal and human nutrition. Because of these health benefits, there is considerable interest in identifying the genes involved in tocopherol biosynthesis to allow transgenic alteration of both tocopherol levels and composition in agricultural crops. While most biochemical reactions leading to tocopherol biosynthesis were identified more than two decades ago (Soll and Schultz, 1980; Soll et al., 1980, 1983, 1985), genes encoding enzymes for tocopherol biosynthesis were identified relatively recently (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Porfirova et al., 2002; Savidge et al., 2002; Cheng et al., 2003; Rohmer, 2003; Van Eenennaam et al., 2003). The
first committed reaction in tocopherol biosynthesis is the condensation of homogentisic acid (HGA) with phytyldiphosphate or geranylgeranyldiphosphate, catalyzed by the homogentisate phytlytransferase (VTE2) or by the homogentisate geranylgeranyl transferase (HGGT) followed by cyclization and methylation reactions. Homogentisate phytlytransferase (HPT) performs the first committed step in this pathway, the phytylation of HGA. Homogentisic acid and phytyldiphosphate (PDP) serve as precursors for tocopherol biosynthesis. In plants, homogentisic acid is formed from Tyr via Tyr transaminase and p-hydroxyphenylpyruvate dioxygenase (Norris et al., 1998). PDP for tocopherol biosynthesis is thought to originate from direct reduction of geranylgeranyldiphosphate (GGDP) by geranylgeranyl diphosphate reductase (GGR). GGDP is proposed either to be directly reduced by GGR to PDP or to serve as substrate for chlorophyll synthase to form geranylgeranyl chlorophyll a. GGR reduces geranylgeranyl chlorophyll a to form chlorophyll a. Hydrolysis of chlorophyll by the action of chlorophyllase releases phytol, which can be reassimilated through two subsequent phosphorylation reactions with phytol kinase (VTE5) catalyzing a CTP-dependent phosphorylation of free phytol. Another enzyme, phytosphosphate kinase, is proposed for the second phosphorylation reaction to obtain PDP. PDP can enter the tocopherol biosynthetic pathway, be utilized for chlorophyll biosynthesis, or enter vitamin K (phylloquinone) biosynthesis.

**Vitamin E pathway gene 5 (VTE5)**

Phytol is the most abundant acyclic isoprenoid compound in the biosphere and it constitutes approximately one-third of the mass of chlorophyll (Volkman and Maxwell, 1986). Phytol acts as a hydrophobic membrane anchor of chlorophyll in the photosynthetic membrane and it plays essential roles in the assembly, structure, and function of both plant and microbial photosynthetic reaction centers (Ben-Shem et al., 2003). In photosynthetic organisms, free phytol is generated during chlorophyll catabolism by Chlorophyllase. During senescence, fruit ripening and abiotic stress huge amount of chlorophyll is hydrolysed by Chlorophyllase in Chlorophyllide a and phytol. While the subsequent fate of the chlorophyll breakdown product chlorophyllide a and its derived pigments has been well characterized, little is known about the metabolic fate of phytol. Previously it was known that phytol derived from chlorophyll breakdown is reesterified to acetic acid or other fatty acids (Peisker et al., 1989; Patterson et al., 1993), while a portion is also irreversibly destroyed by photodegradation (Rontani et al., 1996). Recent work of Ischebeck et al., 2005 and Valentín et al., 2005 identified a salvage
pathway for phytol metabolism. They suggested that phytol which is generated from the degradation of chlorophyll during senescence and abiotic stress can be incorporated in chlorophyll, tocopherol, phylloquinone and phytol esters (Fig. VIII). Ischebeck et al., 2005 also suggested that, presence of two pathways for chlorophyll biosynthesis in plants. (I) geranylgeranyl diphosphate dependant pathway and (II) geranylgeranyl diphosphate independent pathway, in which chlorophyll degradation products are recycled for again chlorophyll biosynthesis (Fig. IX).

By analysing low tocopherol Arabidopsis thaliana mutant, vitamin E pathway gene5-1 (vte5-1), with seed tocopherol levels reduced to 20% of the wild type, Valentin (Valentin et a., 2006) reported a major pathway (Fig. VIII) for the production of phytyldiphosphate and he identified a new gene Vitamin E pathway gene 5-1 (VTE5-1) encoding a phytol kinase which is involved in Chl, tocopherol and vitamin K biosynthesis. According to this pathway chlorophyll a is hydrolysed by chlorophyllase and pheophytinase in to chlorophyllide a and phytol. Chlorophyllide a is used for the synthesis of chlorophyll a by chlorophyll synthase and released phytol is used for the synthesis of phytyldiphosphate through two phosphorylation steps. First the phytol is converted in to phytyl phosphate by CTP-dependent phytol kinase followed by again phosphorylation of phytyl phosphate in to phytyldiphosphate by an unidentified kinase phytlyphosphate kinase. Now this phytyldiphosphate and chlorophyllide a which comes from chlorophyll degradation form chlorophyll a by chlorophyll synthase, ie: phytyldiphosphate is intermediate of both chlorophyll biosynthesis and chlorophyll degradation. Phytyldiphosphate also act as a precursor for tocopherol biosynthesis and vitamin K1 (phylloquinone) biosynthesis.
FIGURE VIII. Schematic Drawing of the Tocopherol Biosynthetic Pathway.

The metabolic fate of GGDP is shown in detail. Enzyme designations are shown in italicized bold text. GGDP is proposed either to be directly reduced by GGR to PDP or to serve as substrate for chlorophyll synthase to form geranylgeranyl chlorophyll a. GGR reduces geranylgeranyl chlorophyll a to form chlorophyll a. Hydrolysis of chlorophyll by the action of chlorophyllase releases phytol, which can be reassimilated through two subsequent phosphorylation reactions with phytol kinase (VTE5) catalyzing a CTP-dependent phosphorylation of free phytol. Another enzyme, phytyl phosphate kinase, is proposed for the second phosphorylation reaction to obtain PDP. PDP can enter the tocopherol biosynthetic pathway, be utilized for chlorophyll biosynthesis, or enter vitamin K (phylloquinone) biosynthesis. MEP, methylerthritol phosphate pathway. (Adapted from Valentin et al., 2005)
FIGURE IX. Phytol metabolism in Arabidopsis. A, phytanyl-PP can be synthesized de novo via the mevalonate or methyl-erythritol-phosphate pathway. Subsequently, phytanyl-PP is the substrate for the synthesis of chlorophyll, phyloquinone, and tocopherol. B, phytol released from chlorophyll by chlorophyllase can be phosphorylated by two sequential kinase reactions. Furthermore, free phytol may be degraded or acylated, resulting in the production of fatty acid phytol esters. (Adapted from Ischebeck et al., 2005)

Bioinformatic analysis of the deduced VTE5 amino acid sequence revealed homology to the archaea type phosphatidate cytidylyltransferase (EC 2.7.7.41) and yeast dolichol kinase (EC 2.7.1.108). TMHMM algorithm (Krogh et al., 2001), analysis revealed that it is a transmembrane protein having six transmembrane helices corresponding to amino acids 29 to 51, 66 to 88, 105 to 122, 127 to 149, 168 to 187, and 226 to 248. A putative paralog (gi
number 51970322, ATG ID At5g58560) of VTE5 is also present in Arabidopsis genome which shows 38.7% homology at amino acid level. At5g58560 was also found to contain the PFAM domain of integral membrane protein cytidylyltransferases (CTP_transf_1) and also includes six TMHMM-predicted transmembrane helices. Its homologous sequences are also present in archaea, eubacteria, cyanobacteria, and plants. Sequence analysis of the deduced amino acid sequences of plant-derived VTE5 orthologs using ChloroP (Emanuelsson et al., 1999) predicted chloroplast targeting for all plant genes.

Heterologous Expression of *Arabidopsis* VTE5 and *Synechocystis* Vte5 Proteins in Escherichia coli clearly demonstrate its phytol kinase activity. E. coli cells transformed with the plasmid expressing *Arabidopsis* VTE5 (pET30b-VTE5) contained >70-fold more phytol monophosphate (PMP) than the vector control in the presence of exogenously added phytol. Enzyme assay of washed E. coli membrane fractions from strains expressing *Synechocystis* Vte5 or Arabidopsis VTE5 confirmed that both enzymes exhibited highest activity using CTP as cosubstrate. ATP and GTP did not support the phytol kinase reaction.

Analysis of ATGENEXPRESS 22K Affymetrix ATH1 transcript profiling data using GENEVESTIGATOR software revealed that VTE5 mRNA expression is relatively high in 6-week-old senescent *Arabidopsis* leaves compared with slightly younger vegetative plants (Valentin et al., 2005). The gene is also highly expressed early in seed development (Valentin et al., 2005). VTE5 expression is sensitive to metal induced oxidative stress. After prolonged incubation of *Arabidopsis* leaves in Cd stress resulted upregulation of VTE5 transcript and under Cu stress its expression was slightly decreased (Collin et al., 2008).

Biotechnological approaches to modify vitamin E content have to take into account the complexity of plastid prenyl lipid synthesis, because geranylgeranyl diphosphate, phytot diphosphate and homogentisic acid are not only the substrates for vitamin E synthesis, but are also critical for the production of many other compounds important for plant development. Two additional substances with vitamin activity, β-carotene (provitamin A) and phylloquinone (vitamin K1), are synthesized from geranylgeranyl diphosphate and phytot diphosphate, respectively. Furthermore, the synthesis of photosynthetic pigments and electron acceptors (chlorophyll, carotenoids, plastoquinone-9) and of two phytohormones (gibberellins, abscisic acid) depends on the plastid isoprenoid pathway.

Tobacco ChIP (geranylgeranyl reductase) which converts geranylgeranyl diphosphate to phytot diphosphate, substrate for Chl and tocopherol biosynthesis, antisense plants had increased accumulation of geranylgeranyl Chl and reduction in total Chl and tocopherol content. Reduced total Chl content in these plants resulted reduced electron
transport chain without a concomitant effect on the stoichiometry, composition and activity of both photosystems. These ChlP antisense plants were much more sensitive to light stress and had high qL (photoinhibitory quenching) and decreased Fv/Fm in the high light condition, it was due to decreased tocopherol content (Grabes et al., 2001).

Tocopherols limit nonenzymatic lipid peroxidation during germination and early seedling development (Sattler et al., 2006). This was confirmed by studying Arabidopsis vte2 (homogentisate phytyltransferase) mutant. VTE2 catalyzes condensation of homogentisic acid and phytyle diphosphate or geranylgeranyl diphosphate the first committed step of tocopherol biosynthesis. This mutant lacked all form of tocopherols. This mutant displayed severe seedling growth defects, which corresponded with massively increased levels of the major classes of nonenzymatic lipid peroxidation products: hydroxyl fatty acids, malondialdehyde, and phytoprostanes (Sattler et al., 2006). Antisense expression of HPT1 in Arabidopsis resulted in reduced (10 fold) seed tocopherol levels, whereas seed-specific sense expression resulted in increased (60%) seed tocopherol levels (Savidge et al., 2002). HPT1 overexpression in Arabidopsis resulted in 10-fold increase in HPT specific activity and 4.4 fold increase in total tocopherol content relative to wild type (Collakava et al., 2003). Its overexpression in seeds resulted 4-fold increase in HPT activity and 40% higher total seed tocopherol content primarily because of increase in γ-tocopherol content. This enlarged pool of γ-tocopherol was almost entirely converted to α-tocopherol in HPT1 and VTE4 (γ-tocopherol methyltransferase) double transgenic plants. HPT1 (VTE2) and VTE4 double transgenic had 12-fold increased vitamin E activity to wild type. These results indicate that HPT activity is limiting in various Arabidopsis tissues (Collakava et al., 2003).

Arabidopsis vte3 (2-methyl-6-phytylbenzoquinol methyltransferase) which converts 2-methyl-6-phytylbenzoquinol to 2,3-Dimethy-5-phytylbenzonquinol, mutant had 25 fold increased δ-tocopherol and this change in δ-tocopherol was accompanied by a reduction of γ-tocopherol in the seeds, resulting in no net change in total tocopherol levels (Eenennaam et al., 2003).

Arabidopsis vte1 (tocopherol cyclase) which converts 2,3-Dimethy-5-phytylbenzonquinol in to γ-tocopherol, mutant lacks all forms of tocopherol. Growth of the vte1 mutant, chlorophyll content, and photosynthetic quantum yield were similar to wild type under optimal growth conditions. Therefore, absence of tocopherol has no large impact on photosynthesis or plant viability, suggesting that other antioxidants can compensate for the loss of tocopherol. During photo-oxidative stress, chlorophyll content and photosynthetic quantum yield were slightly reduced in vte1 as compared with wild type indicating a potential
role for tocopherol in maintaining an optimal photosynthesis rate under high-light stress (Porfirova et al., 2002). VTE1 overexpression in Arabidopsis plants resulted 7 fold increase in leaves total tocopherol in which γ-tocopherol was the major tocopherol form. Tocopherol deficiency in vte1 resulted in the increase in ascorbate and glutathione, whereas accumulation of tocopherol in VTE1 overexpressing plants led to a decrease in ascorbate and glutathione. Deficiency in one antioxidant in vte1, vtc1 (ascorbate deficient), or cad2 (glutathione deficient) led to increased oxidative stress and to the concomitant increase in alternative antioxidants. vte1, vtc1, cad2, or vte1vtc1 mutant had similar growth, slightly reduced chlorophyll content and similar photosynthetic quantum yield as that of wild type. Whereas vte1cad2 mutant has reduced growth, Chl, carotenoids and photosynthetic quantum yield suggesting that reduction of entire photosynthetic unit. These mutants study showed that the simultaneous loss of tocopherol and glutathione results in moderate oxidative stress that affects the stability and the efficiency of the photosynthetic apparatus (Kanwischer et al., 2005).

The mutant Arabidopsis vte4-1, γ-tocopherol methyltransferase involved in the conversion of γ-tocopherol to α-tocopherol, contains high level of accumulated γ-tocopherol, whereas α-tocopherol was absent (Bergmüller et al., 2003). Over-expression of the γ -TMT cDNA in vte4-1 restored wild-type tocopherol composition. During oxidative stress (high light, high temperature, cold treatment) the amounts of α-tocopherol and γ -tocopherol increased in wild type, and γ -tocopherol in vte4-1. However, Chl content and photosynthetic quantum yield were very similar in wild type and vte4-1. This suggest that α-tocopherol can be replaced by γ -tocopherol in vte4-1 to protect the photosynthetic apparatus against oxidative stress. Fatty acid and lipid composition were very similar in WT, vte4-1 and vte1, an Arabidopsis mutant previously isolated which is completely devoid of tocopherol. Therefore, a shift in tocopherol composition or the absence of tocopherol has no major impact on the amounts of specific fatty acids or on lipid hydrolysis (Bergmüller et al., 2003). Overexpression of Arabidopsis γ-tocopherol methyltransferase in Brassica leads to 6-fold increase in α-tocopherol in seeds (Yusuf et al., 2007). These transgenic plants showed tolerance to salt, heavy metal and osmotic stress. Photosynthetic performance of these transgenic was also better in stress condition due to higher tocopherol content (Yusuf et al., 2010).

Carotenoids and tocopherols functionally interact or have complementary or overlapping roles in protecting Synechocystis sp.strain PCC 6803 from lipid peroxidation and HL stress (Maeda et al., 2005). α-tocopherol has a continuous turnover as a scavenger of the
Singlet oxygen induced oxidative stress in plants

The activation or reduction of oxygen gives rise to reactive oxygen species (ROS) that includes the singlet oxygen ($^{1}\text{O}_2$), superoxide ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$) and hydroxyl radical (HO). Plants and other living organisms in the oxidizing environment constantly produce ROS in chloroplasts, mitochondria, peroxisomes and other sites of the cell because of their metabolic processes such as photosynthesis and respiration. The generation of ROS in plants increases by different kinds of environmental stresses, such as high light, high or low temperature, salinity, drought, nutrient deficiency and pathogen attack. (See Pareek et al. (eds), 2009 for a book on abiotic stress in plants.) Plants and other living organisms have evolved a host of anti-oxidants and anti-oxidative enzymes and other small molecules to harmlessly dissipate ROS. Imbalance between ROS production and their detoxification by enzymatic and non-enzymatic reactions causes oxidative stress. As a result of higher net ROS formation, there is photooxidative damage to DNA, Proteins and lipids and ultimately cell death. Recent studies also indicate that ROS can act as signaling molecules involved in growth and developmental processes, pathogen defense responses such as hypersensitive reaction and systemic acquired resistance, stress hormone production, acclimation and programmed cell death (Apel and Hirt, 2004).

Formation of Singlet Oxygen in Plants

Oxygen in its ground state is not very reactive and does not have any deleterious effect. The ground state molecular oxygen is a triplet state ($^3\text{O}_2$) and in fact a diradical, as it has two unpaired electrons. Its two unpaired electrons have parallel spins (↑↑) that does not allow
them to react with most molecules. However, if the triplet oxygen absorbs sufficient energy, the spin restriction is removed and the spin of one of its unpaired electrons is reversed. As a result, there is generation of singlet oxygen ($^1\text{O}_2$), whose outermost pair of electrons has antiparallel spins (↑↓). Singlet oxygen molecules are also formed when superoxide radicals interact with hydroxyl radicals. The excitation energy required to produce $^1\text{O}_2$ from the triplet oxygen is 94 kJ mol$^{-1}$ (Skovsen et al., 2005). It has a short half-life of about 200 ns in cells with a possible diffusion distance of about 270 nm and could even diffuse out of the chloroplast into the cytosol (Skovsen et al., 2005).

In plants, $^1\text{O}_2$ is mainly produced by the chlorophyll (Chl) and its tetrapyrrolic intermediates in the presence of light (Fig. X). Chl, the most abundant pigment in land plants, is the main light absorbing pigment and is present both in the light harvesting complex (LHC) and the photosynthetic reaction centers. These molecules have extra benefit that their excited states are long lived to allow conversion of the excitation energy into an electrochemical potential via charge separation. Inefficient transfer of energy results in the generation of triplet state Chl that reacts with triplet oxygen to produce the highly reactive $^{3}\text{O}_2$ (Fig. X). In the light harvesting complex, the $^1\text{O}_2$ is quenched by the carotenoids. (For further information on the photochemistry of carotenoids, see Frank et al. (Eds), 1999.)

Singlet oxygen ($^1\text{O}_2$) is produced near the reaction centers of the photosystems (Fig. X). With increase in light intensity i.e., from the early morning to the noon, light absorption by leaves increases almost linearly. However, the rate of photosynthesis reaches the maximum value much before the linear increase in light absorption ceases. Therefore, the plants end up in absorbing more light than they could utilize in photosynthesis. This results in an overexcitation of the photosynthetic apparatus. In the presence of excess light energy, the Q$_A$ and Q$_B$ (the first and second plastoquinone electron acceptors of Photosystem II, PS II) in the electron transport chain are over reduced (Barber and Andersson, 1992) and because of that, charge separation cannot be completed between P680 and pheophytin and the triplet state of the reaction center Chl P680 ($^3\text{P}_680$) is favored (Aro et al., 1993; Ohad et al., 1994) leading to the formation of $^1\text{O}_2$ (Foote et al., 1984). Normally when excess light is absorbed, an alternative dissipating pathway is activated that safely returns $^1\text{Chl}$* to its ground state before it can convert to $^3\text{Chl}$*. The excitation energy of excess $^1\text{Chl}$* is dissipated by zeaxanthin or other carotenoids as heat in Chl and/or in carotenoid binding protein complexes (Baroli and Niyogi, 2000; Baroli et al., 2003, 2004; Davison et al., 2002; Pogson and Rissler, 2000). The carotenoids, which quench the excited state of Chl, must be in close proximity with triplet
Chl i.e., within maximum distance of 3.6 Å. In this spin exchange reaction, the triplet state of carotenoid is formed that can dissipate the excess energy as heat. In the reaction center, distance between Chl and carotenoid is too large to allow triplet quenching. $^1$O$_2$, produced in the reaction center, directly reacts with carotenoids. The release of $^1$O$_2$ is also detected in isolated PS II particles (Macpherson et al., 1993) and in thylakoids (Chakraborty and Tripathy, 1992; Fryer et al., 2002, Hideg et al., 1998). $^1$O$_2$ is also generated from the cytochrome b6f complex (Suh et al., 2000) (Fig. 8).

**FIGURE X.** Intracellular generation of $^1$O$_2$ from chlorophyll and heme biosynthesis intermediates and photosystems. (Adopted from Tripathy and Pattanayak, 2010)
Generation of Singlet Oxygen from Chlorophyll Biosynthesis Intermediates

Upon illumination, Chl biosynthesis intermediates i.e., protochlorophyllide (Pchlide) or protoporphyrin IX (Proto IX) produce $^{1}\text{O}_2$ in plants and cause oxidative damage (Chakraborty and Tripathy, 1992; Op den Camp et al., 2003; Tripathy et al., 2007). Formation of active oxygen species, from Chl biosynthesis intermediates, was proposed by several others (Jung et al., 2008; Lermontova and Grimm, 2006; Mock and Grimm, 1997; Rebeiz et al, 1984, 1988, 1990; Shalygo et al., 1998; Tripathy and Chakraborty 1991). The site of generation of $^{1}\text{O}_2$ is mostly thylakoids. This is because Chl biosynthesis intermediates are partially hydrophobic, and consequently are loosely attached to the thylakoid membranes (Mohapatra and Tripathy, 2002, 2007; Tripathy et al., 2004). Although they are associated with the thylakoid membranes, these tetrapyrroles do not form pigment protein complexes and hence are not connected to the reaction center. Although some of the carotenoids are present in the lipid bilayer, a lot more are located in the pigment-protein complexes and they are spatially too far from Chl biosynthesis intermediates to quench their triplet states (Havaux et al., 2007; Mozzo et al., 2008). Synthesis of Chl biosynthetic intermediates are highly regulated and are not overproduced in plants. However, Chl biosynthesis intermediates that are normally present in plants are capable of producing $^{1}\text{O}_2$ that cause oxidative damage in high light and several other stress conditions (Chakraborty and Tripathy, 1992).

Type I and Type II Photosensitization Reactions of Tetrapyrroles

In type II photosensitization, the sensitizer can transfer its excitation energy to a ground state oxygen molecule, resulting in singlet oxygen ($^{1}\text{O}_2$). This highly reactive form of oxygen can oxidize substrates of biological importance such as lipids and proteins (Foote et al., 1984) and affect the metabolic pathways. ALA induced accumulation of non-phototransformable Pchlide in presence of light generates singlet oxygen through type II photosensitization reaction, which destroys the plant (Tripathy and Chakraborty, 1991; Chakrabory and Tripathy, 1990, 1992a, 1992b). However, $^{1}\text{O}_2$ is quite selective and fails to react with molecules that are not electron rich enough and simply returns to the ground state.

Type I photosensitization involves hydrogen atom or electron transfer from the sensitizer to the substrate (Spikes & Bommer, 1991). The resulting free radicals can subsequently react with $\text{O}_2$ to produce oxidized products or other reactive species. The
products are often peroxides, which can in turn break down to induce free radical chain autoxidation, leading to further oxidation in a non-photochemical step.

\[ \text{LOO}^- + \text{LH} \rightarrow \text{LOOH} + \text{L} \]

Sensitizers can produce superoxide radical (\( \text{O}_2^- \)) by undergoing electron transfer processes with the substrate or \( \text{O}_2 \), as shown below.

\[ ^3\text{Sens} + \text{Subs} \rightarrow ^1\text{Sens}^- + \text{Subs}_{\text{ox}} \]

\[ ^1\text{Sens}^- + \text{O}_2 \rightarrow ^1\text{Sens} + \text{O}_2^- \]

or

\[ ^3\text{Sens} + \text{O}_2 \rightarrow ^1\text{Sens}^+ + \text{O}_2^- \]

These reactions produce \( \text{O}_2^- \), which can subsequently give rise to the highly reactive hydroxyl radical (\( \text{OH}^- \)). The hydroxyl radicals thus produced can react with organic molecules in a variety of ways or can initiate radical chain autoxidation.

**Intracellular Destruction of Singlet Oxygen**

The most efficient mechanism of detoxification of \( ^1\text{O}_2 \) in plants involves carotenoids. The carotenoids reach the triplet excited state by absorbing the excess energy of \( ^1\text{O}_2 \) and return it to its triplet ground state \( ^3\text{O}_2 \), and finally dissipates the excess acquired energy as heat (Edge et al., 1997). The physical quencher has to be lipid soluble and needs to be in very close proximity with the photosensitizer. Carotenoids, because of their conjugated double bonds, are most abundant quenchers of \( ^1\text{O}_2 \) in the pigment bed of photosynthetic apparatus. Carotenoids are usually not destroyed or oxidized while quenching the \( ^1\text{O}_2 \). Photosynthetic antenna systems have several xanthophylls i.e., lutein, violaxanthin, neoxanthin, and zeaxanthin. Out of these, lutein is most abundant as it is needed for efficient quenching of \( ^3\text{Chl}^* \). Zeaxanthin is synthesized from violaxanthin under high-light stress by the violaxanthin deepoxidase enzyme and is involved in energy dependent quenching of Chl a fluorescence (see e.g., Demmig-Adams et al. (eds), Mozzo et al., 2008). Tocopherol is lipid soluble and is a minor but significant component of \( ^1\text{O}_2 \) quenchers present in the thylakoid membrane. The suppression of both zeaxanthin and tocopherol in the \( npq1/vte1 \) double mutant results in \( ^1\text{O}_2 \)-mediated lipid peroxidation in high light (Havaux et al., 2005; Triantaphylides et al., 2008). In the PS II reaction center, especially in high light regime, \( ^1\text{O}_2 \) is quenched by \( \beta \)-carotene and \( \alpha \)-tocopherol (Trebst, 2003).
On the other hand, the scavengers of $^{1}\text{O}_2$ are usually water soluble and are themselves oxidized or destroyed while detoxifying the ROS. The oxidized scavenger is re-reduced by a set of biochemical reduction reactions at a tremendous cost to the cell. The cell has only limited capability to resynthesize the destroyed scavengers. Therefore, the cells become extremely prone to $^{1}\text{O}_2$ attack. Ascorbate is an example of $^{1}\text{O}_2$ scavenger that is oxidized after detoxification. It is predominantly present in the plastids. The $^{1}\text{O}_2$ reacts with ascorbate to produce Dehydroascorbate (Kramarenko et al., 2006). The latter is converted back to ascorbate by the dehydroascorbate reductase, and glutathione reductase enzymes involved in the Halliwell-Asada pathway. The vitamin B6 (pyridoxine, pyridoxal, pyridoxamine) can efficiently scavenge $^{1}\text{O}_2$ (Bilski et al., 2000; Ehrenshaft et al., 1999). The fungus Cercospora secretes cercosporin, a $^{1}\text{O}_2$-generating photosensitizer into the extracellular matrix during plant infection (Ehrenshaft et al., 1999). The cercosporin secreted to the host cell by the fungus absorbs solar energy and transfers its energy to oxygen to generate $^{1}\text{O}_2$ that kills the host cell. However, the fungus itself is protected against $^{1}\text{O}_2$-mediated damage by the $^{1}\text{O}_2$-scavenger vitamin B6. In the same vein, the pyridoxine synthase is involved in tolerance to the oxidative stress (Chen and Xiong, 2005). Similarly, exogenous vitamin-B6 protects protoplasts of the flu (fluorescence) mutants of *Arabidopsis thaliana* that generated $^{1}\text{O}_2$ (Danon et al., 2005). Flavonoids that are present in plants in high concentrations in the cytoplasm and isoprene that is mostly synthesized in the chloroplasts could also function as $^{1}\text{O}_2$ quenchers (Affek and Yakir, 2002; Agati et al., 2007; Nagai et al., 2005; Velikova et al., 2004). The water soluble chlorophyll binding protein (WSCP) binds to the free Chl molecules as well as its biosynthesis pathway intermediates and does not allow them to get photoactivated to produce $^{1}\text{O}_2$. It acts as a physical barrier in between the free Chl molecules and the molecular oxygen (Schmidt et al., 2003).

**Singlet Oxygen-mediated Oxidative Damage to the Photosynthetic Apparatus**

Although, plants may have the capacity to detoxify $^{1}\text{O}_2$, they have limited ability to do so. Moreover, unlike $\text{O}_2$, where extensive net works of enzymes are available in plants for its detoxification, there are no known enzymatic means for the detoxification of $^{1}\text{O}_2$. Therefore, if excess $^{1}\text{O}_2$ is generated, plants fail to detoxify the system.
Generation of Tetrapyrrole-induced Singlet Oxygen in Chloroplasts

The Diphenyl ether (DPE) compounds accumulate Proto IX, instead of Pchlide as photosensitizer (Ensminger and Hess, 1985; Gupta and Tripathy, 1999, 2000; Haworth and Hess, 1988; Kunert and Boger, 1981; Yoshimoto and Matsunaka, 1982; Witkowski and Halling, 1987). Pchlide (Chakraborty and Tripathy, 1992) or Proto IX (Tripathy et al., 2007) produced in response to treatments by ALA or DPEs, respectively, generate excess $^{1}O_2$ via type II photosensitization reactions. ALA-induced oxidative stress is photosensitized by the Chl biosynthetic intermediate Pchlide (Tripathy and Chakraborty, 1991) whereas that induced by AF-Na is mediated by the tetrapyrrole Proto IX (Duke et al., 1991; Gupta and Tripathy, 1999). $^{1}O_2$ is generated by both the photo-sensitizers i.e., Pchlide and Proto IX. As 98% of Pchlide and 65% of Proto IX are localized in the thylakoid membranes, they are the primary sites of generation of $^{1}O_2$ inside the plastid. As these photosensitizers are partially partitioned to the chloroplast envelope membranes (Mohapatra and Tripathy, 2002, 2007) they too could be a potential site for generation of $^{1}O_2$.

Singlet Oxygen-induced Impairment of the Electron Transport Chain

Photosystem I (PS I) and Photosystem II (PS II) are two major functional units of thylakoid membrane (see Golbeck, 2006; and Wydrzynski and Satoh, 2005, for PS I and PS II, respectively). Therefore, any damage to the thylakoid membrane is likely to affect the activities of the above photosystems. $^{1}O_2$ impairs the PS I- and PS II-dependent electron transport reactions within a few hours of light exposure of ALA or DPE-treated plants. However, the ALA-induced damage to the thylakoid membranes is more severe than that induced by DPE. The differential extent of injury to the thylakoid membrane caused by the photosensitizer Pchlide or Proto IX is due to increased generation of $^{1}O_2$ induced by Pchlide as compared to Proto IX and their differential localization in the cell. Pchlide is mostly bound to thylakoid membrane, whereas Proto IX is distributed among soluble and membrane fractions (Tripathy et al., 2004; Mohapatra and Tripathy, 2007). $^{1}O_2$ generated by the thylakoid membrane-localized photosensitizer Pchlide could immediately penetrate the membrane and cause quick damage to the photosynthetic apparatus. On the other hand, $^{1}O_2$ produced in the stroma is significantly quenched in the aqueous phase before they reach the membranes. Therefore, AF-Na-induced oxidative stress is probably slower than that of ALA.

The PSII activity of thylakoid membranes, isolated from ALA-treated plants, when exposed to moderate light (250 μmoles photons m-2 s⁻¹) for 30 min is impaired by 60%. The
exogenous scavengers of $^{1}\text{O}_2$, histidine and NaN$_3$, protect the PSII activity demonstrating that the damage to the thylakoid membrane is caused by this active oxygen species (Chakraborty and Tripathy, 1991). Scavengers of the hydroxyl radical formate and O$_2^-$ detoxifier superoxide dismutase fail to protect the thylakoid membranes suggesting that these active oxygen species may not be involved in the tetrapyrrrole-sensitized oxidative damage.

**Impact of $^{1}\text{O}_2$ on Chlorophyll a Fluorescence**

The $^{1}\text{O}_2$ generated in the plastids, due to photosensitization reaction of Chl biosynthesis intermediates, induces damage to the thylakoid membranes. In the ALA-treated plants exposed to sunlight for 30 min and 1 h, the apparent minimum Chl a fluorescence level (Fo) remains almost the same as that of the control. However, the apparent variable Chl a fluorescence ($F_v= F_{\text{max}}$ (or $F_p$) minus Fo) is reduced by 60% after 30 min and 78% after 1 h of exposure to sunlight (Tripathy and Chakraborty, 1991). Plants exposed to 15 min of sunlight do not exhibit any physical symptoms of oxidative damage. However, the same plants when kept in dark for 12 h show physical injuries which include prominent necrotic patches. This results in the decline in the Fo fluorescence. (For a description of Chl a fluorescence, see Papageorgiou and Govindjee, 2004.) The exogenous electron donors to PS II reaction center, MnCl$_2$, diphenylcarbazide and hydroxylamine fail to restore the loss of variable fluorescence ($F_v$) suggesting that the damage to PS II is very close to the reaction center (Tripathy and Chakraborty, 1991; Tripathy, 1993).

The PS II and PS I photochemical reactions are impaired due to oxidative stress induced by $^{1}\text{O}_2$ (Krieger-Liszzkay et al., 2008). Due to gross perturbation of the thylakoid membrane structure, a spectral shift in the Chl a fluorescence spectra of thylakoid membranes is observed at low temperature (77K). The low temperature fluorescence spectra have peaks at 685 nm (F685) and at 695 nm (F695), which mostly originate from the PS II CP 43 and CP 47 respectively (Govindjee, 1995, 2004) and a large F735 peak that originates mostly from PSI (Mullet et al., 1980). If LHCI is removed from PS I by detergent treatment, the inner antenna of reaction center I (RCI) fluoresces at 722 nm (Kuang et al., 1984). Isolated LHCI fluoresces around 735 nm (Haworth et al., 1983). Thus it is apparent that the inner antenna of RCI emits F722 and LHCI emits F735 (Briantais et al., 1986). The relative decline in F735 by $^{1}\text{O}_2$-induced damage may be due to damage to LHCI. $^{1}\text{O}_2$ produced by photosensitization reaction of ALA-induced Pchlide or DPE-treated Proto IX cause almost similar structural alteration of the thylakoid membrane i.e., disintegration of LHCI (Tripathy and Chakraborty, 1991, Tripathy et al., 2007). This is evident from the peak shift from 735 nm (attributed to
LHCl) to 728 nm (Fig. 5). The fluidity of thylakoid membranes isolated from ALA-treated leaves decreases. The decrease in delayed Chl fluorescence intensity coincides with an increase in P700\(^+\) formation, which indicates disturbance in electron transfer between the two photosystems (Hartel et al., 1993a, 1993b). Formation of \(^1\)O\(_2\) from isolated LHClI was also observed (Rinalducci et al., 2004).

Due to membrane lipid peroxidation by \(^1\)O\(_2\), the MDA (malondialdehyde) content in ALA-treated as well as DPE-treated light-exposed plants increases. Scavengers of \(^1\)O\(_2\), i.e., L-histidine and sodium azide are able to protect the oxidative damage to the thylakoid membrane and consequently reduce the MDA production (Chakraborty and Tripathy, 1992). Among superoxide detoxifying enzymes, present in the stroma, superoxide dismutase and ascorbate specific peroxidase are not affected by \(^1\)O\(_2\), whereas glutathione reductase activity is impaired suggesting that superoxide-detoxifying capability of chloroplasts is downregulated due to generation of \(^1\)O\(_2\) (Tripathy and Singhal, 1999).

**Effect of Singlet Oxygen on Thermoluminiscence**

Thermoluminescence (TL) from plants, discovered by Arnold and Sherwood (1957) is a useful probe of the back reaction of PS II (Demeter and Govindjee, 1989; Horváth, 1986; Sane and Rutherford, 1986; Vass, 2005; Vass and Govindjee, 1996). The TL bands at 12°C, 25°C and 48°C are due to Q, B and C bands respectively. The Q (also called D) band is due to charge recombination of the reduced primary quinone acceptor \(Q_A^-\) and S2/3 states whereas the B band arises due to charge recombination of the reduced secondary quinone acceptor \(Q_B^-\) with S2/3 states and the C band emanates from charge recombination of \(Q_A^-\) with the S state, S1 (see e.g., De Vault et al., 1983; Rutherford et al., 1982). The intensification of Q band by the addition of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is accompanied by the loss of the B band and increase of the C band. A residual B band, observed in the presence of DCMU, is due to its incomplete penetration into the leaves and/or due to the presence of \(Q_B^-\) in dark-adapted leaves (Rutherford et al., 1984). In cotyledons, harvested from light exposed AF-Na-treated seedlings, the B band intensity sharply decreases by around 50% and Q and C bands also decline by 50% demonstrating uniform 50% damage to PS II reaction centers by Proto IX-sensitized \(^1\)O\(_2\)-induced oxidative damage (Tripathy et al., 2007). The characteristic oscillation of TL band is affected when water oxidation is damaged, for example, when 33 kDa protein involved in water oxidation is removed (Ono and Inoue, 1985). In both the control and the treated samples, the TL yield observes a periodicity of 4 (Inoue and Shibata,
1978) and the maximum yield is obtained on the 2nd and 6th flashes (Tripathy et al., 2007). This shows that non-damaged PS II centers behave normally. No significant changes are seen in any of the temperature maxima of the various bands (Tripathy et al., 2007) suggesting no change in the activation energies of the various back reactions of PS II (De Vault and Govindjee, 1990).

Singlet Oxygen-induced Oxidative Damage in Mutants

A. Chlorophyll Anabolic Mutants

Tigrina mutant of barley accumulates 2-10 times more Pchlide in dark than the wild type (Nielsen, 1974). Homozygous tigrina-d mutants are fully green and viable if grown in continuous weak light, but show a green-white banded phenotype, when grown under light/dark cycles. They have normal level of POR (Protochlorophyllide oxidoreductase) and when illuminated, the excess Pchlide causes photodynamic damage resulting in the formation of necrotic patches (Von Wettstein et al., 1995). Runge et al. (1995) isolated xantha mutants of Arabidopsis and classified them in two groups, mutants that are blocked in various steps of the Chl biosynthetic pathway prior to POR, and mutants that accumulate Pchlide in the dark. The etiolated PORA and PORB mutant seedlings accumulate significant amounts of non-phototransformable Pchlide in dark and upon light exposure they show bleaching effect and germination defect (Armstrong et al., 1995). However, overexpression of PORA and PORB results in the efficient transformation of non-phototransformable Pchlide to chlorophyllide (Chlide). Transgenic seedlings grown under far red light when transferred to white light are more resistant to photobleaching because of high POR proteins (Frick et al., 2003).

The isolation and studies on Arabidopsis flu mutant by Klaus Apel’s group confirm the role of Pchlide in $^{1}O_2$ generation; it leads to oxidative damage. In flu mutant there is a massive accumulation of Pchilde if those plants are grown under constant dark/light cycle and there is growth arrest and cell death because of generation of $^{1}O_2$. However if the plants are grown under continuous light, there is no accumulation of Pchlide, no generation of $^{1}O_2$ and plants behave like wild type plants. The FLU protein interacts with GluTR (HEMA1) and regulates the accumulation of Pchlide in dark (Meskauskiene and Apel, 2002). In the flu mutant there is no regulatory point at the GluTR level and there is a massive accumulation of Pchlide. Lee et al. (2003) have revealed that TIGRINA d gene of barley is an ortholog of the FLU gene of Arabidopsis thaliana.. Pchlide-mediated $^{1}O_2$ formation leads to induction of early stress-responsive gene (Op den Camp et al., 2003). There is no change in amounts of
other photosensitizers i.e., Proto IX, Mg-proto IX and MPE in the flu mutant. Two major stress reactions were observed when dark-grown flu plants were returned to light: a cell death response and a rapid inhibition of growth. Oxygenation derivatives of linolenic acid, by far the most prominent polyunsaturated fatty acid of chloroplast membrane lipids, start to accumulate rapidly in the flu mutant after the dark/light shift. The oxidation of linolenic acid is not caused by direct interaction with $^{1}\text{O}_2$ but instead occurs enzymatically. Thus, the development of stress symptoms in the flu mutant seems not to be attributable to cell damage caused by $^{1}\text{O}_2$ but rather appears to result from the more indirect role of this ROS. Vitamin B6 that quenches $^{1}\text{O}_2$ in fungi was able to protect flu protoplasts from cell death (Danon et al., 2005); further, protoplasts of flu mutant depleted of both ethylene and salicylic acid had reduced cell death. However, when the gene Executer1 (exe1) was mutated in flu background, the exe1/flu double mutant accumulated free Pchlide in the dark like the flu mutant, but unlike the wild type plants. After transfer to light, exe1/flu generated $^{1}\text{O}_2$ in amounts similar to those of flu but grew like wild type when kept under non-permissive light-dark cycles (Wagner et al., 2004). In flu plants, the growth rate was reduced immediately after the beginning of re-illumination. The exe1/flu plants, however, grew like wild-type plants. Growth inhibition of flu plants was particularly striking when plants were transferred to repeated light-dark cycles, whereas the exe1/flu mutant continued to grow like wild-type plants (Wagner et al., 2004). Both assays demonstrate that the rapid bleaching of flu seedlings and the inhibition of growth after the release of $^{1}\text{O}_2$ are not because of the toxicity of this ROS; further, these do not reflect photooxidative damage and injury, but instead result from the activation of genetically controlled responses that require the activity of the Executer1 gene. The isolation of the Executer 2 protein also shows a similar kind of response in flu back ground (Lee et al., 2007). Inactivation of executer proteins blocks the $^{1}\text{O}_2$-mediated signaling from chloroplast to nucleus that affects the normal plastid development in germinating seeds (Kim et al., 2009). Coll et al. (2009) have isolated another $^{1}\text{O}_2$-linked death activator (soldat8) that encodes the SIGMA6 factor of the plastid RNA polymerase, specifically abrogate $^{1}\text{O}_2$-mediated stress responses in young flu seedlings without grossly affecting $^{1}\text{O}_2$-mediated stress responses of mature flu plants. The other protein named PRL1 (Pleiotropic response locus 1) also affect the expression of $^{1}\text{O}_2$- responsive genes in Arabidopsis (Baruah et al., 2009).

Apart from Pchlide, early intermediates i.e., coproporphyrin also acts as a photosensitizer (Ishikawa et al., 2001; Kruse et al., 1995a, 1995 b). The antisense coproporphyrinogen oxidase (that converts coproporphyrinogen III to protoporphyrinogen IX) of tobacco plants have an excessive amount of coproporphyrin. This precursor gives rise to
photodynamic reactions, which affect cellular processes resulting in retarded growth and necrotic leaves (Kruse et al., 1995a, 1995b). The Arabidopsis coproporphyrinogen oxidase mutants (*lin2*; lesion initiation 2) had pale leaves and developed lesions on the young leave (Ishikawa et al., 2001). 3,3-Diamino benzidine and trypan blue staining of the mutant leaves shows H$_2$O$_2$ accumulation and cell death. Seedlings homozygous for a null mutation in the cpx1 gene of maize completely lack chlorophyll and develop necrotic lesions in the light (Williams et al., 2006). The accumulation of Uroporphyrin I in the Uroporphyrinogen III cosynthase antisense barley plants results in necrotic leaves and ultimately cell death because of accumulation of ROS (Ayliffe et al., 2009). Like Uroporphyrin I, Uroporphyrin III, an intermediate of chlorophyll biosynthesis pathway also acts as photosensitizer. Accumulation of Uroporphyrin III leads to light-dependent necrosis in tobacco (Mock and Grimm, 1997; Mock et al., 1999) and in maize (Hu et al., 1998). Antisense tobacco plants of Uroporphyrinogen decarboxylase have stunted growth with necrotic leaves and high PR1 gene expression. The maize lesion mimic mutant, coding for Uroporphyrinogen decarboxylase, have necrotic spots in leaves. Inhibition of protox in Arabidopsis leads to production of lesion-mimic phenotype, high endogenous level of salicylic acid and PR1 gene expression (Molina et al., 1999). Overexpression of plastidic protox leads to resistance to the DPE herbicide acifluorfen. The overexpressed plants did not show any necrotic leaves (Lemontova and Grimm, 2000). Tobacco plants having reduced ferrochelatase activity also show necrotic leaves in a light intensity dependent manner (Papenbrock et al., 2001).

### B. Chlorophyll Catabolic Mutants

Intermediates involved in Chl degradation pathway also produce ROS. Squash plants expressing the mature (lacking the N-terminal 21 amino acids) citrus chlorophyllase protein, display a lesion-mimic phenotype when grown under natural light. The caused phenotype is because of the accumulation of chlorophyllide, which is a photodynamic porphyrin molecule (Harpaz-Saad et al., 2007). Arabidopsis Pheophorbide a oxygenase (PAO, also called *acdl*, Accelerated cell death 1) mutant shows cell death phenotype that is because of the accumulation of the Chl degradation pathway intermediate pheophorbide a. The latter gets photoactivated in presence of light and generates ROS that forms lesions in the mutant plants. The lesions form in *acdl* mutant leaves mostly start at the tip of the leaf and subsequently run down the leaf blade (Pruzinska et al., 2003). Hirashima et al, 2009, also observed that the accumulation of Pheophorbide a in dark grown *acdl* antisense plants caused cell death, which is very surprising. LLS1 (lethal leaf spot 1), the homologue of ACD1 (Accelerated cell death
Pheophorbide a oxygenase) in maize is responsible for Chl catabolism. The maize lls1 mutant formed lesions when grown in light (Gray et al., 1997). Similarly the Arabidopsis Red chlorophyll catabolite reductase (RCCR, also called acd2, Accelerated cell death 2) mutant showed lesion formation in leaves and spontaneous cell death phenotype (Mach et al., 2001). It is observed that the accumulation of $\text{H}_2\text{O}_2$ in the acd2 mitochondria is causal for its cell death phenotype (Yao and Greenberg, 2006). The lesion formation in acd2 is because of the accumulation of red chlorophyll catabolite (RCC) in dark that generates $^1\text{O}_2$ in the presence of light (Pruzinska et al., 2007). Further work should be done to check whether the generation of $\text{H}_2\text{O}_2$ and $^1\text{O}_2$ are independent events or one lead to the other.

Photodynamic Herbicides

Certain compounds in presence of light directly interact with the molecular oxygen and produce active oxygen species (AOS). Such compounds are toxic to living beings because of their photodynamic nature. Plant tetrapyrroles are photodynamic compounds which act as photosensitizers to generate active/reactive oxygen species (AOS/ROS) endogenously in presence of light. So there is a strong regulation against accumulation of intermediates of the chlorophyll (Chl) and heme biosynthetic pathway in plants (Figure IV). Application of certain herbicides (ALA, the substrate of chlorophyll biosynthesis pathway) results in over accumulation of tetrapyrroles in dark, which in presence of light absorb excess sunlight to produce triplet-excited state that interacts with molecular oxygen to generate active oxygen species. These are called photodynamic herbicides.

There are two types of porphyrin generating photodynamic herbicides. One consists of 5-aminolevulinic acid (ALA) whose application results in the overaccumulation of protochlorophyllide in dark (Granik, 1959; Tripathy and Rebeiz, 1985, 1986; Chakraborty and Tripathy, 1990, 1992a, 1992b; Tripathy and Chakraborty, 1991) and the other consists of diphenyl ethers which act as inhibitors of protoporphyrinogen oxidase (Matringe et al, 1989, 1992a; Jacobs and Jacobs, 1993; Camadro et al, 1991; Arnould and Camodaro, 1998; Lermontova and Grimm, 2000; Lee et al., 2000; Gupta and Tripathy, 2000; Graham, 2005). Rebeiz et al., 1984 reported a novel approach for design of new herbicides. For modern agricultural practice herbicide treatment is essential. An ideal selective herbicide should have following characteristics. a) It should kill the weeds without damaging crop plant b) high environmental safety and c) should be highly degradable resulting in minimal presence in soil. 5-Aminolevulinic acid (ALA) is an ideal selective experimental herbicide, which is selective,
environmentally safe as it degrades within 48 h leaving no traces in the soil. Theoretically, ALA may be expected to be able to function only as a non-selective herbicide, because it acts via Chl biosynthetic pathway common to all green plants. However, ALA was found to have selective herbicidal effect (Rebeiz et al., 1988). It kills mostly broad leaf weeds.

**Porphyrin-Generating Compounds**

There are two important types of porphyrin-generating compounds. One consists of 5-aminolevulinic acid (ALA), the substrate of tetrapyrroles and the other is a group of diphenyl ethers that inhibit protoporphyrinogen oxidase activity, thereby deregulating the tetrapyrrole metabolism. Besides other compounds like cercosporin, rose bengal and several other compounds could generate $^{1}O_2$ and $O_2$ (Daub and Hangarter, 1983; Haworth and Hess, 1988, Ito and Kobayashi, 1977; Wilson, 1966).

**A. 5-Aminolevulinic Acid**

5-Aminolevulinic acid (ALA) is the sole precursor of all the tetrapyrroles i.e., Chl, hemes, sirohemes, and phytochromobilins. Tetrapyrrole intermediates are photosensitizers and generate radicals and ROS especially $^{1}O_2$ in the presence of light. So plants regulate their own tetrapyrrole biosynthesis and degradation pathway to avoid the consequence of the excess generation of ROS. The major regulatory point is at the production of the initial precursor ALA. So ALA synthesis is the rate-limiting step of the tetrapyrrole biosynthetic pathway. ALA is formed from Glutamyl-tRNA by the enzyme Glutamyl-tRNA reductase (GluTR). In *Arabidopsis* this enzyme has three isoforms (HEMA1, HEMA2 and HEMA3) and their expression levels are different in different plant tissues. Pchlide, which accumulates in the dark, repress the ALA synthesis by downregulating the ALA synthesis by feed-back inhibition (Stobart and Ameen-Bukhrai, 1984, 1986). However, when ALA is applied externally, green plants bypass the regulatory feedback inhibition of Pchlide pool and induce excess accumulation of Mg-tetrapyrroles in dark (Granik, 1959, Rebeiz et al., 1984, 1988). When only ALA is applied externally, Pchlide is the major porphyrin that accumulates. But in ALA + Modulator (structurally related to tetrapyrrole molecule) treatments, several other types of porphyrins accumulate, depending upon the target site of the modulator (Rebeiz et al., 1991). ALA is added along with the modulator for providing the carbon skeleton to accumulate porphyrins. Eight molecules of ALA are required to form one molecule of tetrapyrrole. The mode of action of some of the modulators has been attributed to their metal chelating properties. Enzymes in the porphyrin synthesizing pathway essentially require
certain metal ions for their activity, which is made unavailable. Another way by which some of these modulating chemicals may be acting is by stimulating enzyme activity, i.e. by behaving as cofactor analogs.

**B. Diphenyl ether herbicides (DPEs)**

Diphenyl ether herbicides (DPEs) are photodynamic herbicides and inhibitors of the enzyme Protox (Matringe et al., 1989), belonging to the Chl and heme biosynthetic pathway. Though DPE entry into cells is light independent, light and pigments are mandatory for their herbicidal action. Initial symptom of DPE damage is isolated water soaked spots seen on tissue, followed by loss of leaf turgidity, bleaching and necrosis. Diphenyl ethers like oxyfluorfen and RH-8817 do not affect germination of imbibed corn seeds (Devlin et al., 1983). However, these seeds when grown in light develop deformed and dwarfed, with primary leaf being tightly curled, giving it a spike like appearance. More lipophilic DPEs like oxyfluorfen and acifluorfen-methyl have greater potency as herbicides than the more polar acifluorfen, which suggests a membrane site of action. This correlation may also be partly due to greater ease of penetration through cuticle by the more lipophilic molecules. DPE herbicides are known to have several molecular sites of action, each probably with its own quantitative structure-activity relationship (Nandihalli & Duke, 1994). DPEs cause plants to overaccumulate a large quantity of Proto IX (Duke et al., 1991). Pchlide, Mg-Proto IX and Mg-Proto IX monomethylester are also found to be elevated, but to significantly less extent compared to Proto IX, in tissues with inhibited Protox activity. Plasmamembrane, mitochondrion and chloroplast have been implicated as sites of DPE action (Duke et al., 1984). Earliest perceptible damage is the disruption of Plasmamembrane, tonoplast and structural abnormalities at the chloroplast envelope (Duke et al., 1991). There is broad agreement that the light dependent lethal effect of DPEs is a result of lipid peroxidation, which leads to destruction of polyunsaturated fatty acid in membranes (Duke and Kenyon, 1986, 1987). The first detectable damage in Acifluorfen (AF) treated and light exposed cucumber cotyledon discs, is cellular leakage, followed by inhibited photosynthesis, ethylene, ethane and malondialdehyde evolution - all characteristic of membrane lipid peroxidation (Kenyon et al., 1985). Degradation of membrane structure may be a primary herbicidal effect of DPEs (Orr and Hess, 1981). DPEs can inhibit carotenoid synthesis, ATP formation, photosynthetic electron transport, and induce membrane peroxidation by causing massive accumulation of Proto IX (Kunert et al., 1987; Lydon and Duke, 1988). Chemical nature of the toxic species causing peroxidation in the case of DPE herbicides is debated. Probably,
superoxide radical is not of primary importance in development of DPE toxicity (Ensminger & Hess, 1985; Kunert and Boger, 1981; Yoshimoto and Matsunaka, 1982). However, Duke et al., 1984, produced evidence that superoxide and H2O2 accumulated in mitochondria of AF treated cucumber cotyledons. Haworth and Hess, 1988 added oxyfluorfen to isolated thylakoid membranes in vitro, which generated \( \cdot \text{O}_2 \) during illumination.

When barley plastids were incubated with diphenylether herbicide, it was found that protoporphyrin IX formation by the plastids was completely abolished (Jacobs and Jacobs, 1993). The Protox gene was isolated from yeast and showed that this protein is the molecular site of the action of DPEs (Camadro et al., 1994). Overexpression of tobacco protoporphyrinogen IX oxidase leads to resistance to the diphenyl-ether herbicide acifluorfen (Lermontova and Grimm, 2000). It was also seen that Arabidopsis chll-1 mutant is resistant to acifluorfen herbicide that inhibits Protox (Soldatova et al., 2005). When acifluorfen-sprayed and dark incubated cucumber plants were exposed to light the plants were died because of oxidative stress (Gupta and Tripathy, 1999, 2000). The diphenylether herbicide lactofen induces cell death and expression of PR1, PR5 and PR10 protein in soyabin plants. The anthocyanin biosynthesis pathway genes i.e., CHS and CHR were also induced in lactofen treated samples (Graham, 2005).

**Mechanism of action of photodynamic herbicides**

Chlorophyll molecules are the major associated pigments of light-harvesting complex, but these are potent endogenous photosensitizers in plants. Absorption of light causes Chl to \( ^1\text{Chl} \) and ultimately \( ^3\text{Chl} \) that interact with O2 to produce singlet oxygen (\( ^1\text{O}_2 \)) (Foote, 1976). Because the average lifetime of \( ^1\text{Chl} \) in the PS II LHC is several times longer than in the PSI LHC, the potential for generation of \( ^1\text{O}_2 \) is greater in the PS II LHC. The PSII reaction center consists of the heterodimer D1 and D2 protein, which binds to the Chl dimer (P680) and a pheophytin molecule (Pheo). Trapping of excitation energy by the reaction center results in charge separation between P680 and pheophytin and finally leads to reduction of QA to QB (Barber and Andersson, 1992). When the QA and QB are over reduced because of excess light energy, charge separation cannot be completed and triplet state of P680 (\( ^3\text{P680} \)) is favored (Aro et al., 1993; Vass et al., 1992; Ohad et al., 1994) leading to the formation of singlet oxygen due to energy transfer. The release of singlet oxygen was first detected in isolated PSII particles (Macpherson et al., 1993) and also detected in vivo (Fryer et al. 2002, Hideg et al., 1998).
ALA-treated plants, when transferred to light exhibit symptoms of photodynamic damage, which is due to triplet state of Pchlide. The Pchlide molecules are attached to the thylakoid membrane. So the primary target of photodynamic reaction should be the thylakoid membrane (Tripathy and Chakraborty, 1991; Gupta and Tripathy, 1999). The light-harvesting-chlorophyll-protein complex (LHCP) remains functionally attached but both photosystem II (PSII) and photosystem I (PSI) photochemical reactions are impaired. The photodynamic damage to PSII was higher than that to PSI. The PSII reaction center gets affected that is confirmed by PSII light saturation curve and from the observation that, failure of NH$_2$OH to donate electrons to PSII. But there is severe damage to LHCP I. The thylakoid spectra of the damaged plants showed a peak shift from 735nm (attributed to LHCI) to 728nm. It has been seen that the herbicide effect in initiated by ALA in presence of light and even continued in dark and irreversible. Hartel et al., 1993a, 1993b showed that the fluidity of thylakoid membranes isolated from ALA-treated leaves decreased and there was decrease in delayed Chl fluorescence intensity that coincided with an increase in P700$^+$ formation, which indicates disturbance in electron transfer between the two photosystems. ALA-induced accumulation of non-phototransformable Pchlide is a reasonable candidate for type II photosensitization reaction, which could produce singlet oxygen in presence of light, leading to the photodynamic damage.

There was significant increase in MDA in ALA-treated light-exposed plants, which indicated degradation of membrane lipid resulting in increase in conductivity of the bathing medium of photodynamically damaged leaf discs. Reduction of MDA production in chloroplasts isolated from ALA-treated light-exposed plants in presence of L-histidine and sodium azide further confirmed that photodynamic damage was mediated by singlet oxygen. Photodynamic damage to the photosynthetic electron transport reactions was light intensity dependent and also occurred in dark demonstrating that photodynamic action is primary in nature and not due to secondary effect (Chakraborty & Tripathy, 1992a). Scavengers of singlet oxygen, like L-histidine and sodium azide was able to protect the thylakoid membrane linked to photodynamic damage (Chakraborty & Tripathy, 1992b) but scavengers of hydroxyl radical (i.e., formate) or superoxide radical (i.e., superoxide dismutase) failed to protect the same. Out of the three superoxide detoxifying enzymes present in the stroma, superoxide dismutase and ascorbate specific peroxidase were not affected by ALA and light induced photodynamic reactions, whereas glutathione reductase was partially affected. These results suggest that superoxide-detoxifying capability of chloroplasts is impaired due to
photodynamic damage. There is loss of Chl and protein content in photodynamically damaged plants (Chakraborty & Tripathy, 1990).

**Mutant Studies**

*Tigrina* mutant of barley accumulates 2-10 times more Pchlide in dark than wild type (Nielsen, 1974). Homozygous *tigrina-d* mutants are fully green and viable if grown in continuous weak light, but show a green-white banded phenotype, when grown under light/dark cycles. It has normal level of POR, and when illuminated, the excess Pchlide causes photodynamic damage resulting in the formation of necrotic patches (von Wettstein et al., 1995). Runge et al., 1995 isolated xantha mutants of Arabidopsis and classified them in two groups, mutants that are blocked in various steps of the Chl biosynthetic pathway prior to POR, and mutants that accumulate Pchlide in the dark.

In *flu* mutant, Pchlide mediated singlet oxygen formation leads to induction of early stress-responsive gene i.e., ascorbate peroxidase (op den Camp et al., 2004). There is no change in amounts of other photosensitizers i.e, protoporphyrin IX, Mg_2-protoporphyrin IX and Mg_2-protoporphyrin monomethylester in the *flu* mutant. Two major stress reactions were observed when dark-grown *flu* plants were returned to the light: a cell death response and a rapid inhibition of growth. Oxygenation derivatives of linolenic acid, by far the most prominent polyunsaturated fatty acid of chloroplast membrane lipids, start to accumulate rapidly in the *flu* mutant after the dark/light shift. The oxidation of linolenic acid is not caused by direct interaction with singlet oxygen but instead occurs enzymatically. Thus, the development of stress symptoms in the *flu* mutant seems not to be attributable to cell damage caused by singlet oxygen but rather appears to result from the more indirect role of this ROS. Vitamin B6 that quenches \(^1\)O_2 in fungi was able to protect *flu* protoplasts from cell death (Danan et al., 2005). They have also shown protoplasts of *flu* mutant depleted of both in ethylene and salicylic acid had reduced cell death. Recently, it has been revealed that TIGRINA d gene of barley is an ortholog of the FLU gene of A. thaliana (Lee et al., 2003). However, when the gene executor1 was mutated in *flu* background, in contrast to wild-type plants but like *flu*, the executor1/*flu* double mutant accumulated free protochlorophyllide (Pchlide) in the dark. After transfer to the light, executor1/*flu* generated singlet oxygen in amounts similar to those of *flu* but grew like wild type when kept under nonpermissive light-dark cycles (Wagner et al., 2004). In *flu* plants, the growth rate was reduced immediately after the beginning of reillumination. The executor1/*flu* plants, however, grew like wild-type plants. Growth inhibition of *flu* plants was particularly striking when plants were transferred to repeated light-dark cycles, whereas executor1/*flu* continued to grow like wild-type plants.
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(Wagner et al., 2004). Both assays demonstrate that the rapid bleaching of flu seedlings and the inhibition of growth after the release of singlet oxygen are not because of the toxicity of this ROS and do not reflect photooxidative damage and injury, but instead result from the activation of genetically controlled responses that require the activity of the EXECUTER1 gene.

Except Pchlide the other intermediate coproporphyrin was also acts as photosensitizer (Kruse et al., 1995a, b; Ishikawa et al., 2001). The antisense coproporphyrinogen oxidase (converts coproporphyrinogen III to protoporphyrinogen IX) tobacco plants have excessive amount of coproporphyrin. This precursor gives rise to photodynamic side reactions which affects cellular process resulting in retarded growth and necrotic leaves (Kruse et al., 1995a, b). The Arabidopsis coproporphyrinogen oxidase mutants (lin2) had pale leaves and developed lesions on the young leaf (Ishikawa et al., 2001). DAB and trypan blue staining of the mutant leaves showed H$_2$O$_2$ accumulation and cell death. The expression of PR1 gene was also more. Seedlings homozygous for a null mutation in the cpx1 gene of maize completely lack chlorophyll and develop necrotic lesions in the light (Williams et al., 2006). Uroporphyrin III, another intermediate of chlorophyll biosynthetic pathway is also acts as photosensitizer. Accumulation of Uroporphyrin III also leads to light-dependent necrosis in tobacco (Mock and Grimm, 1997; Mock et al., 1999) and in maize (Hu et al., 1998). Antisense tobacco plants of Uroporphyrinogen decarboxylase resulted in stunted growth with necrotic leaves and high PR1 gene expression. The maize lesion mimic mutant (lesion mimic gene codes for Uroporphyrinogen decarboxylase) resulted in the production of necrotic spots in leaves. Accumulation of protoporphyrinogen IX also leads to photodynamic damage in Arabidopsis (Molina et al., 1999). Inhibition of protoporphyrinogen oxidase in Arabidopsis resulted in lesion-mimic phenotype and the mutant had high endogenous level of salicylic acid and PR1 gene expression (Molina et al., 1999). Overexpression of plastidic protoporphyrinogen IX oxidase leads to resistance to the diphenyl-ether herbicide acifluorfen. The overexpressed plants did not show ant necrotic leaves (Lermontova and Grimm, 2000). Tobacco plants having reduced ferrochelatase activity also showed necrotic leaves in a light intensity dependent manner (Papenbrock et al., 2001). The amount of protoporphyrinogen IX was more in the ferrochelatase antisense plants, which is a photosensitizer.

The enzymes ACD1 and ACD2 also play crucial role in lesion formation in plants. LLS1, the homologue of ACD1 (Accelerated cell death 1/ Pheophorbide a oxygenase) in maize is responsible for Chl catabolism. The maize lls1 mutant formed lesions when grown in light (Gray et al., 1997; Pruzinska et al., 2003). The lesion formation mostly starts at the tip of
the leaf and subsequently run down the leaf blade. Similarly the *Arabidopsis acd2* (Accelerated cell death 2/ Red chlorophyll catabolite reductase) mutant showed lesion formation in leaves (Mach *et al.*, 2001). In both the cases the lesion formation might be because of the accumulation of porphyrin molecules that produce free radicals in presence of light. Disruption of Chl catabolism by the *acd2* mutant is likely to cause the accumulation of red chlorophyll catabolite and Pheophorbide a, which are phototoxic porphyrin molecules. These molecules create free radicals in presence of light. Overexpression of *ACD2* showed increased tolerance to cell death (Mach *et al.*, 2001) by altering the flux of Chl catabolites that may normally accumulate during disease and trigger cell death.

**Salt Stress induced oxidative stress in plants**

Soil salinity is one of the major abiotic stresses that adversely affect crop productivity and quality. It is estimated that 6% of the world’s total land and 30% of the world’s irrigated areas already suffer from salinity problems (Unesco Water Portal, 2007). Hence developing salt tolerant crops is essential for sustaining food production. Salinity is a soil condition characterized by a high concentration of soluble salts. According to USDA Salinity Laboratory, soils are considered as saline when the ECe is ≥ 4 dS m⁻¹. Photosynthesis, together with cell growth, is among the primary processes to be affected by salinity (Munns *et al.*, 2006; Sudhir *et al.*, 2004). Salt stress can affect photosynthesis of the plants directly by decreasing CO₂ availability due to diffusion limitation through the stomata and the mesophyll (Flexas *et al.*, 2007, 2004). Salt stress also alters the photosynthetic metabolism of the plants (Lawlor and Cornic, 2002). Secondary effect of salt stress on photosynthesis is generation of reactive oxygen species (ROS) causing oxidative stress which leads to cell death. Oxidative stress is the result of most of the stress conditions (Chaves and Oliveira, 2004) and it adversely affect the photosynthetic machinery (Ort, 2001). ROS, such as \(^1\text{O}_2, \text{O}_2^-\), HO' and, H₂O₂ are toxic molecules capable of causing oxidative damage to DNA, proteins and lipids (Apel & Hirt 2004). Under optimal growth conditions, ROS are mainly produced at a low level in organelles such as chloroplasts, mitochondria and peroxisomes. However, during stress, their rate of production is dramatically elevated. It is important to note that whether ROS will act as damaging, protective or signaling factors depends on the delicate equilibrium between ROS production and scavenging at the proper site and time (Gratao *et al.*, 2005). Plants have different antioxidant defense machinery to protect themselves against oxidative stress damages. Plants possess very efficient enzymatic (superoxide dismutase, catalase,
ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione peroxidase, guaicol peroxidase and glutathione-S-transferase) and non-enzymatic (ascorbic acid, glutathione, phenolic compounds, alkaloids, non-protein amino acids and tocopherols) antioxidant defense systems which protect plant cells from oxidative damage by scavenging of ROS (Gill et al., 2010). Salt stress has various effects on photosynthetic electron transport activities due to the marked alterations in the composition of thylakoid membrane proteins for examples D1 protein of PSII 47-kDa chlorophyll protein (CP) and 94-kDa protein, and accumulation of a 17-kDa protein resulting decreased energy transfer from light harvesting antenna to PSII (Sudhir et al., 2005).

Plants respond to salt stress by closing stomata to avoid excessive water loss resulting decrease in the internal CO₂ concentrations and slows down the reduction of CO₂ by Calvin cycle. This response leads to depletion of the oxidized NADP⁺, final acceptor of electrons in PSI, and alternatively increases the leakage of electrons to O₂ forming O₂⁻ (Hsu et al., 2003). Furthermore, Na⁺/Cl⁻ toxicity resulting from salt stress could disrupt the photosynthetic electron transport and provoke electron leakage to O₂ (Gossett et al., 1994; Borsani et al., 2001; Slesak et al., 2002). The decrease in internal CO₂ concentrations slows down the reactions of Calvin cycle and induces photorespiration particularly in C₃ plants, resulting in generation of more H₂O₂ in the peroxisomes (Leegood et al., 1995; Wingler et al., 2000; Ghannoum et al., 2009). The cell membrane-bound NADPH oxidase and the apoplastic diamine oxidase are supposed to be activated during salt stress and therefore contribute to generation of ROS (Cross et al., 1993; Hernandez et al., 2001; Lin et al., 2001; Mazel et al., 2004; Tsai et al., 2005). Salt stress increases the rates of respiration with the consequence of respiratory electron leakage to O₂ (Fry et al., 1986; Moser et al., 1991; Jeanjean et al., 1993).