Modulation of Chlorophyll Biosynthesis by Environmental Cues

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Summary

Environmental signals control diverse physiological processes in plant growth and development. Plants tend to adapt the structure of photosynthetic apparatus and pigment composition in response to several environmental factors. Tetrapyrroles play vital roles in various biological processes, including photosynthesis and respiration. Expression of genes encoding enzymes of tetrapyrrol biosynthesis as well as the abundances and activities of the enzymes

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are severely impacted by availability of water, soil salinity, low or high temperature and low or high light intensity. Plastids share many cellular metabolic pathways and alterations of plastid functions by environmental signals are known to affect various aspects of plant development. The generation of reactive oxygen species (ROS) in plants is triggered by different kinds of environmental parameters, such as high light, high or low temperature, salinity, drought and nutrient deficiency. Imbalance between production of ROS and their detoxification by enzymatic and non-enzymatic reactions causes oxidative stress. Suitable genetic manipulation of the chlorophyll (Chl) biosynthetic pathway might lead to tolerance towards environmental stresses leading to oxidative stress at the cellular level, and efficient adaptation of the photosynthetic apparatus to low and high light intensities. The present review deals with environmental modulation of Chl biosynthesis and its impact on plant productivity.

I. Introduction

Environmental cues including light, water, temperature, soil and nutrient content have a profound impact on plant growth and development. For example, light has significant effects on morphogenesis of seedlings during the transition from heterotrophic to photoautotrophic growth. Plants tend to adapt the structure of the photosynthetic apparatus and the pigment composition to light quality and

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Abbreviations: ALA – 5-Aminolevulinic acid; ALAD – 5-Aminolevulinic acid dehydratase; CAO – Chlorophyllide A oxygenase; Chl – Chlorophyll; Chlide – Chlorophyllide; Coprox – Coproporphyrinogen oxidase; CPO – Coproporphyrinogen oxidase; DV – Divinyl; DV-Pchlide – Divinyl protochlorophyllide; DVR – Divinyl reductase; FLU – Fluorescence; GGPP – Geranyl geranyl pyrophosphate; GluRS – Glutamyl-tRNA synthetase; GluTR – Glutamyl-tRNA reductase; GSA – Glutamate 1-semialdehyde; GSA-AT – Glutamate 1-semialdehyde aminotransferase; GUN – Genome uncoupled; LHC II – Light-harvesting complex II; Lin2 – Lesion initiation 2; lip1 – Light-independent photomorphogenesis 1; MPE – Mg-protoporphyrin IX monomethylester; MTF – Mg-Protoporphyrin IX methyltransferase; MV – Monovinyl; MV-Pchlide – Monovinyl protochlorophyllide; PBG – Porphobilinogen; PBGD – Porphobilinogen deaminase; Pchlide – Protochlorophyllide; PhPP – Phytyl diphosphate; PLBs – Prolamellar bodies; POR – Protochlorophyllide oxidoreductase; PPIX – Protoporphyrin IX; Protogen IX – Protoporphyrinogen IX; Protox – Protoporphyrinogen oxidase; ROS – Reactive oxygen species; SAM – *S*-adenosyl-methionine; SDR – Short chain dehydrogenases/reductases; tRNAglu – Glutamate conjugated tRNA; Urogen III – Uroporphyrinogen III; UROS – Uroporphyrinogen III synthase

quantity and other environmental factors. Tetrapyrroles play vital roles in various biological processes, including photosynthesis and respiration (Rebeiz et al. 1994; Papenbrock and Grimm 2001; Block et al. 2007; Tanaka and Tanaka 2007; Masuda 2008; Biswal et al. 2012; Phung et al. 2012). Plants are exposed to various abiotic stresses such as low temperature, high temperature, salinity, drought, flooding, oxidative stress and heavy metal toxicity etc. either during their entire life cycle or a part thereof. Plant growth, development, photosynthesis and productivity are severely affected due to environmental stresses, particularly during early seedling growth. When seeds germinate beneath the soil, their seedlings remain in near-darkness for a while. Therefore, etiolated rice seedlings beneath the soil do not synthesize Chl and contain a special form of plastids called etioplasts or etiochloroplasts. As seedlings come out of soil, they are exposed to light and light-mediated Chl biosynthesis and other associated greening processes are initiated resulting in transformation of etioplasts to chloroplasts (See Solymosi and Aronsson, Chap. 3). During chloroplast biogenesis in the light, proplastids in meristematic tissue and etioplasts in dark-grown seedlings develop into the mature, photosynthetic chloroplast of the green leaf (Waters and Pyke 2005).

Chloroplast biogenesis and development in seedlings can be described as the differentiation process from the plastid progenitor, a proplastid, to a mature chloroplast. Plastids carry out many essential metabolic pathways and alteration of plastid functions affects various

aspects of plant growth and development. Chloroplasts are responsible for the biosynthesis of carbohydrates, fatty acids, pigments, and the synthesis of amino acids and proteins from inorganic nitrogen (Staehelin and Newcomb 2000). Chloroplast development involves the biosynthesis of components of the photosynthetic apparatus involving synthesis of Chl and carotenoids, lipids and proteins which is governed in a coordinated manner by chloroplast and nuclear genomes (Leon et al. 1998; Gray et al. 2003; Nott et al. 2006). Biosynthesis of porphyrins, particularly that of Chl, during early greening stages of seedlings is elucidated in detail (Tripathy and Rebeiz 1985, 1986, 1988; Meskauskiene et al. 2001; Goslings et al. 2004; Bollivar 2006; Tanaka and Tanaka 2007; Wu et al. 2007; Wang et al. 2010; Tripathy and Pattanayak 2012).

Chl biosynthesis and chloroplast development during irradiation of dark-grown plants is impacted by external and internal factors such as light quality, temperature, nutrition, leaf age, leaf water potential, salt etc. as they influence transcription, translation and post-translational modification of proteins involved in chloroplast biogenesis (Virgin 1965; Bengtson et al. 1978; Bhardwaj and Singhal 1981; Eskins et al. 1986; Tewari and Tripathy 1998, 1999; Le Lay et al. 2000, 2001; Sood et al. 2004, 2005; Mohanty et al. 2006; Dutta et al. 2009; Mohanty and Tripathy 2011; Dalal and Tripathy 2012).

All enzymes of the Chl biosynthetic pathway are nuclear encoded and post-translationally imported into chloroplasts. Chl synthesis is synchronized with the formation of other pigments such as carotenoids and with pigment-binding proteins; Chl synthesis is also involved in the coordination between chloroplast and nucleus (Nott et al. 2006).

II. Chlorophyll Biosynthetic Enzymes and Their Modulation by Environment

A. Biosynthesis of 5-Aminolevulinic Acid

Unlike animals where one enzyme 5-aminolevulinic acid (ALA) synthase could form ALA by condensation and decarboxylation of succinyl-CoA and glycine, the synthesis of ALA in plants requires three different enzymes located in the chloroplast stroma. These are glutamyl-tRNA synthetase (GluRS) [EC 6.1.1.17], glutamyl-tRNA reductase (GluTR) [EC 1.2.1.70] and glutamate semialdehyde aminotransferase (GSA-AT) [EC 5.4.3.8] (Fig. 27.1a).

GluRS, also known as aminoacyl-tRNA synthetase, ligates glutamate to tRNA^{GLU} (Huang et al. 1984; Kannangara et al. 1984, 1994) (Fig. 27.1a). Unlike class I aminoacyl-tRNA synthetases, GluRS avoids the aminoacyl-AMP formation in the absence of tRNA. In eukaryotic cells chloroplastic GluRS is post-translationally imported into the chloroplast where it ligates glutamate to tRNA^{GLU} that contains the UUC anticodon (Schön et al. 1986, 1988).

GluTR, the second enzyme of the pathway, uses NADPH to reduce the activated α-carboxyl group of glutamyl-tRNA (Glu-tRNA) to synthesize glutamate 1-semialdehyde (GSA) (Hoober et al. 1988). The GluTR is a homopentamer of identical subunits of 54 kDa (Pontoppodian and Kannangara 1994). This enzyme is subject to feedback regulation by heme and appears to be a major control point of porphyrin biosynthesis (Kannangara et al. 1988). In *A. thaliana* GluTR interacts with FLU, a negative regulator of the Chl biosynthesis pathway (Meskauskiene et al. 2001; Meskauskiene and Apel 2002). FLU is a nuclear-encoded chloroplast protein, and the *flu* mutant has a higher level of ALA synthesis and protochlorophyllide (Pchlide) accumulation than that of wild-type plants. Probably FLU is a component of negative regulatory system for ALA synthesis when cells have high Pchlide contents. A FLU-like protein is also present in barley (Lee et al. 2003). GluTR is encoded by the *HEMA* gene. It has two isoforms in barley and cucumber, whereas in *A. thaliana* it has three isoforms.

The formation of 5-aminolevulinate/ALA from GSA is catalyzed by GSA-AT, the third and the last enzyme required for ALA biosynthesis. This enzyme is functionally an aminomutase, which transfers the amino group from carbon 2 of GSA to the neighboring carbon atom i.e., carbon 5 to form ALA (Fig. 27.1a). The enzyme is inhibited by gabaculine (Gough et al. 1992).
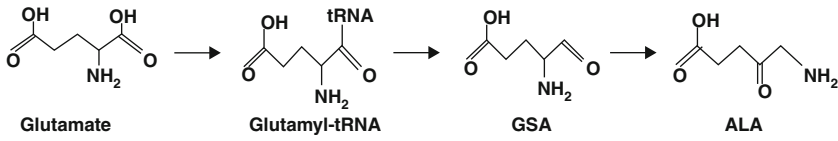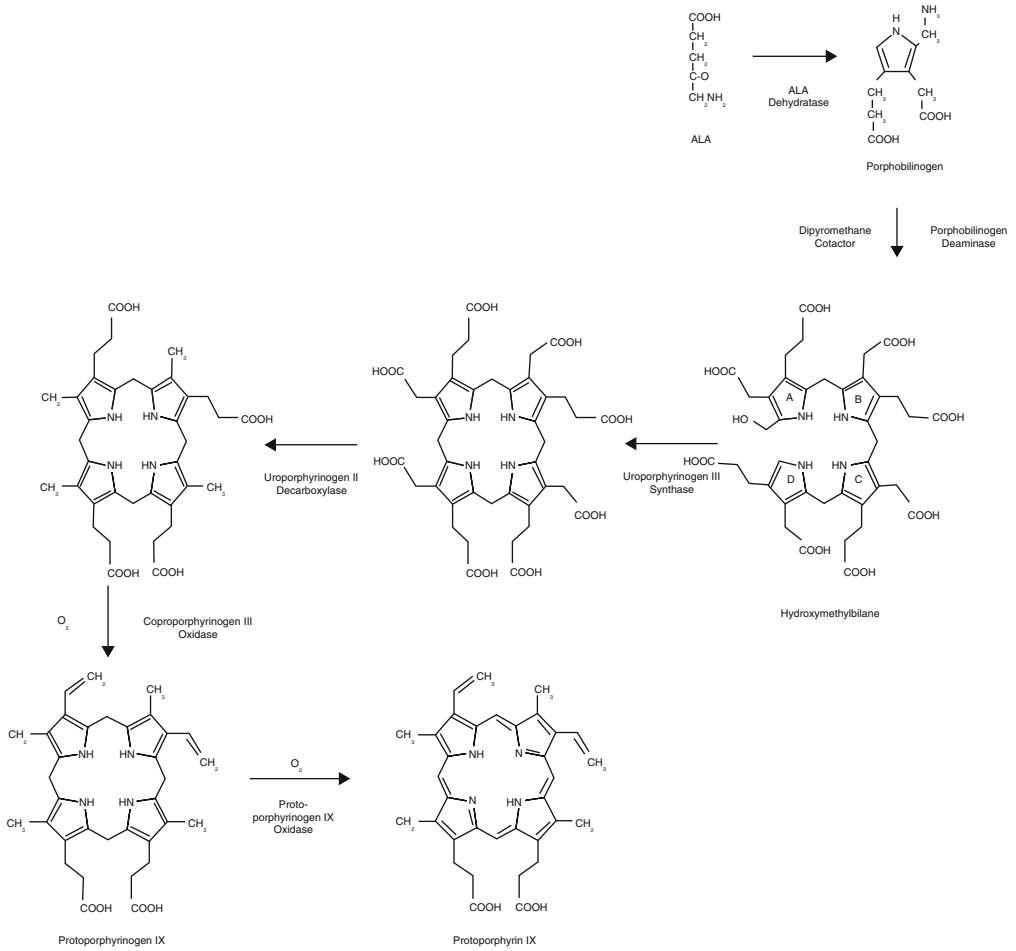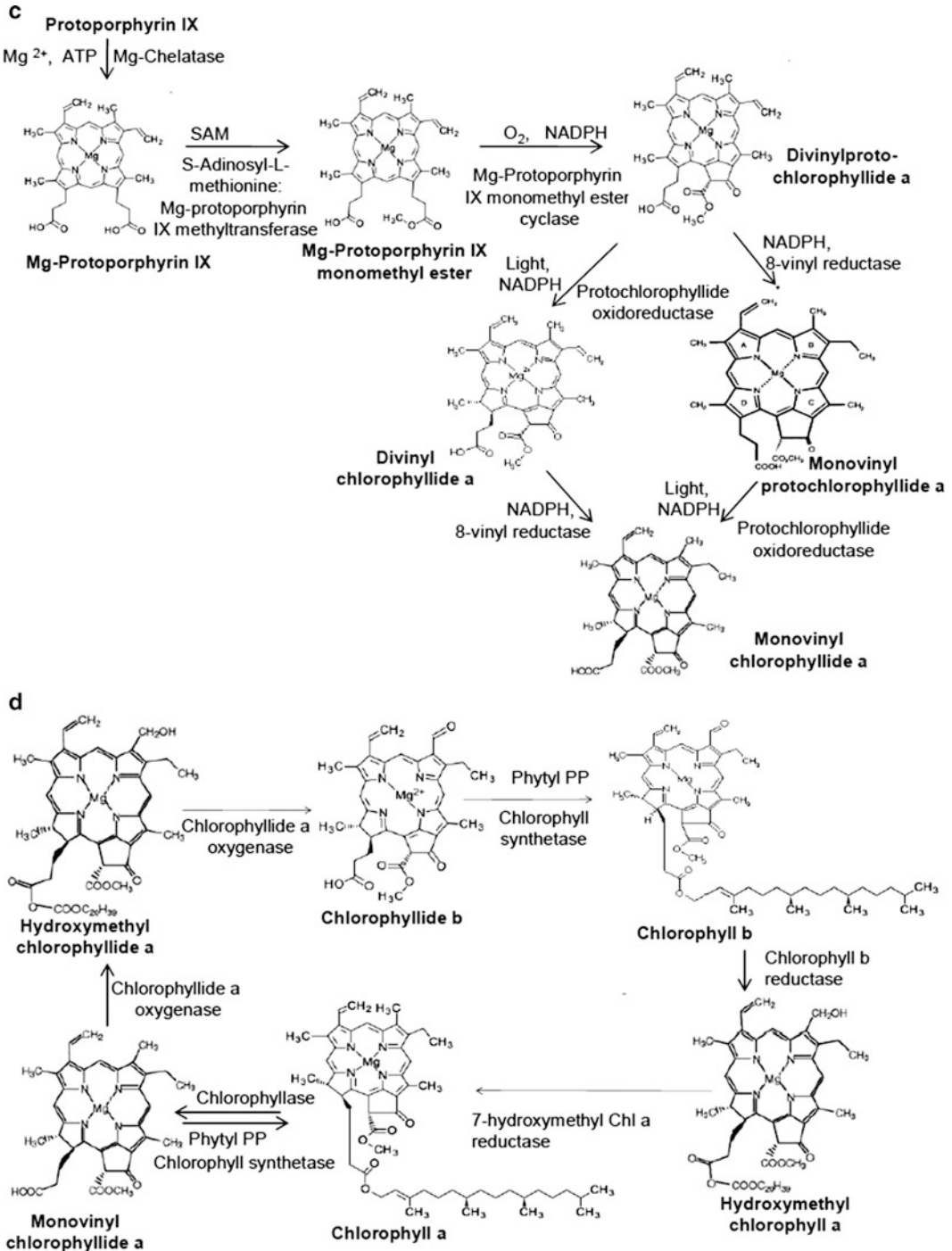
Fig. 27.1. Chl biosynthesis pathway in higher plants. (**a**) Biosynthesis of ALA from glutamic acid (**b**) Biosynthesis of protoporphyrin IX from ALA (**c**) Biosynthesis of monovinyl chlorophylide a from protoporphyrin IX (**d**) Biosynthesis of Chl a and Chl b from monovinyl chlorophylide a.

*1. Environmental Modulation of ALA
Biosynthesis*

a. Light Regulation of ALA Biosynthesis

In cucumber and *Arabidopsis thaliana*, the
HEMA1 gene is expressed in photosynthetic
tissues and is induced by illumination, but no
transcripts were detectable in roots (Tanaka
et al. 1996; Ilag et al. 1994). Gene expression
of *HEMA1,* and the corresponding protein
abundance, increases in response to light
treatment of dark-grown seedlings suggest-
ing that an increased demand for Chl biosyn-
thesis stimulates its expression and the gene
promoter may have light-responsive elements
(Mohanty et al. 2006). On the other hand,
HEMA2 is preferentially expressed in non-
photosynthetic tissues, and its expression is
light-independent (Tanaka et al. 1996; Nagai
et al. 2007). A third *HEMA* gene, *HEMA3*,
has been identified in *A. thaliana*, but its
expression is low (Matsumoto et al. 2004).

In *A. thaliana* light stimulates transcription
of *GSA* (Ilag et al. 1994). The gene expres-
sion is also activated by the hormone kinetin
(Yaronskaya et al. 2006). The expression
of *GSA* and protein abundance of GSA-AT
increases when etiolated seedlings are
transferred to light demonstrating that it is a
light-inducible gene that significantly contrib-
utes to Chl synthesis (Mohanty et al. 2006). In
soybean also, the *GSA* is light inducible. It con-
tains a light-regulated cis element (containing
GAGA) that is found to be involved in tran-
scriptional control (Frustaci et al. 1995). The
mRNA level is high in soybean leaves (Sangwan
and O'Brian 1993) whereas the mRNA is not
detectable in roots (Frustaci et al. 1995).

b. Modulation of ALA Biosynthesis
by Temperature

Environmental factors such as chill- or heat-
stress influence gene expression, translation
and post-translational modification of pro-
teins involved in chloroplast biogenesis
(Tewari and Tripathy 1998, 1999; Mohanty
et al. 2006; Abdelkader et al. 2007a, b; Dutta
et al. 2009). When 5-day old etiolated wheat
seedlings grown at 25°C are transferred to
7°C (chill-stress), 42°C (heat-stress) or 25°C
(control) and exposed to cool white fluorescent
light (50 μmoles m^{-2} s^{-1}) for 24 h, the Chl
content gradually increases in control seed-
lings. In chill- and heat-stressed seedlings Chl
biosynthesis is severely down-regulated. A lag
period up to 12 h is observed, both in chill-
and heat-stressed wheat seedlings before Chl
accumulation accelerates (Fig. 27.2).

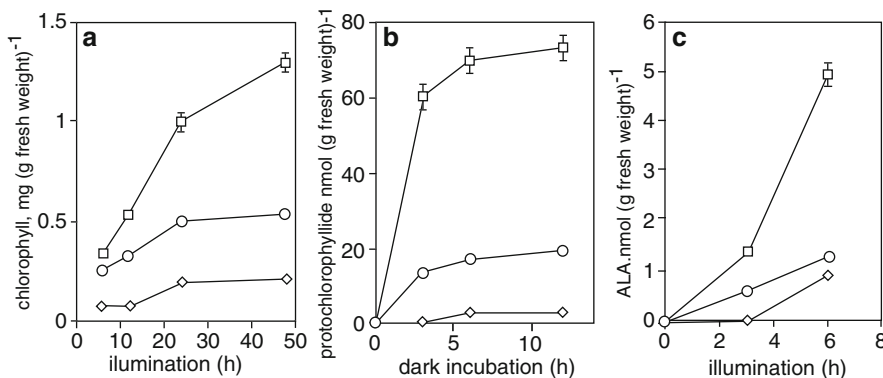*Fig. 27.2.* Biosynthesis of Chl (*left panel* (**a**)), Pchlide (*central panel* (**b**)), and ALA (*right panel* (**c**)) in control
(25°C, squares), chill-stressed (7°C, diamonds), or heat-stressed (42°C, circles) cucumber seedlings. Each data
point is the mean of three replicates; error *bars* represent SD. Missing error bars indicate that they are smaller
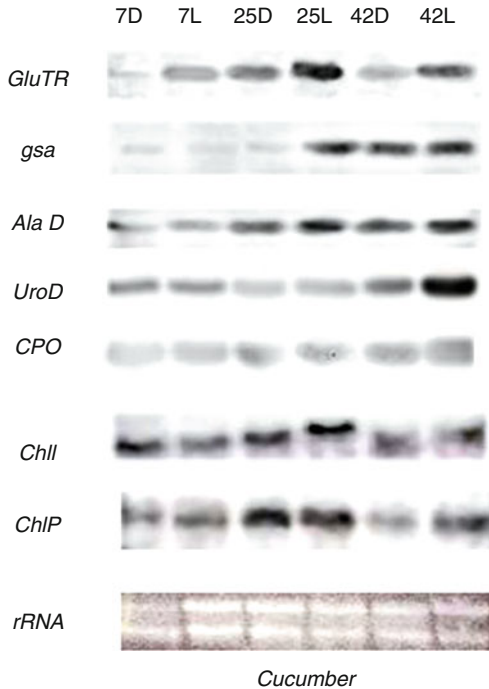than the symbols (Tewari and Tripathy 1998).

Fig. 27.3. Modulation of gene expression (Northern blot) of Chl biosynthesis pathway enzymes by temperature stress in cucumber. Five-day old etiolated seedlings grown at 25°C were transferred to light (50 mmol m^{-2} s^{-1}) or dark at 7°C, 25°C or 42°C for 24 h and Northern blotting was performed. *Numbers* denote temperature and D and L denote *dark* and *light* respectively (Mohanty et al. 2006).

ALA synthesis in the presence of LA is almost linear up to 6 h of illumination in control and heat-stressed seedlings (Tewari and Tripathy 1998). For the first 3 h, ALA synthesis is completely inhibited in chill-stressed cucumber seedlings. As compared with the controls, the net synthesis of ALA is severely reduced in chill- and heat-stressed seedlings, respectively (Fig. 27.2) (Tewari and Tripathy 1998). Among ALA biosynthetic enzymes, the expression of *HEMA* is light-inducible in cucumber i.e., its expression increases in response to light in cucumber seedlings (Fig. 27.3). Its expression was down-regulated both in chill- and heat-stressed seedlings (Mohanty et al. 2006).

The expression of *GSA* increases upon light exposure of etiolated control seedlings. However, heat-stressed etiolated seedlings display a higher *GSA* expression level than etiolated control seedlings. *GSA* expression further increases in illuminated heat-stressed seedlings and is significantly reduced in cold-treated cucumber seedlings (Fig. 27.3) (Mohanty et al. 2006).

Reduced ALA biosynthesis in cucumber at low temperature could be due to reduced gene and/or protein expression of two ALA biosynthetic enzymes GluTR and GSA-AT.

The reduced Chl synthesis was also reported in temperature-stressed maize/Pinus seedlings mostly due to down-regulation of early intermediates of Chl biosynthesis i.e., GSA and ALA (Hodgins and Van Huystee 1986; Hodgins and Oquist 2006).

c. Regulation of ALA Biosynthesis by Salinity

The Chl biosynthesis and chloroplast biogenesis are substantially regulated by salt-stress. ALA content was reduced in sunflower leaves on treatment with salt stress (Santos 2004) that may be due to reduction in the ALA precursor glutamate (Santos and Caldeira 1999; Santos et al. 2001).

d. Water-Stress and ALA Biosynthesis

In response to water-stress, Chl biosynthesis is down-regulated. The reduced Chl synthesis in water-stressed seedlings is mostly due to down-regulation of early intermediates of Chl biosynthesis i.e., GSA and ALA (Dalal and Tripathy 2012).

Reduced GSA synthesis in water-stressed rice seedlings is due to down-regulation of *HEMA1* transcript abundance (Fig. 27.4). The protein/transcript abundance of GSA-AT increased (Fig. 27.4) in water-stressed rice seedlings, however the ALA contents declined suggesting that the GSA-AT, the next enzyme involved in ALA biosynthesis, may be inactivated by post-translational modification. These results show that the Chl biosynthesis pathway is down-regulated at the early steps under stress conditions to prevent the accumulation of harmful singlet oxygen generating tetrapyrroles (Dalal and Tripathy 2012).
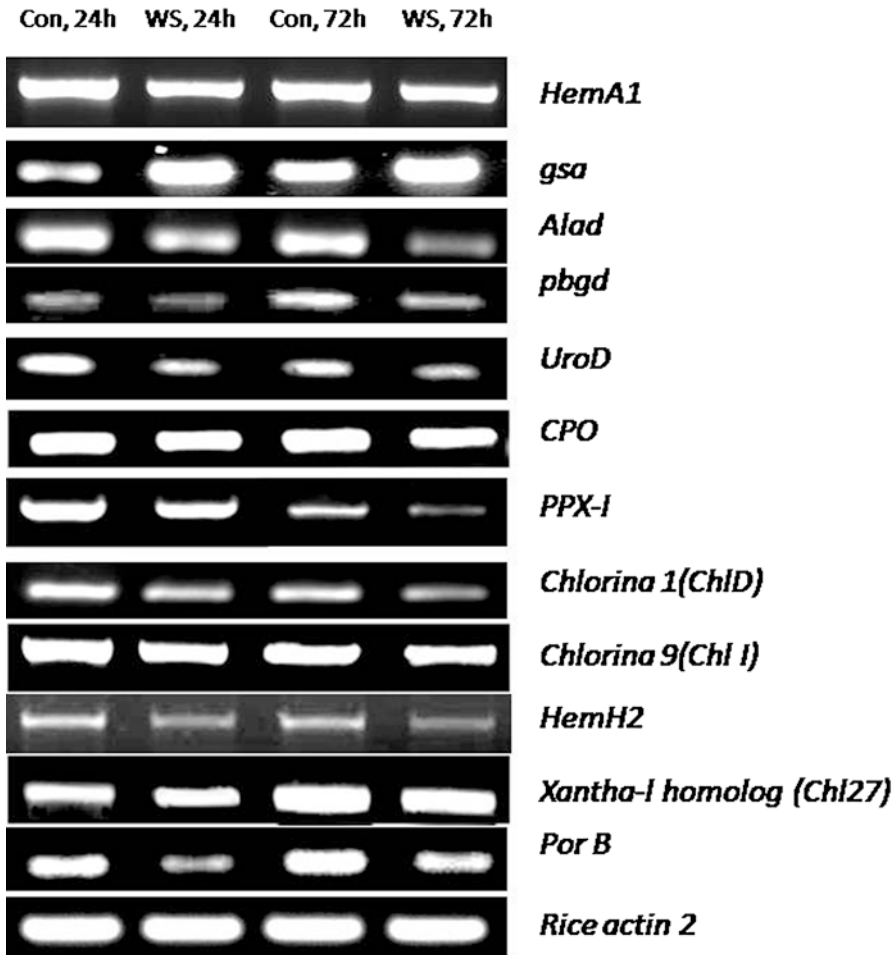
Fig. 27.4. Modulation of gene expression of chlorophyll biosynthetic enzymes due to water stress in seedlings of drought sensitive rice cultivar Pusa Basmati 1, after 24 h and 72 h of greening. Con denotes control and WS denotes water-stressed seedlings, respectively, that were treated with 50 mM PEG 6000, dissolved in nutrient solution, 16 h prior to transfer to 100 μmol m^{-2} s^{-1} white light (Dalal and Tripathy 2012).

Micromolar concentrations of Zn^{2+}, Cu^{2+} and Cd^{2+} inhibit ALA biosynthesis in barley by impairing the activity of GluTR (Pontoppidan and Kannangara 1994).

B. Biosynthesis of Protoporphyrin IX

Protoporphyrin IX (PPIX) is synthesized from eight molecules of ALA by a series of enzymatic biochemical reactions that are largely common to plants and animals. PPIX synthesis involves several enzymes.

5-Aminolevulinic acid dehydratase (ALAD), also known as porphobilinogen (PBG) synthase is a homo-octameric metal-loenzyme that catalyzes the condensation of two ALA molecules to form PBG (Fig. 27.1b) (Shemin 1976; Spencer and Jordan 1995). The ALAD of spinach is a hexamer with molecular weight of 300 kDa (Liedgens et al. 1980). The enzyme from radish cotyledons has a pH optimum of 8.0 (Shibata and Ochiai 1977) and requires Mg^{2+} and Mn^{2+} for activity. It is most active at slightly alkaline pH and shows a maximal binding of three Mg (II) per subunit (Kervinen et al. 2000).

The gene encoding ALAD is cloned from various plant sources. It has been isolated from pea, spinach, soybean and tomato.

The enzyme porphobilinogen deaminase (PBGD) is a soluble chloroplast protein (Castelfranco et al. 1988) that catalyzes the formation of the linear tetrapyrrole, hydroxymethylbilane, from four molecules of PBG (Fig. 27.1). Molecular weights from different plants range from 34 to 44 kDa. The *PBGD* gene has been isolated and cloned from pea (Witty et al. 1993) and *A. thaliana* (Lim et al. 1994). In *A. thaliana*, PBGD was found in both leaves and roots (Lim et al. 1994). Sequence comparison from different species shows that specific Arg and Cys residues are well conserved, and that these are implicated in catalysis and dipyrromethane cofactor binding (Witty et al. 1993). The synthesis and activity of PBGD are regulated by light and differ among cell types (Smith 1988; Shashidhara and Smith 1991; Spano and Timko 1991; He et al. 1994).

The Uroporphyrinogen III Synthase (UROS) enzyme, in concert with PBGD, catalyses formation of uroporphyrinogen III (Urogen III) from hydroxymethylbilane, a product of PBGD activity. This enzyme helps in maintaining the formation of the biologically active isomer III by inverting the ring D; in its absence, hydroxymethylbilane spontaneously cyclizes to uroporphyrinogen I (Urogen I). Inversion of ring D probably involves the production of a spiro-cyclic intermediate (Crockett et al. 1991). This enzyme has been purified from wheat germ (Higuchi and Bogorad 1975). The enzyme was found to be heat labile and the activity was enhanced by Na^+ and K^+. The enzyme PBGD and UROS may be present as a complex (Tsai et al. 1987). The *UROS* gene was isolated from *A. thaliana* (Tan et al. 2008). The localization of the protein in the chloroplast was confirmed by an in vitro protein import study and confocal microscopy (Tan et al. 2008). The barley *uros* mutant showed a necrotic phenotype in a developmental manner because of Urogen I accumulation (Ayliffe et al. 2009). The mutation in *UROS* also suppressed the expression of genes involved in the light reactions of photosynthesis (Ayliffe et al. 2009).

The Uroporphyrinogen III Decarboxylase (UROD) enzyme catalyzes stepwise decarboxylation of Urogen III to yield coproporphyrinogen III (Coprogen III). The enzyme catalyzes decarboxylation of all four carboxyl residues of Urogen III to yield Coprogen III. The order of Urogen III decarboxylation is substrate concentration dependent and under normal conditions enzymatic decarboxylation begins at the ring-D acetate group, in a clockwise manner (Luo and Lim 1993). Although all four isomers of Urogen are accepted by the enzyme, aromatic porphyrins are not decarboxylated (Castelfranco and Beale 1981). The discrimination between isomers, Urogen I and Urogen III for conversion into Coprogen occurs principally at the first step. Porphyrins, especially oxidation products of the substrates, have been shown to inhibit the enzyme (Smith and Francis 1981). The *UROD* was cloned from tobacco and barley (Mock et al. 1995). The in vitro translational product of *UROD* was imported into pea chloroplasts and processed into a 39 kDa product (Mock et al. 1995). Martin et al. (2001) reported the first crystal structure of a plant (tobacco) UROD.

Coproporphyrinogen oxidase (Coprox, CPOX) catalyses the oxidative decarboxylation of propionate side chains on ring A and B of Coprogen III to yield protoporphyrinogen IX (Protogen IX). In aerobic organisms, oxygen is utilized as the sole electron acceptor for enzymatic activity. The enzyme activity was found to be activated by Fe^{2+}, Mn^{2+} and inhibited by EDTA and o-phenanthroline (Hsu and Miller 1970). The corresponding gene, *CPOX*, was isolated and characterized from soybean (Madsen et al. 1993), tobacco and barley (Kruse et al. 1995a, b), *A. thaliana* (Ishikawa et al. 2001) and maize (Williams et al. 2006). The *CPOX* mRNA is highly expressed in soybean root nodules followed by leaves, but no mRNA for *CPOX* was detectable in soybean roots (Madsen et al. 1993). The level of mRNA reached its maximum in developing cells and decreased drastically when cells were completely differentiated. The Coprox isoform, CPO1 fused with green fluorescent protein showed that it was localized in the plastids, whereas CPO2 appeared to localize to mitochondria (Williams et al. 2006). However, mitochondria lack CPOX activity (Smith 1988).

Protoporphyrinogen oxidase (Protox) catalyzes the oxygen-dependent aromatization of Protogen IX to protoporphyrin IX (PPIX, Proto IX). This enzyme catalyzes the six-electron oxidation of Protogen using a flavin cofactor, and molecular oxygen as terminal electron acceptor (Poulson and Polglasse 1974). Protogen is unstable and spontaneously undergoes oxidation in the presence of oxygen and its oxidation is enhanced by light (Jacobs and Jacobs 1979). Koch et al. (2004) reported on the crystal structure of mitochondrial Protox from tobacco and revealed that it contains an FAD-binding domain, a substrate-binding domain and a membrane-binding domain (Koch et al. 2004). Protox forms a loosely associated dimer that folds into an FAD-binding and substrate-binding domain. The substrate-binding domain of Protox also helps in forming a complex with the ferrochelatase enzyme. Protox has been purified from barley etioplasts (Jacobs and Jacobs 1987) and found to be localized in the envelope (stromal side) and thylakoid membranes (stromal side) of chloroplasts (Matringe et al. 1992a; Che et al. 2000). The envelope and thylakoid membranes fail to synthesize Proto IX from the substrate ALA, whereas the stromal fraction could synthesize a small amount. When however, all three components were mixed together the PPIX synthesizing capacity increased. The PPIX synthesizing capacity was reduced by oxidizing agents, and increased in the presence of reductants like dithiothreitol (DTT). ATP also increased PPIX synthesis (Manohara and Tripathy 2000).

Protox has been isolated from spinach, tobacco and *A. thaliana* (Narita et al. 1996; Lermontova et al. 1997; Che et al. 2000; Watanabe et al. 2001). In all these plant species, Protox was encoded by two genes, namely *PPOX1* and *PPOX2*, and was found in both the chloroplast and mitochondria, respectively. In *A. thaliana* the levels of transcripts of plastid Protox were very high in leaves, whereas it was low in roots and floral buds (Narita et al. 1996). In tobacco, both transcripts were accumulated synchronously during diurnal and circadian growth (Lermontova et al. 1997). The spinach PPOX1

are preferentially localized to the stromal side of the thylakoid membrane and inner envelope membrane (Che et al. 2000). The spinach *PPOX2* codes for two proteins of 59 kDa (PPOX2 L) and a 55 kDa (PPOX2 S) by using two in-frame start codons. PPOX2 L is associated with the chloroplast inner envelope membrane and PPOX2 S is associated with inner mitochondrial membranes (Watanabe et al. 2001). As it is folded into an extremely compact form, the Protox is highly resistant to proteases i.e., trypsin, endoproteinase Glu-C, or carboxypeptidases (Arnould and Camadro 1998). However, structurally bicyclic herbicides i.e., diphenyl ether-type herbicides, were shown to inhibit Protox activity in chloroplasts (Camadro et al. 1991; Matringe et al. 1992b). It has also been shown that the fungal toxin cyperin inhibits Protox activity (Dayan et al. 2008).

1. Developmental Modulation of ALAD

In cucumber and wheat *ALAD* expression increases upon transfer of etiolated seedlings to light (Mohanty et al. 2006). However, in pea, expression of *ALAD* is high in dark-grown samples as compared to light-grown samples (Li et al. 1991). In contrast, the corresponding protein level is significantly lower in dark-grown seedlings as compared to light-grown seedlings (He et al. 1994). ALAD is detectable in embryonic leaves whether the plants are grown in darkness or under continuous white-light illumination (He et al. 1994). In pea, *ALAD* transcript abundance is highly dependent on leaf developmental age; the transcript abundance increases with time until the leaf is fully expanded. Subsequently, its mRNA abundance decreases sharply (He et al. 1994). However, a significant amount of the protein is detected even in the matured leaves despite the mRNA abundance of *ALAD* being extremely low. The activity of ALAD significantly decreases during senescence (Hukmani and Tripathy 1994).

ALAD from tobacco leaves and radish cotyledons is inhibited by Zn^{2+} and Fe^{2+} (Shetty and Miller 1969; Shibata and Ochiai 1977), and arsenic inhibits its activity in

maize leaves (Jain and Gadre 2004). $PbCl_2$ and $CdCl_2$ inhibit ALAD in *Amaranthus lividus* (Bhattacharjee and Mukherjee 2003).

2. Developmental Regulation of PBGD

The *PBGD* mRNA is slightly higher in the dark than in the light, even though the protein level is significantly lower in dark (He et al. 1994). The *PBGD* transcript abundance is dependent on leaf developmental age; i.e., the transcript abundance increases with increased age until the leaf is fully expanded and after that its mRNA level declines (He et al. 1994). Despite an extremely low level of *PBGD* mRNA, a significant amount of protein is detected even in matured leaves. Furthermore, PBGD activity rapidly declines during senescence (Hukmani and Tripathy 1994).

The enzyme is heat stable and maintains its activity at temperatures ranging from 55°C to 70°C. The PBGD enzyme from pea chloroplasts was inhibited by Fe^{2+}, Mn^{2+} and Zn^{2+} whereas Ca^{2+} and Mg^{2+} were only weakly inhibitory at physiological concentrations (Spano and Timko 1991).

3. Environmental Regulation of UROD

The expression of the *UROD* gene and the corresponding protein level increase during illumination in barley (Mock et al. 1995) and cucumber (Mohanty et al. 2006).

In heat-stressed plants UROD activity, its gene and protein expression are substantially upregulated in heat-stressed seedlings whereas it is downregulated in chill-stressed plants (Tewari and Tripathy 1998; Mohanty et al. 2006) (Fig. 27.3).

The enzyme activity is inhibited by metals such as Fe^{2+}, Co^{2+}, Pb^{2+}, Ni^{2+} and Mg^{2+} (Chen and Miller 1974), but stimulated by ATP (Manohara and Tripathy 2000).

4. Modulation of CPO

The *CPO* gene expression is not affected by light or heat stress in wheat and cucumber, however the gene expression is down-regulated by chill stress (Mohanty et al. 2006) (Fig. 27.3).

In vitro protein import assays of tobacco and barley CPO protein showed that it was imported into the pea chloroplast and accumulated in the stroma. There are two isoforms of CPO (CPO1 and CPO2) in maize. *A. thaliana* mutant defective in the *lesion initiation 2 (LIN2)* gene encoding CPO develops lesions on leaves, in a developmentally regulated and light-dependent manner (Ishikawa et al. 2001).

5. Environmental Modulation of Protox

The Protox activity substantially decreases in response to chill-stress (Tewari and Tripathy 1998). Its transcript and protein abundance decrease in water- stressed seedlings (Fig. 27.4) (Dalal and Tripathy 2012).

6. Environmental Regulation of Proto IX Biosynthesis

Proto IX biosynthesis is modulated by several environmental factors. In chill-stressed seedlings Proto IX synthesis from Urogen III is severely reduced whereas in heat-stressed seedlings the activity is substantially increased (Tewari and Tripathy 1998). Proto-IX synthesis from Coprogen III is reduced in chill-stressed seedlings, however in heat-stressed seedlings, Proto IX synthesis from Coprogen III is not affected (Tewari and Tripathy 1998). In heat-stressed seedlings, ALAD and PBGD were partially inhibited.

Reduced ALAD activity and gene expression were observed in water-stressed rice and chill- and heat-stressed cucumber seedlings (Figs. 27.3 and 27.4) (Mohanty et al. 2006; Dalal and Tripathy 2012). Limitation of ALA, a substrate for ALAD probably reduced its gene expression in water stress and other stress conditions. The increased or decreased availability of the substrate of the enzyme could positively or negatively regulate the gene expression of the enzyme. The enzymatic activity of PBGD that deaminates PBG to form Urogen III is reduced (Dalal and Tripathy 2012) due to down-regulation of its transcript abundance in water-stressed rice seedlings (Fig. 27.4).

UROD protein abundance decreases in water-stressed seedlings, which well

correlates with the declined message abundance of *UROD* in response to water stress (Fig. 27.4). The UROD protein and transcript abundance also decline in chill-stressed wheat seedlings (Mohanty et al. 2006). This is in contrast to the earlier observations in cucumber and wheat where UROD activity and its transcript/protein abundance increased (Fig. 27.3) in response to heat-stress (Tewari and Tripathy 1998; Mohanty et al. 2006). The next two enzymes involved in Proto IX biosynthesis are Coprox and Protox. The enzyme activity of Coprox and Protox decreased in water-stressed rice and chill-stressed cucumber seedlings (Dalal and Tripathy 2012; Tewari and Tripathy 1998) due to down-regulation of their gene/protein abundance (Figs. 27.3 and 27.4).

C. Biosynthesis of Protochlorophyllide

The PPIX synthesis reactions are common to plants and animals. In green organisms the tetrapyrrole biosynthesis pathway branches to the formation of Fe-tetrapyrrole leading to synthesis of heme and Mg-tetrapyrrole that results in the synthesis of Chl. In green plants the Mg-branch involves insertion of Mg to PPIX by Mg-chelatase to synthesize Mg-PPIX, esterification of Mg-PPIX to Mg-protoporphyrin IX monomethylester (MPE) mediated by Mg-Protoporphyrin IX: S-adenosyl methione methyl transferase and formation of the isocyclic ring to synthesize Pchlide by MPE cyclase (Fig. 27.1c).

The insertion of Mg^{2+} into PPIX is catalyzed by Mg-chelatase to synthesize Mg-protoporphyrin IX (Mg-PPIX). In photosynthetic organisms, Mg-chelatase has three subunits (CHLI, CHLD and CHLH) and it catalyses the insertion of Mg^{2+} in two steps; an ATP-dependent activation that is followed by an ATP-dependent chelation step (Walker and Weinstein 1994; Walker and Willows 1997). The optimal ATP concentration for activation is found to be higher than that of chelation step. Out of its three subunits, CHLI is an ATPase and its ATPase activity is repressed when it forms a complex with CHLD (Jensen et al. 1999). The N-terminal halves of subunits

CHLD and CHLI share high sequence similarity suggesting that the CHLD subunit is also an AAA+protein (*A*TPases *A*ssociated with diverse cellular *A*ctivities); however, its ATPase activity has yet not been detected (Jensen et al. 1999).

The *CHLI/Chlorina9* has been cloned from soybean, barley (Jensen et al. 1996), *A. thaliana* (Gibson et al. 1996; Rissler et al. 2002), maize and rice (Zhang et al. 2006). This protein is localized to the stroma. In *A. thaliana*, most of the *CHLI* homozygous mutants have a pale green phenotype (Rissler et al. 2002). A second *CHLI* gene, *CHLI-2* has been reported from *A. thaliana* (Rissler et al. 2002; Huang and Li 2009).

The N-terminus of CHLD shows structural similarities with the AAA domain of CHLI and therefore it is believed to contribute in complex formation and interaction with CHLH (Fodje et al. 2001). The *CHLD/Chlorina1* cDNA sequence has been isolated and cloned from tobacco (Papenbrock et al. 1997) and rice (Zhang et al. 2006). The *CHLD* gene expression changes with respect to the diurnal changes in tobacco (Papenbrock et al. 1999). Virus-induced gene silencing of *CHLH* in tobacco led to the lowering of *CHLD* and *CHLI* mRNAs along with reduction in the Chl contents (Hiriart et al. 2002).

In tobacco, *CHLH* is strongly expressed in young leaves and less expressed in mature leaves and only traces of its transcripts were found in flowering organs (Kruse et al. 1997). *CHLH* expression was found to be light inducible in soybean and rice and its transcript levels were under the control of a circadian oscillation (Nakayama et al. 1998; Jung et al. 2003). CHLH protein was also found to be induced on transferring the Arabidopsis seedlings to white light from dark (Stephenson and Terry 2008). The *CHLH* transcripts undergo diurnal variation in *A. thaliana* and tobacco (Gibson et al. 1996; Papenbrock et al. 1999). Depending upon the concentration of Mg^{2+} in the lysis buffer, the CHLH protein migrated between stroma and the envelope membranes and was localized in the envelope membrane at very high concentrations of Mg^{2+} (Nakayama et al. 1998).

Mutants of *CHLH* have been isolated from *A. thaliana* (Mochizuki et al. 2001). *Genome uncoupled 5* (*GUN5*) gene codes for CHLH subunits of Mg-chelatase. The rice *CHLH* mutants also showed a Chl-deficient phenotype (Jung et al. 2003; Zhang et al. 2006). In *A. thaliana*, the Mg-chelatase subunit CHLH regulates retrograde signaling (Mochizuki et al. 2001). Mutation in the *CHLH* gene results in the repressed expression of *LHCB*. Transgenic tobacco plants expressing antisense RNA for Mg-chelatase *CHLH* were Chl deficient (Papenbrock et al. 2000b). In these plants, less PPIX and heme accumulated, and a decrease in ALA synthesizing capacity was observed. *A. thaliana* protein GUN4 regulates Mg-chelatase activity (Larkin et al. 2003; Davison et al. 2005), and promotes the interactions between CHLH and chloroplast membranes and Chl biosynthesis (Adhikari et al. 2009, 2011).

S-adenosyl-L-methionine:Mg-PPIX methyltransferase (SAM-MgProtoMTF) catalyzes the conversion of Mg-PPIX to Mg-protoporphyrin monomethyl ester (MPE). It transfers a methyl group to the carboxyl group of the C13-propionate side chain of Mg-PPIX (Gibson et al. 1963) where SAM acts as a methyl group donor. The gene (*CHLM*) encoding for the SAM-MgProtoMT is cloned from *A. thaliana* (Block et al. 2002) and tobacco (Alawady and Grimm 2005). In tobacco, the methyltransferase physically interacts with the CHLH subunit of Mg-chelatases (Alawady et al. 2005).

The *A. thaliana* CHLM protein contains an N-terminal plastid transit sequence. The mature protein (without transit peptide) contains two functional regions, the N-terminal hydrophobic region that enhances the association of the protein with the envelope and thylakoid membranes and the C-terminal region that binds to Ado-met (Block et al. 2002). The *A. thaliana*, *CHLM* T-DNA insertion mutant shows albino phenotype; there is accumulation of Mg-PPIX and reduction in major Chl protein complexes in this mutant (Pontier et al. 2007). Down-regulation of the CHLM protein in antisense *CHLM* tobacco plants results in reduced ALA-synthesis and Mg-chelatase activities (Alawady and Grimm 2005).

Mg-protoporphyrin IX monomethylester cyclase catalyzes the formation of the isocyclic ring E of the Mg-protoporphyrins and converts MPE to Pchlide. There are two pathways for the formation of the isocyclic ring, i.e., aerobic cyclization and anaerobic cyclization. The former pathway is predominant in plants, green algae and cyanobacteria where the ketone oxygen of divinyl Pchlide (DV-Pchlide) is derived from molecular oxygen (Walker et al. 1989). The pH optimum of the cyclase activity is approximately 9.0 and the enzyme activity was found to be inhibited by CN^- and N_3^- (Whyte and Castelfranco 1993). The cyclase reaction with the two barley mutants *xantha l* and *viridis K* revealed that at least two plastid proteins (a membrane bound protein and a soluble protein) are required for cyclization (Walker et al. 1991; Walker and Willows 1997). Biochemical and genetic studies have demonstrated that the gene responsible for the *xantha-l* mutant encodes a membrane-bound cyclase subunit and it needs a soluble fraction for the cyclization reaction (Rzeznicka et al. 2005). *Xantha l* mutants accumulated less Chl, high MPE and had no cyclase activity (Rzeznicka et al. 2005). Three *Xantha l* mutants were characterized. In the leaky mutant *xantha-l*[35], a C-to-T point mutation resulted in an exchange of amino acid residue Ser-181 to Phe. In the non-leaky mutant *xantha-l*[81], a G-to-A point mutation resulted in the exchange of Gly-155 to Glu. Sequence alignment showed that Gly-155 is a highly conserved residue. In the non-leaky mutant *xantha-l*[82], point mutation resulted in truncation as a TGG codon corresponding to Trp-291 turned into a TGA stop codon and the truncated protein was not stable. However, the level of the protein found in the *xantha-l*[35] and *xantha-l*[81] mutants was similar as in the wild-type (Rzeznicka et al. 2005).

The gene responsible for the aerobic cyclization reaction has been isolated and characterized from different plants i.e., *CHL27* from *A. thaliana* and *xantha l* from barley (Tottey et al. 2003; Rzeznicka et al. 2005). Antisense *A. thaliana* and tobacco

plants with reduced amounts of CHL27 show chlorotic leaves with reduced abundance of all Chl-proteins and accumulate MPE (Tottey et al. 2003; Peter et al. 2010). The *A. thaliana chl27* T-DNA mutant is pale green with an elevated Chl *a/b* ratio, and has unstacked thylakoid membranes with reduced LHCII protein. Their photosynthetic activity is reduced due to damaged Photosystem II (PS II) reaction centers (Bang et al. 2008; Hansson and Jensen 2009). In tobacco, the co-suppression of the *NTZIP,* which includes coding region for a di-iron motif, resulted in reduced Chl level and lower photosynthetic activity (Liu et al. 2004).

1. Environmental Regulation of Protochlorophyllide Synthesis

The accumulation of Mg-PPIX and pheo-phorbide inhibits Mg-chelatase activity in pea (Popperl et al. 1997). Mg-chelatase activity and the expression of the genes encoding this enzyme are up-regulated by light (Mohanty et al. 2006) (Fig. 27.3). The *CHLI* mRNA is induced by light (Gibson et al. 1996; Jensen et al. 1996; Nakayama et al. 1998) and constitutively expressed in matured leaves. It is also regulated by diurnal rhythm but not regulated by circadian rhythm (Matsumoto et al. 2004). CHLI could be a target for chloroplastic thioredoxin and the in vivo reduction process is light dependent (Ikegami et al. 2007).

The Mg-chelatase activity is severely down-regulated in chill- and heat- stressed cucumber seedlings (Tewari and Tripathy 1998). In wheat light treatment in control and heat-stressed seedlings leads to higher accumulation of *CHLI/Chlorina9* transcripts. Its expression diminishes in cold- and heat-treated wheat seedlings. *CHLI/Chlorina9* expression is also down-regulated by salt-stress in cucumber/wheat/rice seedlings.

Mg-chelatase activity is down-regulated in water-stressed rice seedlings (Dalal and Tripathy 2012) (Fig. 27.4). Moreover, the gene/protein expression of *CHLI/Chlorina9* and *CHLD/Chlorina1* subunits of Mg-chelatase (Zhang et al. 2006) partially declined in water-stressed seedlings. CHLI1

subunit is post-translationally regulated by chloroplastic thioredoxin (Ikegami et al. 2007) and therefore could have impaired function in altered redox environment in water-stressed seedlings. Stoichiometric imbalance among the subunits of Mg-Chelatase decreases the Mg-chelatase activity as seen in CHLI over-expressing or under-expressing transgenic Arabidopsis plants (Papenbrock et al. 2000a). Inadequate proportion of all subunits is known to hamper the correct assembly of active Mg-chelatase (Guo et al. 1998; Hansson et al. 1999; Jensen et al. 1999); therefore, nonstoi-chiometric abundance among its subunits may have led to decreased enzyme activity of Mg-chelatase in water stressed seedlings.

D. Phototransformation of Protochlorophyllide to Chlorophyllide

In angiosperms, Protochlorophyllide oxi-doreductase (POR) has the obligate require-ment of light for photo-converting Pchlide to chlorophyllide (Chlide). It catalyzes the con-version of Pchlide to Chlide in light using NADPH as reductant (Fig. 27.1c). POR con-verts Pchlide to Chlide, by adding two hydrogen atoms at C17 and C18 on ring D. In the POR catalytic cycle, a ternary enzyme-NADPH-Pchlide complex is formed. Light energy absorbed by the Pchlide in the com-plex may produce torsional strain in the molecule that provides a favorable condition for hydride/hydrogen transfer from NADPH (Begley and Young 1989). POR is a member of a large family of enzymes known as short chain dehydrogenases/reductases (SDR) (Wilks and Timko 1995), which generally catalyze NADP(H)- or NAD(H)-dependent reactions involving hydride and proton transfers. A tyrosine (Tyr) and a lysine (Lys) residues are both conserved throughout all members of the SDR family. In POR, it was also seen that Tyr and Lys residues are impor-tant for its activity (Wilks and Timko 1995; Lebedev et al. 2001). The Tyr may be depro-tonated, acting as a general acid to facilitate hydride transfer to or from $NAD(P)^+/H$ (Bohren et al. 1994). The proton at the C-18 position of Pchlide is derived from Tyr 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 pK_a of the phenolic group of the Tyr (Wilks and Timko 1995). 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 that 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* sp. PCC 6803 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 product (Chlide) and subsequent binding of Pchlide have taken place (Heyes and Hunter 2004). Monovinyl Pchlide (MV-Pchlide) and 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.

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 mutations within the central core prevent proper attachment to the thylakoid. It is a peripheral membrane protein that accumulates to a 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. 2001) and for NADPH and pigment binding (Townley et al. 2001; Reinbothe et al. 2006). 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 dark-grown bean seedlings indicated the existence of 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 (Schoefs and Franck 2003; Masuda and Takamiya 2004). Plastids isolated from dark-grown wheat seedlings exhibit a smaller 77 K fluorescence emission 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 a 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 (Schoefs and Franck 1993; He et al. 1994; Schoefs et al. 1994, 2000a, b). The dimer has an absorption maximum at 638 nm and an 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, illumination for more than a minute usually converts non-active Pchlide to photo-active Pchlide.

Full-length cDNA clones of *POR* were isolated from barley (Holtorf et al. 1995), pea (Spano et al. 1992), *A. thaliana* (Armstrong et al. 1995; Oosawa et al. 2000), tobacco (Masuda et al. 2002), cucumber (Kuroda et al. 1995) and many other higher plants. The high degree of sequence similarity among PORs from different taxonomic groups implies a common mechanism of enzyme action.

A characteristic feature of POR accumulating in darkness is its sensitivity to illumination. The *POR* mRNA expression was also decreased (Santel and Apel 1981). Red and far-red light treatment also inhibit *POR* gene expression indicating that *POR* expression is controlled by phytochrome (Mosinger et al. 1985). The negative effect of light on the POR enzyme and its mRNA was observed in different dicotyledons like bean, pea, tomato and *A. thaliana* (Forreiter et al. 1991; Spano et al. 1992; Armstrong et al. 1995) and in the monocotyledonous plants maize and barley (Forreiter et al. 1991; Holtorf et al. 1995). However, some flowering plants have isoforms of POR. In *A. thaliana,* (Armstrong et al. 1995; Oosawa et al. 2000; Su et al. 2001; Pattanayak and Tripathy 2002), barley (Holtorf et al. 1995; Holtorf and Apel 1996) and tobacco (Masuda et al. 2002) there are different PORs present. The N-termini of PORA and PORB of barley etioplasts have recently been characterized (Plöscher et al. 2009). In *A. thaliana* 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 decrease upon illumination of etiolated plants (Holtorff and Apel 1996) while that of *PORC* increases and was dominantly expressed in both mature and immature tissues (Oosawa et al. 2000). *PORB* transcript and PORB protein levels remain constant in both darkness and upon illumination (Armstrong et al. 1995; Holtorf et al. 1995; Holtorf and Apel 1996). Both *PORB* and *PORC* of *A. thaliana* exhibit diurnal fluctuation but only the *PORB* mRNA of

A. thaliana exhibits circadian regulation (Su et al. 2001). *PORC* mRNA and PORC protein levels also increased under high light intensity (Su et al. 2001; Masuda et al. 2003). In cucumber the levels of the *POR* mRNA increased in etiolated cotyledons when they were illuminated with continuous light (Kuroda et al. 1995; Fusada et al. 2000). The plant hormone cytokinin regulates cucumber *POR* gene expression by binding to the *cis*-elements present at the 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 1996). *POR* gene expression in cucumber is regulated by phytohormones, particularly by cytokinins and abscisic acid (Kuroda et al. 2001). In the *lip1* mutant of pea, cytokinins restored the formation of PLB and photoactive Pchlide in the dark (Seyedi et al. 2001a), but in *A. thaliana* their application results in loss of PLBs (Chory et al. 1994). In lupine, *POR* gene expression is also regulated by cytokinins and abscisic acid (Kusnetsov et al. 1998).

POR gene expression is also organ specific. *A. thaliana PORB* and *PORC* are expressed in all photosynthetic tissues of the mature plants but not in the root (Armstrong et al. 1995; Oosawa et al. 2000). Cucumber *POR* gene expression is also observed in photosynthetic tissues (Kuroda et al. 1995). Plant age plays a crucial role in *POR* gene expression. In *A. thaliana* and barley *PORA* expression is only observed in young seedlings whereas *PORB* is expressed both in young and matured green tissue (Armstrong et al. 1995). In *A. thaliana* 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, the highest level of expression is observed 8–10 days

after germination of seedlings (Spano et al. 1992). The transcript level of pea *POR* did not decrease after 48 h of light exposure. Immunoblot analysis showed that there was no POR protein detectable after 48 h of light exposure. These results suggested that pchlide reductase activity in pea is primarily regulated post-transcriptionally, most likely at the level of translation initiation/elongation or protein turnover (Spano et al. 1992).

Far-red-light modulation of POR: Etiolated seedlings of *A. thaliana* grown under continuous far-red light are unable to green when subsequently transferred to white light; this is called far-red blocking of the greening (Buhr et al. 2008). 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). Overexpression of *PORA* and *PORB* in specific mutants overcame the photooxidative damage (Sperling et al. 1997, 1998).

Overexpression of a cyanobacterial POR protein in the *A. thaliana porA* mutant could restore 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 but could form prolamellar bodies if treated with cytokinin (Seyedi et al. 2001a): however unlike the *A. thaliana* 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 *A. thaliana* (Frick et al. 2003; Masuda et al. 2003). Single *POR* mutants display no obvious phenotypes at the whole plant or chloroplast ultra-structural levels, except that etiolated *PORB* mutants have less extensive 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 seedlings were able to green to a similar extent as the wild type, and the greening of the *PORC* mutant was repressed under high light conditions.

From a molecular evolutionary perspective, the light-dependent POR (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 early evolution (Martin et al. 2002), and analysis of LPOR proteins in species of conifer shows evidence for loss of enzyme activity (Kusumi et al. 2006). The discovery of genes for LPOR in the plastid genomes of diverse cryptophyte algae suggests that these genes have been lost relatively recently.

In all photosynthetic organisms Pchlide and Chlide are originally formed as 3,8-divinyl derivates. The 8-vinyl reductase reduces the 8-vinyl group on the tetrapyrrole to an ethyl group using NADPH as the reductant. This enzymatic activity has been detected in isolated chloroplasts of barley (Tripathy and Rebeiz 1988), plastid membranes from cucumber (Parham and Rebeiz 1995), and also in solubilized crude extracts derived from etiolated barley leaves (Kolossov and Rebeiz 2001). It has been demonstrated in vitro that the monovinyl (MV) and divinyl (DV) Chl biosynthesis reactions may operate in parallel (Tripathy and Rebeiz 1986). However, the mutant of maize (*Zea mays*) that accumulated only DV-Chl instead of MV-Chl and capable of photosynthetic growth with DV-Chl suggests that a single gene product is responsible for the reduction of the vinyl group of Chlide (Bazzaz 1981). Nagata et al. (2005) followed by Nakanishi et al. (2005) isolated a mutant of *A. thaliana* which accumulates DV- Chl. By map-based cloning they detected that the gene is 8-vinyl

reductase. The recombinant protein was successfully tested for the conversion of the C8-vinyl group of Chlide to an ethyl group on ring B. The 3,8-divinyl-chlide *a* is the major substrate of divinyl reductase (DVR) (Nagata et al. 2007). Starch granules were not found in the mutant chloroplasts, suggesting the reduction of photosynthetic activity in the mutant (Nakanishi et al. 2005). The transcript level of *DVR* expression is high in leaves, stems and flower buds, and low in roots.

The mutant is pale green and the Chl *a*/*b* ratio varies in between 6 and 10 depending on the developmental stage and growth conditions. This mutant is capable of photosynthesizing and growing under low-light conditions (70–90 μmol photons $m^{-2} s^{-1}$); but it rapidly dies under high light conditions (1,000 μmoles photons $m^{-2} s^{-1}$) (Nagata et al. 2005). The thylakoid membranes were in a disorderly fashion having no distinct grana stacks in the mutant and no significant differences in the size and the number of chloroplasts between the wild type and the mutants were observed.

1. Role of POR in Combating Oxidative Stress

Light absorbed by colored intermediates of Chl biosynthesis is not utilized in photosynthesis. Instead, it is transferred to molecular oxygen, generating singlet oxygen ($^1O_2$) (Chakraborty and Tripathy 1992). As there is no enzymatic detoxification mechanism available in plants to destroy $^1O_2$, its generation should be minimized. The concentration of a major Chl biosynthetic intermediate, i.e., Pchlide in Arabidopsis was manipulated by overexpressing the light-inducible PORC that effectively phototransforms endogenous Pchlide to Chlide leading to minimal accumulation of the photosensitizer Pchlide in light-grown plants (Pattanayak and Tripathy 2011). In *PORC* overexpressing (*PORCx*) plants exposed to high-light, the $^1O_2$ generation and consequent malonedialdehyde production was minimal and the maximum quantum efficiency of photosystem II

remained unaffected (Fig. 27.5) demonstrating that their photosynthetic apparatus and cellular organization were intact.

Further, *PORCx* plants treated with 5-aminolevulinicacid, when exposed to light, photo-converted over-accumulated Pchlide to Chlide, reduced the generation of $^1O_2$ and malonedialdehyde production and reduced plasma membrane damage (Fig. 27.6). So *PORCx* plants survived and bolted whereas, the ALA-treated wild type plants perished. Thus, overexpression of *PORC* could be biotechnologically exploited in crop plants for tolerance to $^1O_2$-induced oxidative stress, paving the use of ALA as a selective commercial light-activated biodegradable herbicide (Pattanayak and Tripathy 2011).

Reduced Pchlide content in *PORCx* plants released the Pchlide-mediated feed-back inhibition of ALA biosynthesis that resulted in higher ALA production. Increase of ALA synthesis up-regulated gene expression and protein level of several downstream Chl biosynthetic enzymes elucidating a regulatory net work of expression of genes involved in ALA and tetrapyrrole biosynthesis (Pattanayak and Tripathy 2011).

2. Environmental Modulation of Shibata Shift

In etiolated wheat seedlings, the phototransformable Pchlide peak (F657) is substantially higher than the non-phototransformable peak (F632) (Fig. 27.7a) demonstrating the presence of large aggregates of POR-Pchlide-NADPH ternary complexes. Although chilling arrested *de novo* synthesis of the Chl biosynthetic intermediate Pchlide (Tiwari and Tripathy 1998), it did not affect the ratio of non-phototransformable to phototransformable Pchlide suggesting that low temperature did not affect formation of large aggregates of POR-Pchlide-NADPH ternary complexes (Fig. 27.7a) (Mohanty and Tripathy 2011). In contrast, although heat-stress partially arrested Pchlide synthesis (Fig. 27.7a) in etiolated seedlings (Tewari and Tripathy 1998), it substantially affected the aggregation state of POR-Pchlide-NADPH
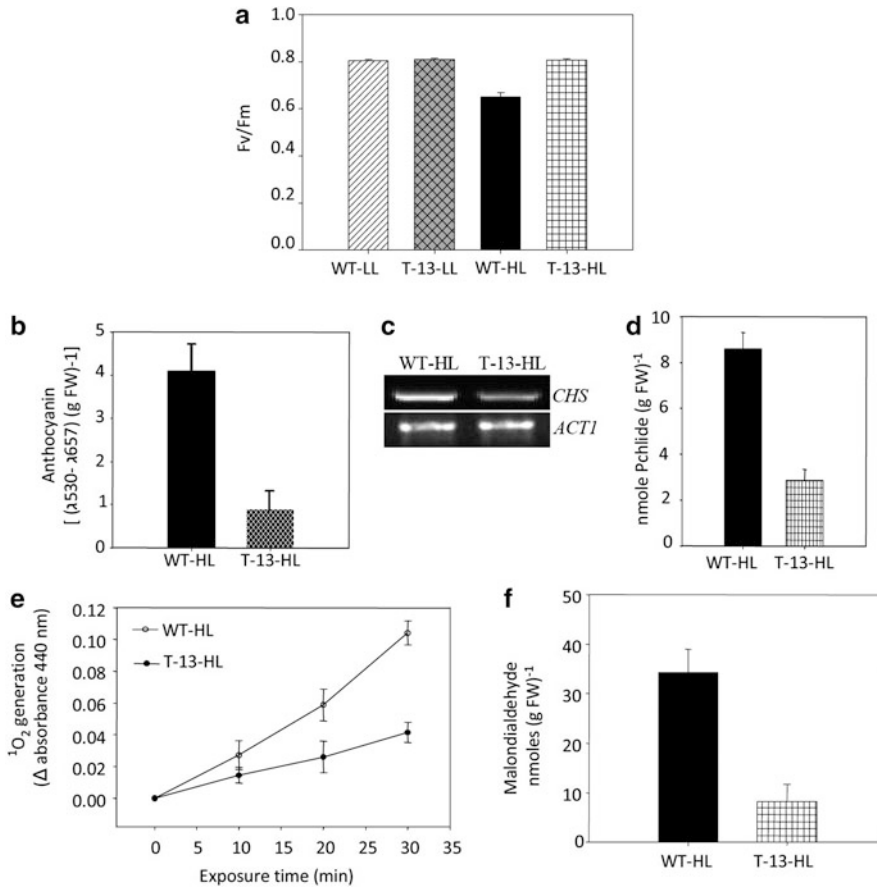
Fig. 27.5. Morphological and physiological responses of WT and PORCx (T-13) plants to light stress. Both WT and T-13 plants were grown in moderate light (100 μmoles photons m^{-2} s^{-1}) for 22–24 days and subsequently transferred to low-light (LL) (50 μmoles photons m^{-2} s^{-1}, 16 h light/8 h dark) or high-light (HL) (330 μmoles photons m^{-2} s^{-1}, 16 h light/8 h dark) regimes for 6-7 d as described in experimental procedures. (**a**) Photosynthetic efficiency (Fv/Fm) of leaves of LL- and HL-exposed plants was monitored by PAM 2100 fluorometer. Values are mean ± SD (n = 20). (**b**) Anthocyanin contents of WT and T-13 plants grown under HL. (**c**) The gene expression study of CHS in HL-grown WT and T-13 plants was done by RT-PCR as described in experimental procedures. *AtACT1* was used as an internal control. (**d**) Pchlide contents of HL-treated WT and T-13 plants measured 10 min after the end of dark period. (**e**) Singlet oxygen ($^1O_2$) contents in WT and T-13 plants. Thylakoid membranes were isolated in complete darkness from HL-exposed plants and the $^1O_2$ production were determined in terms of RNO bleaching using histidine as a trap. (**f**) Malondialdehyde (MDA) production in HL-treated WT and T-13 plants. Each data point represented in all the above experiments is the average of six replicates. The error *bar* represents SD. (Adopted from Pattanayak and Tripathy 2011).

complex as indicated by near absence of 657 nm peak in etiolated heat- stressed seedlings. Within hours of exposure of etiolated seedlings grown at 25°C to heat-stress, a progressive decline of the 657 nm phototransformable peak was observed suggesting the disaggregation of existing large aggregates of POR-Pchlide-NADPH polymeric complexes present in prolamellar bodies to monomeric or dimeric forms. The rate of degradation of polymeric complexes must exceed their formation, if any, in heat-stressed samples. Unlike heat-stress, in vivo application of salt-stress to excised wheat leaves reduced the peak of non-photo-transformable Pchlide and did not affect the
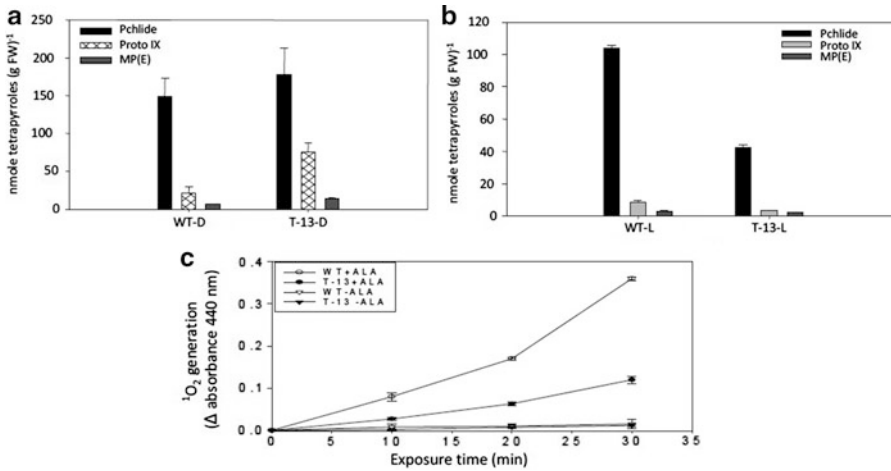
Fig. 27.6. Contents of chlorophyll biosynthetic pathway intermediates and singlet oxygen production in ALA-treated WT and PORCx (T-13) plants. WT and T-13 plants grown for 28–32 days at 220C $\pm$ 20C under 14 h L/10 h D photoperiod (100 μmoles photons m^{-2} s^{-1}) were sprayed with ALA (3 mM), dark incubated for 14 h and exposed to light (100 μmoles photons m^{-2} s^{-1}) for 10 min. Leaves were harvested both from dark incubated and light exposed plants, homogenized and their tetrapyrrole contents were monitored spectrofluorometrically. (**a**) Pchlide, Proto IX and MP(E) contents of ALA-treated (3 mM) and 14 h-dark-incubated WT and T-13 plants. (**b**) After dark incubation both WT and T-13 plants were exposed to light (10 min) and their Pchlide, Proto IX and MP(E) were determined. (**c**) $^{1}O_2$ contents in ALA-treated (+ALA) and untreated (−ALA) WT and T-13 plants. The experiments were repeated five times and each data point is the average of five replicates. The bars represents $\pm$ SD (Pattanayak and Tripathy 2011).

longer wavelength phototransformable forms suggesting that prothylakoids rather than prolamellar bodies were affected by salinity (Abdelkader et al. 2007a).

In addition to changes in the aggregation status of polymeric POR-Pchlide-NADPH complexes, the flash-induced phototransformation and the Shibata shift (Shibata 1957) leading to chloroplast biogenesis is substantially affected in temperature-stressed samples. Upon flash illumination (0.2 s) of etioplasts isolated from control seedlings the phototransformable Pchlide peak at 657 nm emanating from large aggregates of polymeric POR-Pchlide-NADPH complexes almost disappeared due to photo-reduction of Pchlide to Chlide. Transformation of $Pchlide_{655}$ into $Chlide_{692}$ was observed by exposing the leaf primordia of common ash (*Fraxinus excelsior* L.) and Hungarian ash (*Fraxinus angustifolia* Vahl.) (Solymosi and Böddi 2006) and that of Horse chestnut (*Aesculus hippocastanum*) (Solymosi et al. 2006) to a white light flash of 10 s. After 1 and

10 min of illumination, the peak at 692 nm slowly blue shifted to 676 nm (Shibata shift) (Fig. 27.7b). In chill-stressed and heat-stressed seedlings the Shibata shift was significantly arrested (Fig. 27.7a) (Mohanty and Tripathy 2011) probably due to the disaggregation of the PLB membrane particles or of the POR units as well as by their conformational changes (Böddi et al. 1990; Smeller et al. 2003).

Heating of excised etiolated barley leaves resulted in decreased accumulation of Pchlide(650), and a flash could trigger the formation of Chlide(672) instead of the formation of Chlide(684) (Eullaffroy and Popovic 1997). Similar to heat-stress, water-stress affects the Shibata shift, although the phototransformation of Pchlide to Chlide was not impaired by water deficit (Fig. 27.7b) (Le Lay et al. 2001). Both chill-stress and heat-stress affected the Shibata shift (Fig. 27.7a). This is variance to in vitro heating (40°C) of excised barley leaves where the Shibata shift was not affected (Eullaffroy et al. 1995).
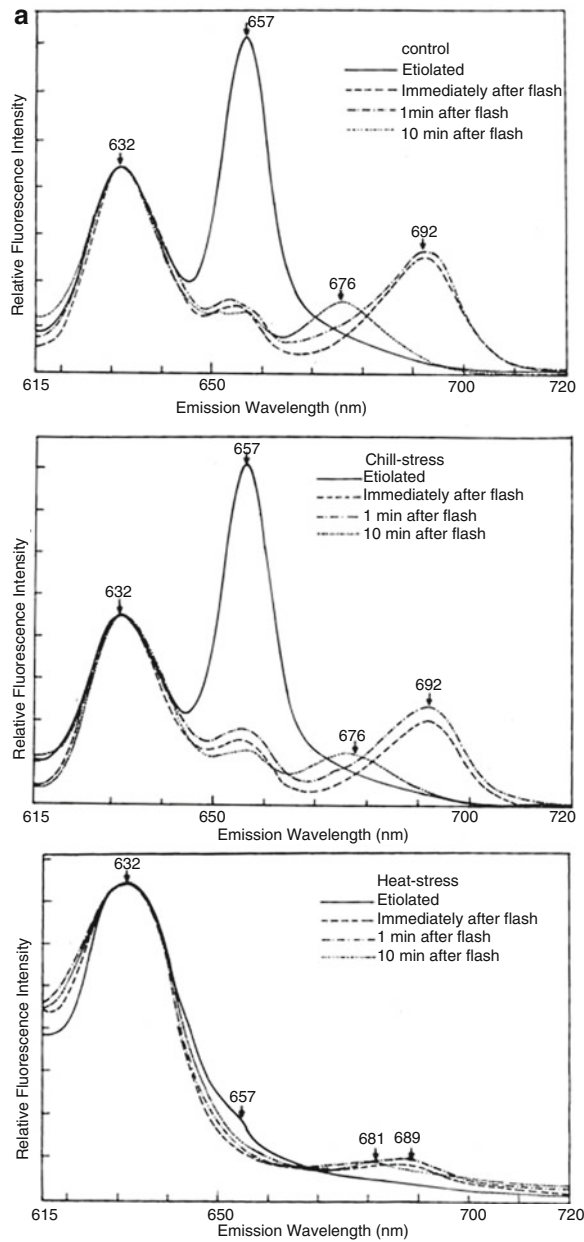
Fig. 27.7. (continued)

Upon 16 h of water-stress treatment, the etiolated seedlings displayed an emission fluorescence peak (77 K) at 632 nm due to non-phototransformable Pchlide and a peak at 657 nm due to phototransformable Pchlide (Fig. 27.7b). However, as compared to the control, the ratio of non-photo-transformable/photo-transformable Pchlide (F632/F657) increased from 0.10 to 0.15 in stressed seedlings suggesting an impairment of aggregation of monomeric POR-Pchlide-NADPH to 16-mer or larger aggregates of POR-Pchlide-NADPH complex, i.e. $(POR-Pchlide-NADPH)_n$. This may be due to reduced assembly or due to degradation of polymeric complexes in the stressed environment (Dalal and Tripathy 2012).
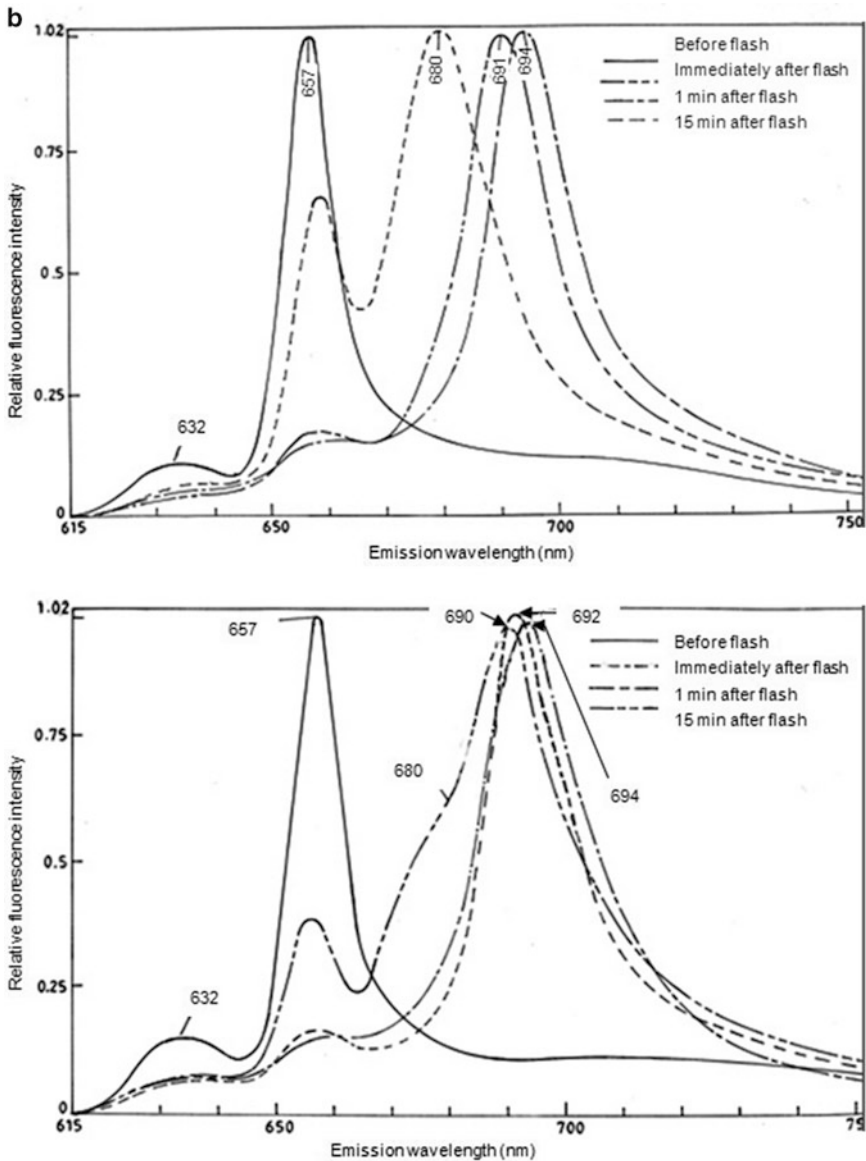
Fig. 27.7. (**a**) Low temperature (77 K) fluorescence emission spectra (E440) of plastids isolated from 6-day old etiolated control, (*upper panel*) chill-stressed (*middle panel*) and heat-stressed (*lower panel*) wheat seedlings showing Shibata-shift. Five-day old seedlings grown at 25°C were transferred to 7°C and 42°C in dark for 24 h. Low temperature fluorescence emission spectra were recorded before flash, immediately after flash (0.2 s) and after 1 min and 15 min post-flash incubation (Mohanty and Tripathy 2011). (**b**) Low temperature (77 K) fluorescence emission spectra (E440) of leaves from 6-d old etiolated control (*upper panel*) and water-stressed (*lower panel*) rice (PB1) seedlings, showing Shibata-shift. For water-stress, seedlings were treated with 50 mM PEG 6000, dissolved in nutrient solution, 16 h prior to taking spectra. Low temperature fluorescence emission spectra were recorded before the flash, immediately after a flash of 0.2 s and after 1 min and 15 min post-flash incubation (Dalal and Tripathy 2012).

The flash-induced photo-transformation and Shibata shift leading to chloroplast biogenesis was substantially affected in 16 h-water stressed samples. Upon red light flash illumination (0.2 s) of control leaves the phototransformable Pchlide peak at 657 nm emanating from large aggregates of polymeric (POR-Pchlide-NADPH)$_n$ complexes almost disappeared due to photo-reduction of Pchlide to Chlide, and a new peak appeared at 691 nm due to formation of Chlide-LPOR-NADP$^+$ complexes (El Hamouri et al. 1981; Oliver and Griffiths 1982; Franck 1993; Wiktorsson et al. 1993; Franck et al. 1999). Transformation of Pchlide 658 into Chlide692 was observed by exposing the leaf primordia of common ash (*Fraxinus excelsior* L.) and Hungarian ash, *Fraxinus angustifolia* Vahl. (Solymosi et al. 2006), wheat (Franck et al. 1999) and that of Horse chestnut (*Aesculus hippocastanum*) (Solymosi et al. 2006) to light flash. One min after flash, 691 nm-peak shifted to 694 nm (Fig. 27.7b) in control leaves due to the formation of Chlide-LPOR-NADPH complexes (El Hamouri et al. 1981; Oliver and Griffiths 1982; Franck et al. 1999). Subsequently, this peak blue-shifted to 680 nm after 15 min post-flash incubation of control leaves due to the release of Chlide from the active site of LPOR and disaggregation of multimeric complexes, a process called Shibata shift (Shibata 1957; Böddi et al. 1990; Franck 1993).

In water-stressed leaves, upon red light flash illumination of etiolated leaves the phototransformable pchlide peak at 657 nm disappeared and a new peak appeared at 692 nm due to formation of Chlide-LPOR-NADP$^+$ complexes (El Hamouri et al. 1981; Oliver and Griffiths 1982) demonstrating that phototransformation of Pchlide to Chlide could still take place in 16 h-water-stressed samples (Dalal and Tripathy 2012). After 1 min post-flash incubation this peak shifted to 694 nm due to the formation of Chlide-LPOR-NADPH complexes (Fig. 27.7b). In water stressed leaves the shift to lower wavelengths was substantially delayed. A shoulder appeared at 680 nm after 15 min of dark incubation, in contrast to complete shift to 680 nm in control seedlings, suggesting a slow release of Chlide from the active site of LPOR (Shibata 1957; Böddi et al. 1990). In a non-physiological environment i.e. after desiccation of detached barley leaves a slow-down of Shibata was earlier reported (Le Lay et al. 2000, 2001). Upon 15 min of dark incubation after flash illumination, a good amount of phototransformable Pchlide (F657) was regenerated in control seedlings (Fig. 27.7b) and substantially less in water-stressed seedlings (Fig. 27.7b) demonstrating the down-regulation of synthesis of Pchlide and its conversion to photo-transformable form.

E. Synthesis of Chlorophyll *a* and Chlorophyll *b*

Chlorophyllide *a* oxygenase (CAO) catalyzes the oxidation of Chlide *a* to Chlide *b* (Fig. 27.1d). During conversion of Chlide *a* to Chlide *b* the electron is transferred from the Rieske center to the mononuclear iron with subsequent activation of molecular oxygen for oxygenation of the Chlide *a* methyl group (Beale and Weinstein 1990; Porra et al. 1993). Chlide *b* is synthesized by oxidation/conversion of the methyl group on the D ring of the porphyrin molecule to a formyl group at that position. The CAO enzyme contains domains for a [2Fe-2S] Rieske center and for a mononuclear nonheme iron-binding site and has a tyrosine radical (Eggink et al. 2004). The conserved Rieske center and non-heme-iron binding motifs of CAO are likely to be involved in electron transport from ferredoxin to molecular oxygen. The recombinant CAO protein catalyzes chlide *a* to chlide *b* in the presence of NADPH and reduced ferredoxin (Oster et al. 2000). However, Pchlide *a* is not a substrate for the CAO enzyme (Oster et al. 2000).

The *CAO* was first cloned by Tanaka et al. (1998) from Chlamydomonas and also has been cloned from *A. thaliana* (Espineda et al. 1999) and rice (Lee et al. 2005). Both transcript and protein level of CAO increased when *A. thaliana* plants were transferred from moderate to shade light (Harper et al. 2004). Rice has two CAO isoforms namely *OsCAO1*,

OsCAO2 that are differentially regulated in light and dark. The level of the *OsCAO1* transcript is less in the dark and is higher in the light whereas the *OsCAO2* mRNA levels are higher in dark conditions and are reduced by exposure to light (Lee et al. 2005).

Overexpression of the *CAO* gene in *A. thaliana* led to an increase in the Chl *b* levels leading to reduction of the Chl *a/b* ratio from 2.85 to 2.65 in full green rosette leaves and at the same time there was 10–20% increase in antenna size (Tanaka et al. 2001). Overexpression of *A. thaliana CAO* in *Synechosystis* sp. PCC 6803 resulted in production of Chl *b* up to about 10% of total Chl content and the resulting Chl *b* pigments were efficiently incorporated into the Photosystem I Chl-protein complex (Satoh et al. 2001). Simultaneous overexpression of both *CAO* and *LHC II* genes in *Synechosystis* sp. PCC 6803 resulted in an increase in Chl *b* content up to 80% of total Chl (Xu et al. 2001). High light grown transgenic *A. thaliana* plants also showed decreased Chl *a/b* ratio under high light (Tanaka and Tanaka 2005). When the *CAO* gene of *Prochlorothrix hollandica* was overexpressed in *A. thaliana,* it was observed that approximately 40% of Chl *a* of the core antenna complexes was replaced by Chl *b* in both photosystems (Hirashima et al. 2006). The CAO sequence has been divided into four parts, the N-terminal sequence predicted to be a transit peptide, the subsequent conserved sequence unique in land plants (A-domain), a less-conserved sequence (B-domain) and the C-terminal conserved sequence common in chlorophytes and prochlorophytes (C-domain) (Nagata et al. 2004). The C-domain is sufficient for catalytic activity and the N-terminal 'A' domain confers protein instability by sensing the presence of Chl *b* and regulates the accumulation of the CAO protein (Yamasato et al. 2005). Chloroplast Clp protease is involved in regulating Chl *b* biosynthesis through the destabilization of CAO in response to the accumulation of Chl *b* (Nakagawara et al. 2007). The B domain alone is not involved in the regulation of CAO protein levels

(Sakuraba et al. 2007). Further work on domain analysis also indicated that transgenic *A. thaliana* plants overexpressing CAO from which the A-domain had been deleted, accumulated an excess amount of Chl *b* during greening and the etiolated transgenic plants either died or were retarded when exposed to continuous light immediately after etiolation (Yamasato et al. 2008). This was most likely due to deregulated Chl b synthesis that reduced the energy transfer rate between photosynthetic pigments (Sakuraba et al. 2010).

Chl synthetase encoded by *CHLG* catalyzes the esterification of Chlide *a* and Chlide *b* to Chl (Fig. 27.1d) (Rüdiger et al. 1980). Pchlide is not the substrate for this enzyme, which indicates that reduction of the 17, 18 double bond on ring D is essential for esterification (Benz and Rüdiger 1981b). Compounds which have the 13(2)-carbomethoxy group at the same side of the macrocycle as the propionic side chain of ring D are neither substrates nor competitive inhibitors (Helfrich et al. 1994). Only compounds having the 13(2)-carbomethoxy group at the opposite site are substrates for the enzyme. Esterification of Chlide is a rapid phase, leading to esterification of 15% of total Chlide within 15–30 s, followed by a lag-phase of nearly 2 min and a subsequent main phase (Schmid et al. 2002). It has been shown that the conversion of Chlide to Chl is a four-step process including three intermediates, i.e., Chlide geranylgeraniol, Chlide dihydrogeranylgeraniol and Chlide tetrahydrogeranylgeraniol before the formation of Chlide phytol or Chl (Schoefs et al. 2000a, b).

In etioplasts, geranyl-geranyl pyrophosphate (GGPP) is used as a substrate (Rüdiger et al. 1980), while in chloroplasts the preferential substrate is phytyl diphosphate (PhPP) (Soll et al. 1983). Chl synthetase in chloroplast thylakoid membranes incorporates phytol in the presence of ATP and a stromal kinase (Benz and Rüdiger 1981a). The enzyme was not affected by the developmental stage of the plastids. In etiolated wheat, the enzyme was found in latent form in PLBs (Lindstein et al. 1990).

The *CHLG* gene was isolated from *A. thaliana*, *Avena sativa*, rice and tobacco (Gaubier et al. 1995; Schmid et al. 2001; Wu et al. 2007; Shalygo et al. 2009). In *A. thaliana*, the *CHLG* transcript has been detected in green or greening tissues (Gaubier et al. 1995), whereas in *A. sativa*, the gene is expressed equally both in dark- and light-grown seedlings (Schmid et al. 2001). Sequence analysis of cDNAs from rice yielded a putative Chl synthase homolog (Scolnik and Bartley 1996); however, the biochemical properties and physiological functions remained unknown until Wu et al. (2007) characterized a rice mutant with inactivated *CHLG*. The young rice Chl synthase mutant plants have yellow-green leaves with decreased Chl synthesis (Wu et al. 2007). In the mutated plants, there is accumulation of tetrapyrrole intermediates, reduced expression of *LHCB1* and delayed chloroplast development.

1. Modulation of Chlorophyll b Synthesis Confers Tolerance to Low Light and High Light

Overexpression of *CAO* in tobacco plants resulted in a decreased Chl *a*/*b* ratio i.e., from 3.38 in wild-type plants to 2.33 in transgenic plants when grown in high light and from 2.8 to 2.4 in low light-grown plants (Pattanayak et al. 2005). The overexpression of full length *CAO* in tobacco (*Nicotiana tabacum*) resulted in an increased Chl synthesis and a decreased Chl *a/b* ratio in low-light-grown (LL) as well as in high-light-grown (HL) tobacco plants; this effect was more pronounced in HL-plants. The potential of Chl biosynthesis and the POR activity increased compensating for the usual loss of Chl when plants were grown in high light. Increased Chl *b* synthesis in CAOx plants was accompanied by an increased abundance of light-harvesting chlorophyll-proteins (LHCPs) and other proteins of electron transport chain that led to an increase in capture of light, as well as enhanced (40–80%) electron transport rates of Photosystem I and Photosystem II at both limiting and saturating light intensities. However, the increase in the whole chain electron transport was somewhat lower (20–50%). The light-saturated photosynthetic carbon assimilation, starch content and the dry matter accumulation increased in CAOx plants grown in both low and high-light regimes (Figs. 27.8 and 27.9).

These results from the laboratory of the author (Biswal et al. 2012) demonstrate that controlled up-regulation of Chl b biosynthesis co-modulates the expression of chloroplast proteins that increase the antenna size and electron transport rates and enhances CO_2 assimilation, starch contents and dry matter accumulation.

Chl *b* reductase catalyzes the conversion of Chl *b* to Chl *a*. It reduces the formyl group of Chl *b* to a hydroxymethyl group. It was observed that barley etioplast had Chlide *b* reductase activity and the enzyme needs NADPH and reduced ferredoxin for its activity (Scheumann et al. 1996, 1999). The gene encoding Chl *b* reductase was isolated from rice and it belongs to a family of short-chain dehydrogenase/reductases (Kusaba et al. 2007). It encodes a protein of 504 amino acids and contains a dinucleotide binding motif (TGXXXGXG) and a catalytic site (YXXXK) and uses NADPH as a cofactor. Interestingly, two genes for Chl *b* reductase were found in the genomes of *A. thaliana* and rice (Kusaba et al. 2007; Sato et al. 2009). It was also observed that disruption of the genes encoding Chl *b* reductase in *A. thaliana* resulted in non-degradation of Chl *b* and LHC II (Horie et al. 2009). In the presence of recombinant CAO enzyme, the Chlide *a* gets converted to Chlide *b* using NADPH, molecular oxygen and ferredoxin (Oster et al. 2000). In this in vitro assay, a small amount of 7-hydroxymehtyl Chlide *a* was also formed. When the 7-hydroxymethyl Chlide *a* was used as a substrate for the in vitro enzymatic assay, the recombinant enzyme also efficiently converted 7-hydroxymehtyl Chlide *a* to Chlide *b* (Oster et al. 2000). Then, Chl synthase converts Chlide *b* into Chl *b*. Chl *b* is further converted to hydroxymethyl Chl *a* by the enzyme Chl *b* reductase (Kusaba et al. 2007). This enzyme converts the formyl group of Chl *b* to a hydroxymethyl group using NADPH as a reductant.
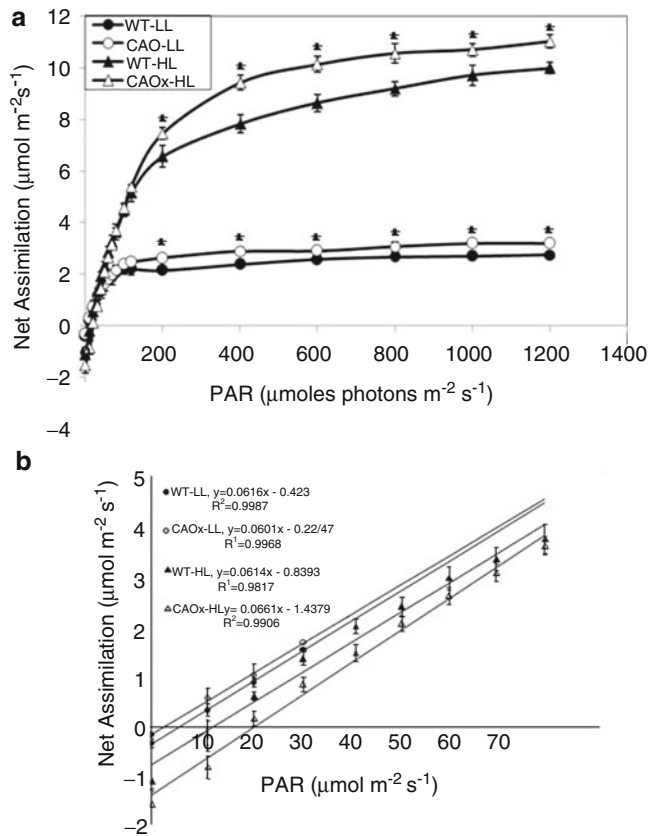
Fig. 27.8. Photosynthesis (net CO_2 assimilation rate) light response curves and quantum yield of leaves from attached WT and CAOx plants grown in LL and HL intensities. (**a**) Net CO_2 assimilation rates of attached leaves of WT and CAOx plants were monitored by IRGA (Licor 6400-XT portable photosynthetic system) in ambient CO_2 at different light intensities. Light response curves were measured up to 1,800 μmol of photons m^{-2} s^{-1} at 28°C. (**b**) Relative quantum yield of CO_2 fixation by leaves from WT and CAOx plants grown in LL or HL regimes. Quantum yield was measured from the above photosynthetic rate after the chamber reached to a steady-state. Light intensity curves at LL-intensities upto 80 μmol of photons m^{-2} s^{-1}; the slopes of these curves provide relative quantum yield of CO_2 fixation by leaves. Leaves were pre-exposed for 15 min at 700 μmol photons m^{-2} s^{-1} and 200 μmol photons m^{-2} s^{-1} for LL and HL grown plants respectively prior to CO_2 assimilation measurement. These experiments were done three times with similar results. Each data point is the average of five replicates and the error *bar* represents SE. *Asterisks* indicate significant differences determined by ANOVA followed by Tukey's test (*P < 0.05) (Biswal et al. 2012).

Geranyl-geranyl reductase mediates the reduction of geranylgeranyl diphosphate to phytyl diphosphate. The cDNA encoding a pre-geranyl-geranyl reductase from *A. thaliana* has been isolated and characterized (Keller et al. 1998). The recombinant protein catalyzes the reduction of geranyl-geranyl-Chl *a* into phytyl-Chl *a,* as well as the reduction of free geranyl-geranyl diphosphate to phytyl diphosphate, suggesting this is a multifunctional enzyme.

2. Modulation of Phytol Synthesis and Its Impact on Plant Development, Photosynthesis, Tocopherol Contents and Oxidative Stress

a. Modulation by CHLP

Antisense expression of *CHLP* coding for geranyl-geranyl reductase affects the Chl and tocopherol contents in tobacco (Tanaka et al. 1999). The reduced tocopherol and Chl
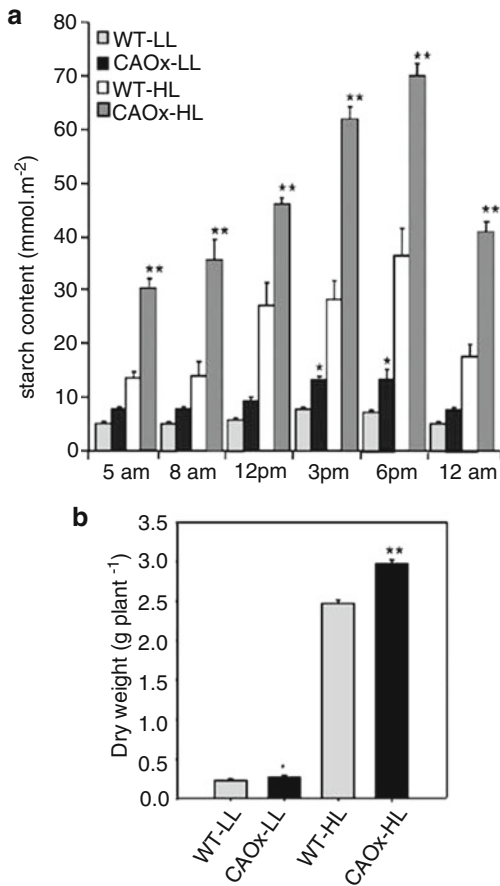
Fig. 27.9. Diurnal starch content and dry weight measurement in WT and CAOx plants. (**a**) Starch content was measured from mature leaves of WT and CAOx plants grown under LL and HL at various times over a diurnal cycle as described in methods. Note the diurnal starch accumulation was maximum between 3 and 6 PM and CAOx-HL plants showed maximum starch accumulation. (**b**) Dry weight of WT and CAOx plants was measured after aerial parts of the plant were dried at 70°C for 5 days. HL-grown WT and CAOx plants showed significant increase in dry matter accumulation in comparison to WT-LL and CAOx-LL plants. *Asterisks* indicate significant differences determined by ANOVA followed by Tukey's test ($^{*}P<0.05$; $^{**}P<0.001$). These experiments were done three times with similar results. Each data point is the average of four replicates in (**a**) and 15 replicate in (**b**) and the error *bars* represent SD (Biswal et al. 2012).

contents in *CHLP* antisense plants resulted in the reduction of electron transport chains and PS II activity. There are also more lipid peroxidation products in *CHLP* antisense plants. Havaux et al. (2003) found accumula-

tion of xanthophylls cycle pigments in *CHLP* antisense plants which could be a compensatory mechanism for tochopherol deficiency. The *CHLP* transcript levels in peach were abundant in Chl-containing tissues and flower organs however barely detected in roots and mesocarp of the ripening fruits (Giannino et al. 2004). Its transcript level is up-regulated during etioplast to chloroplast and chloroplast to chromoplast development (Keller et al. 1998).

The responses of Chl biosynthetic enzymes to various environmental stresses are examined and summarized in Table 27.1. These stresses broadly downregulate most of the enzymes of Chl biosynthesis pathway. However, gene/protein expression of a certain enzyme i.e., GSA-AT is upregulated in most stresses, i.e., heat, water, salt etc. The expression of UroD is upregulated in high-temperature. As GSA-AT is a crucial enzyme involved in the last step of synthesis of ALA, plants most likely upregulate its expression to compensate for the reduced expression of earlier enzymes of the ALA biosynthesis.

III. Future Prospects

Plant tetrapyrroles play an important role in plant development, growth, productivity and modulation of their biosynthesis and protect plants from environmental stresses. Therefore, genetic manipulation of tetrapyrrole biosynthesis either via molecular marker assisted breeding programs or transgenic approaches will have a potential to protect crop plants from environmental stresses and increase yield.

Acknowledgments

Table 27.1. Impact of various stress-induced alterations in activity, protein and transcript levels of enzymes involved in Chl biosynthesis (Dalal and Tripathy 2012)

	Water stress	Salt	Chill	Heat
GluTR				
Protein			–	–
Transcript	–	+	–	–
GSA-AT				
Protein	+	+	+	+
Transcript	+	+	–	+
ALAD				
Enzyme activity	–	–	–	–
Transcript	–	–	–	–
PBGD				
Enzyme activity	–	–	–	–
Transcript	–	–	–	0
UROD				
Enzyme activity			–	+
Protein	–	–	–	+
Transcript	–	–	–	+
CPO				
Enzyme activity	–	–	–	0
Protein	–		0	0
Transcript	–	–	0	0
Protox				
Enzyme activity	–	–	–	0
Protein	–	–		
Transcript	–	–		
Mg-chelatase				
Enzyme activity	–	–	–	–
Protein	0	–		
Transcript	–	–	–	–
MPE cyclase				
Enzyme activity			–	–
Transcript	–	–	–	–
POR				
Enzyme activity	–	–	0	–
Protein	–		0	0
Transcript	–			
CAO				
Transcript		–		
ChlP				
Protein	–	–		
Transcript			–	–

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