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Review article

Recent advances in chlorophyll biosynthesis and breakdown in higher plants

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Abstract

Chlorophyll (Chl) has unique and essential roles in photosynthetic light-harvesting and energy transduction, but its biosynthesis, accumulation and degradation is also associated with chloroplast development, photomorphogenesis and chloroplast-nuclear signaling. Biochemical analyses of the enzymatic steps paved the way to the identification of their encoding genes. Thus, important progress has been made in the recent elucidation of almost all genes involved in Chl biosynthesis and breakdown. In addition, analysis of mutants mainly in *Arabidopsis*, genetically engineered plants and the application of photo-reactive herbicides contributed to the genetic and regulatory characterization of the formation and breakdown of Chl. This review highlights recent progress in Chl metabolism indicating highly regulated pathways from the synthesis of precursors to Chl and its degradation to intermediates, which are not longer photochemically active.

Abbreviations: ALA, 5-aminolevulinic acid; Chl, chlorophyll; Chlase, chlorophyllase; Chlide, chlorophyllide; GluTR, glutamyl tRNA reductase; FCC, fluorescent chlorophyll catabolite; Fd, ferredoxin; FeCh, ferrochelatase; MgCh, Mg–chelatase; MgProto, Mg–protoporphyrin IX; MgProtoMe, Mg–protoporphyrin IX monomethylester; MTF, Mg–protoporphyrin IX methyltransferase; NCC, nonfluorescent chlorophyll catabolite; PAO, pheophorbide *a* oxygenase; Pchlide, protochlorophyllide; pFCC, primary fluorescent chlorophyll catabolite; Pheide, pheophorbide; Proto, protoporphyrin IX; POR, NADPH-protochlorophyllide oxidoreductase; PPX, protoporphyrinogen oxidase; RCC, red chlorophyll catabolite; RCCR, red chlorophyll catabolite reductase

Introduction

The appearance of chlorophyll (Chl) in terrestrial plants during springtime and its disappearance in autumn demonstrates the season-dependent nature of processes that can even be seen from outer space. Thereby, about 10^9 tons of chlorophyll are seasonally synthesized and degraded per year, most of them in the oceans (Rüdiger, 1997). Thus, the Chl biosynthetic and degradative reactions belong to the most important biochemical pathways known. The localization of the contributing enzymes corresponds to the biochemical nature of the metabolic intermediates: enzymes catalyzing early steps in the synthesis are highly soluble and located mostly in the chloroplast stroma, whereas enzymes of the late steps are associated with thylakoid or inner envelope membranes (Table 1, Figure 1). Chl degrading enzymes are also attached to envelope membranes, before the final catabolites are deposited in vacuoles (Figure 1).

In addition to Chl, other tetrapyrrole end products are synthesized through the same pathway: heme, an Fe-containing porphyrin is involved

Table 1. Overview over the biosynthetic and catabolic enzymes involved in Chl metabolism.

Enzyme (Abbreviation)	Gene(s)	Annotation for *A. thaliana*	Localization	Reference
Chl biosynthesis				
Glutamyl tRNA reductase (GluTR)	*HemA1*	At1g58290	HemA1: plastids, entire plant	McCormac *et al*., 2001
	HemA2	At1g09940	HemA2: plastids, roots and flowers	
	HemA3	At2g31250	HemA3: annotation in the database	
Glutamate-1-semialdehyde aminotransferase (GSA-AT)	*Gsa1*	At5g63570	Plastids, stroma	Ilag *et al*., 1994
	Gsa2	At3g48730		
ALA dehydratase (ALAD)	*Alad*	At1g69740	Plastids, stroma	Kaczor *et al*., 1994
		At1g44318		
Porphobilinogen deaminase (PBD)		At5g08280	Leaf and root plastids, stroma	Lim *et al*., 1994
Uroporphyrinogen III synthase (UroS)	*HemD*	At2g26540	Plastids, stroma (?)	Annotation due to sequence similarity with bacterial HemD proteins.
Uroporphyrinogen III decarboxylase (UroD)	*HemE*	At2g40490		Similar to tobacco *URO D:* Mock and Grimm, 1997
		At3g14930		
Coproporphyrinogen oxidase (CPO)	*LIN2*	At5g63290	Plastids	Ishikawa *et al*., 2001
Protoporphyrinogen oxidase (PPX)	*PPX I*	At4g01690 At5g14220	PPX-1: plastid thylakoid	Watanabe *et al*., 2001
	PPX II		PPX-2S: mitochondria, inner membrane	
			PPX-2L: plastid envelope	
Mg–chelatase (MgCh)	*ChlI*	ChlI1: At4g18480	Plastids, stroma and membranes	Guo *et al*., 1998
		ChlI2: At5g45930		
	ChlD	At1g08520 (At2g19250?)	Plastids, stroma and membranes	Papenbrock *et al*., 1997
	ChlH gun5	At5g13630	Plastids, inner envelope	Mochizuki *et al*., 2001
Mg–protoporphyrin IX methyltransferase (MTF)	*ChlM (?)*	At4g25080	Plastids, thylakoid and envelope	Block *et al*., 2002
Mg–protoporphyrin IX monomethylester cyclase (MTC)	*CHL27*	At3g56940	Plastids, thylakoid and envelope	Tottey *et al*., 2003
8-vinyl reductase (VR)	*BclJ* (?)	?	Plastid membranes	Suzuki and Bauer, 1995
NADPH-protochlorophyllide oxidoreductase (POR)	*PorA*	At5g54190	Light-dependent POR: (pro)plastid membranes	Su *et al*., 2001
	PorB	At4g27440		
	PorC	At1g03630		
Geranylgeranyl reductase	*ChlP*	At1g74470		Keller *et al*., 1998
Chlorophyll synthase (CS)	*ChlG*	At3g51820	Plastid membranes	Gaubier *et al*., 1995c
Chl cycle:				
Chlorophyll *a* oxygenase (CAO)	*Cao*	At1g44446	Plastids, stroma	Oster *et al*., 2000

Chlorophyll *b* reductase (CBR)	–	Enzyme activity	Plastids, thylakoid	Scheumann *et al.*, 1998
Hydroxychlorophyll *a* reductase (HCR)	–	Enzyme activity	Plastids	Scheumann *et al.*, 1998
Chl breakdown:				
Chlorophyllase (Chlase)	*AtCLH1*	At1g 19670	Plastids, vacuole?	Tsuchiya *et al.*, 1999
	AtCLH2	At5g43860		
Mg-dechelatase (MCS)	–	Enzyme activity	Plastids	Suzuki and Shioi, 2002
Pheophorbidase	–	Enzyme activity	Cytosol?	Suzuki *et al.*, 2002
Pheophorbide *a* oxygenase (PAO)	*ACD1*	At3g44880	Plastids, inner envelope	Pružinská *et al.*, 2003
Red chlorophyll catabolite reductase (RCCR)	*ACD2*	At4g37000	Plastids, stroma	Wüthrich *et al.*, 2000

in N_2 fixation (leghemoglobin), in O_2 transport (hemoglobin and myoglobin), in respiratory and photosynthetic electron transport (cytochromes) and in cellular detoxification (e.g. peroxidases and catalases). Heme is also a metabolic intermediate for the synthesis of the linear tetrapyrroles, phytochromobiline and phycobilins.

The degradation of Chl takes place in virtually the same cellular compartment as its biosynthesis. However, the mutual regulation of Chl biosynthesis and breakdown is still unknown. While Chl formation is mainly controlled by light and development, Chl catabolism initially occurs at a basal turnover level but is enhanced during senescence and fruit ripening.

Chlorophyll biosynthesis

Chl synthesis is embedded into a network of pathways leading to the formation of various tetrapyrroles and can be subdivided into three parts, (i) formation of 5-aminolevulinic acid (ALA), the committed step for all tetrapyrroles, (ii) formation of protoporphyrin IX (Proto) from eight molecules of ALA, and (iii) formation of Chl in the magnesium branch. This partition also reflects biochemical properties of intermediates as well as differences in the control of the enzymatic steps (Figure 1). Although the expression of each enzyme is individually and independently controlled, most genes required in Chl biosynthesis show a light-induced and developmental-dependent expression profile. A number of genes at regulatory steps in Chl biosynthesis exhibit diurnal and circadian control (Thimm *et al.*, 2004), resulting in variations of enzymatic activities. In addition, a few regulatory sites mainly at the beginning of each section determine the metabolic flow and are particular targets for endogenous and environmental factors. The enzymes involved in Chl biosynthesis are listed in Table 1. For a detailed and comprehensive overview of the enzymatic steps of Chl biosynthesis see Beale (1999). The review focuses on recent findings with emphasis on pathway control and late steps in Chl biosynthesis.

ALA synthesis

The formation of ALA (Figure 2) is the rate-controlling point of the whole pathway. Thereby,

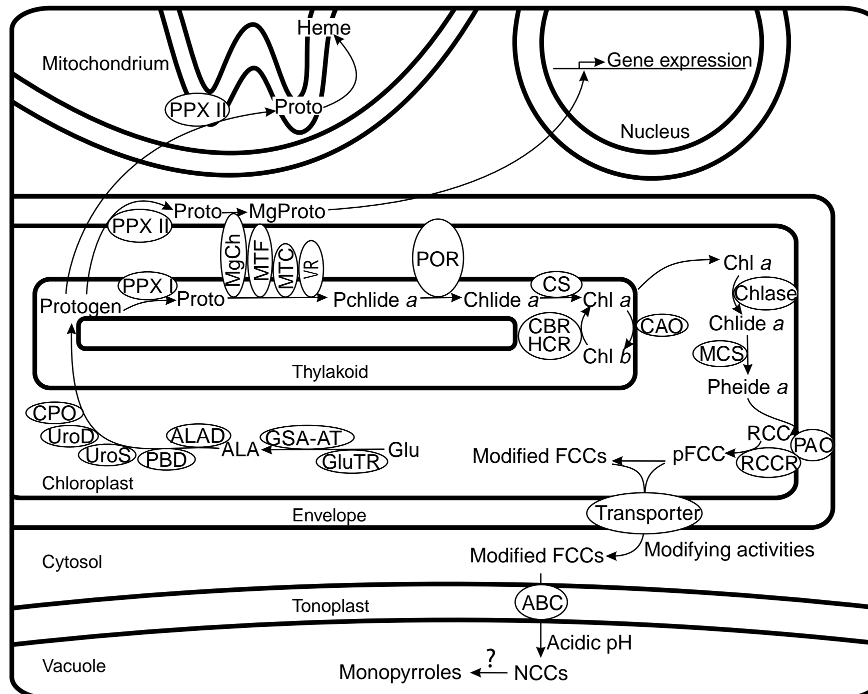

Figure 1. Topographical model of Chl metabolism. Abbreviations of enzymes are found in Table 1. The figure includes localizations of soluble and membrane-bound enzymes of Chl synthesis and degradation, while not all metabolites of the entire pathway are depicted. The formation of tetrapyrrole precursors takes place in the stroma, while late enzymes of Chl synthesis are attached to or integral part of plastid membranes. The model considers transfer of protoporphyrinogen to mitochondria, Mg–porphyrin-mediated plastid signaling and translocation of modified FCCs and NCCs to the cytoplasm and the vacuoles, respectively. Further details are described in the text.

glutamyl tRNA reductase (GluTR) is the key enzyme for metabolic and environmental control. Three genes encoding GluTR (*HEMA1-3,* McCormac *et al.*, 2001) and two genes encoding glutamate-1-semialdehyde aminotransferase (Ilag *et al.*, 1994) were described in *Arabidopsis. HEMA1* is expressed in all tissues and induced by light, whereas *HEMA2* expression was observed in roots only without light induction (McCormac *et al.*, 2001). Heme is an allosteric inhibitor for GluTR. The first 31–34 amino acids at the N-terminus of the mature protein were accounted to be heme-responsive (Vothknecht *et al.*, 1996). Mutants with reduced heme degradation or impaired formation of phytochromobilin showed reduced protochlorophyllide (Pchlide) contents. This effect was accounted to heme-mediated reduction in ALA synthesis and could be compensated by ALA feeding (Terry and Kendrick, 1999).

The crystal structure of GluTR from the archaebacterium *Methanopyrus kandleri* revealed a V-shaped, dimeric enzyme. The role of conserved amino acids in catalysis was elucidated and three domains of the monomers were identified as NADPH-binding-, catalytic- and dimerization sites, respectively (Moser *et al.*, 2001). Bacterial GluTRs, however, do not contain the amino terminal extension of plant enzymes that were accounted to be additionally involved in heme-mediated control (Vothknecht *et al.*, 1996).

In darkness, Chl synthesis in angiosperms is blocked at the light-dependent step of Pchlide reduction. Simultaneously, ALA synthesis is suppressed. The *Arabidopsis flu* mutant, which was identified among mutated etiolated seedlings, accumulate fluorescent Chl precursors. *FLU* encodes a negative, heme-independent regulator of ALA synthesis in darkness (Meskauskiene *et al.*, 2001). A tetratricopeptide repeat domain of FLU allows physical interaction with GluTR (Meskauskiene and Apel, 2002). It is still open from where FLU receives the signal for post-translational

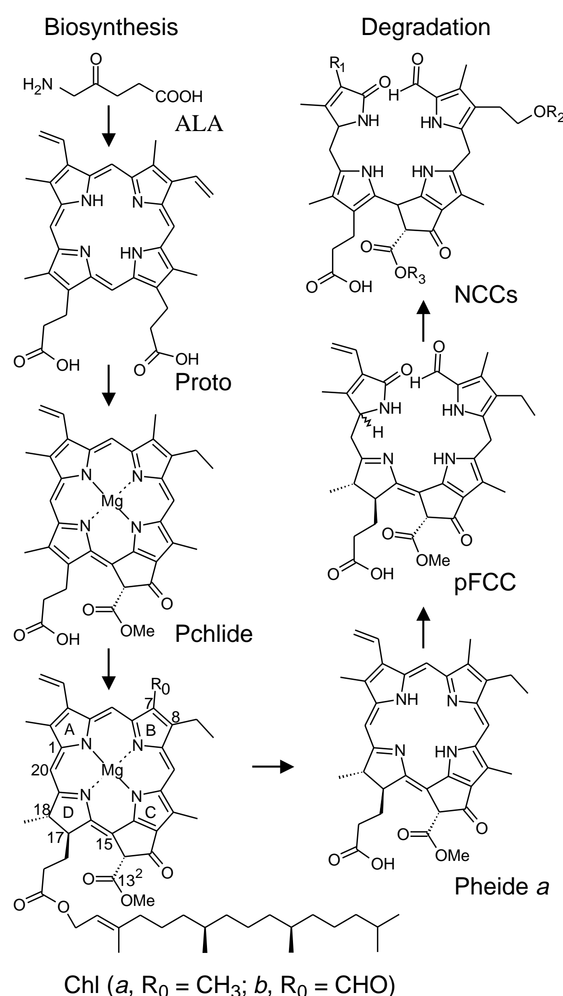

Figure 2. Structures of Chl and of important intermediates of Chl biosynthesis and degradation. Relevant carbon atoms, pyrrole rings (A–D) and methine bridges (α–δ) are labeled. R_1–R_3 in NCCs indicate species-specific modifications.

control of GluTR. A defect in a *FLU* ortholog of barley was found to be responsible for the phenotype of the *tigrina* d^{12} mutant (Lee *et al.*, 2003).

Protoporphyrin synthesis

A total of eight molecules of ALA are condensed to a first porphyrin, uroporphyrinogen III, which then is oxidatively converted to Proto in three steps (Beale, 1999; Figures 1 and 2). Along this second part of the pathway, porphyrins become increasingly hydrophobic and photoreactive. Excited porphyrins can transfer energy and electrons to oxygen, lipids and proteins resulting in rapid peroxidation and degradation (op den Camp *et al.*, 2003), but also in stimulated antioxidative reactions and pathogen defence responses (Mock *et al.*, 1999; Ishikawa *et al.*, 2001). Thus, accumulation of photosensitising porphyrins by metabolic perturbation of tetrapyrrole biosynthesis through e.g. antisense repression of biosynthetic enzymes is extremely hazardous for light-grown plants (Kruse *et al.*, 1995; Mock and Grimm, 1997; see also chapter on 'control of photoreactive intermediates in Chl breakdown')

Up to coproporphyrinogen III oxidase, the enzymes of the pathway are likely to be located in the stroma or loosely attached to the membranes. Protoporphyrinogen oxidase (PPX) activity is located both in mitochondrial and plastidal membranes (Figure 1). Two PPX isoenzymes were described in different species. Spinach PPX I was found in the plastid membrane fraction, whereas the *PPX II* gene codes for two different translation products, which were either targeted to the inner mitochondrial membrane or to the plastid inner envelope (Watanabe *et al.*, 2001). Thus, porphyrin transport is required from plastids to mitochondria to provide substrate for mitochondrial heme synthesis as well as for plastid-derived Mg–porphyrin signalling (see below). Candidates for porphyrin transporters have been proposed, however conclusive evidence for their *in vivo* function in transport has not been presented yet (Møller *et al.*, 2001; Lindemann *et al.*, 2004).

From protoporphyrin to protochlorophyllide

Two metal chelatases, Mg–chelatase (MgCh) and ferrochelatase (FeCh) that possess quite different biochemical properties, compete for the same substrate, Proto. *In vitro*, the insertion of Fe^{2+} into Proto occurs spontaneously and reversibly without the requirement for ATP. FeCh is located in both plastids and mitochondria (Singh *et al.*, 2002), but it was estimated that plastids are the major heme-synthesizing organelles (Cornah *et al.*, 2002; Masuda *et al.*, 2003).

In plants, MgCh consists of three subunits, CHLI, CHLD and CHLH (Papenbrock *et al.*, 1997). Subunits I and D were shown to form an activation complex under ATP hydrolysis (Hansson *et al.*, 1999). In *Arabidopsis* and pea chloroplasts, CHLH and CHLD were Mg^{2+}-dependently distributed between the envelope membrane and the stroma (Nakayama *et al.*,

1998). It was suggested that the CHLI/CHLD-complex provides Mg^{2+} while CHLH carries the substrate. ALA synthesis as well as MgCh activity and transcript levels were maximal in the light, whereas highest FeCh activity was found at the beginning of darkness (Papenbrock *et al.*, 1999).

In vitro, the *Rhodobacter* Mg–protoporphyrin IX (MgProto) methyltransferase (MTF) activity increased sevenfold in the presence of MgCh subunit BchH and a tight interaction between both enzymes was proposed (Hinchigeri *et al.*, 1997). *Arabidopsis* MTF was detected in thylakoid and envelope membranes, probably anchored by a hydrophobic region of its N-terminal half (Block *et al.*, 2002). A physical interaction between tobacco MgCh and MTF was demonstrated by the yeast two-hybrid system. Moreover, in transgenic tobacco with altered MTF expression, MgCh and the ALA synthesizing activities paralleled the MTF activities. This was explained with changes of corresponding mRNA contents: it is proposed that reduced and increased MTF activities in chloroplasts are communicated to the cytoplasm for modulating transcriptional activities of regulatory enzymes of the pathway (A. Alawady and B. Grimm, unpublished).

Cyclization of a fifth ring in MgProto monomethylester (MgProtoMe) is a three-step reaction that is catalyzed by the MgProtoMe cyclase and yields divinyl Pchlide. The *acsF* mutant of the purple bacterium *Rubrivivax gelantinosus* accumulated MgProtoMe (Pinta *et al.*, 2002). AcsF exhibits sequence similarity to the di-iron carboxylate monooxygenases Crd1 from *Chlamydomonas reinhardtii* (Moseley *et al.*, 2000) and CHL27 from *Arabidopsis* (Tottey *et al.*, 2003). As in the *acsF* mutant, antisense repression of *CHL27* led to the accumulation of MgProtoMe upon ALA feeding. It is unclear whether this enzyme is sufficient to carry out all three steps of the cyclization reaction, or if additional components are required.

Late steps in chlorophyll synthesis

Reduction of the C17/C18 double bond in ring D converts Pchlide (Figure 2) to Chlide. This reaction is catalyzed by NADPH-Pchlide oxidoreductase (POR), which exists in two different forms: A light-dependent POR was found in all photosynthetic organisms with the exception of some anaerobic, bacteriochlorophyll-containing bacteria, whereas a light-independent POR, consisting of three subunits is present in all gymnosperms, aerobic photosynthetic bacteria and algae, but not in angiosperms.

The light-dependent POR is a peripheral membrane-associated, monomeric enzyme of 35–38 kDa and is encoded by small gene families in barley (Holtorf *et al.*, 1995) and *Arabidopsis* (Su *et al.*, 2001). In *Arabidopsis*, *PORA* is dark-induced, whereas *PORB* is constitutively expressed and *PORC* is light-induced. A ternary complex of Pchlide, NADPH and POR is essential for normal etioplast development and conversion to chloroplasts in *Arabidopsis* (Sperling *et al.*, 1998). A substrate-dependent import of PORA into plastids has been described (Reinbothe *et al.*, 2000), but could not be confirmed until recently (Kim and Apel, 2004). This study with intact *Arabidopsis* seedlings revealed a substrate-dependent import of a PORA-GFP fusion protein into plastids in cotyledons but not in primary and other true leaves indicating a role of the Pchlide-dependent protein import in the developmental switch of cotyledons from storage organs to photosynthetically active leaves. A number of Ptc (Pchlide-dependent translocation complex) proteins that interact with prePORA during *in vitro* import into barley chloroplasts were identified in cross-linking experiments (Reinbothe *et al.*, 2004a; Reinbothe *et al.*, 2004b). Ptc33 exhibits sequence similarity to Toc33/34, whereas Ptc52 is homologous to Tic55 both of which are Rieske-type oxygenases. The Toc and Tic proteins are assigned to the 'classical' plastid protein import machinery. Ptc47 was shown to be an integral inner envelope protein similar to tyrosin: 2-oxoglutarate aminotransferases, an enzyme of the shikimate pathway (Reinbothe *et al.*, 2004a). Finally, Ptc16 is identical to Oep16, an integral outer envelope protein, which is down-regulated during de-etiolation of dark-grown barley seedlings.

Phytyl pyrophosphate is the substrate for a prenyl-type esterification of the C18 propionyl group of Chlide *a* or *b*. Phytyl pyrophosphate is formed through reduction of three double bonds of geranylgeranyl pyrophosphate by a NADPH-dependent reductase. Geranylgeranyl pyrophosphate serves as substrate for Chl, phylloquinone and tocopherol formation. In higher plants, reduction takes place either on free geranylgeranyl pyrophosphate or on geranylgeranylated Chl

(Keller *et al.*, 1998). Transgenic tobacco plants with antisense repression of the geranylgeranyl reductase gene, *ChlP,* have decreased contents of total and phytylated Chl as well as of tocopherol (Tanaka *et al.*, 1999). A *ChlP* deletion mutant of *Synechocystis* grew only heterotrophically, did not contain tocopherol and had exclusively reduced levels of geranylgeranylated Chl. Photosystem I of this mutant was particularly unstable, possibly by the integration of geranylgeranylated Chl into the reaction center rather than by the lack of tocopherol (A.V. Shpiljov, V. Zinchenko, H. Lokstein and B. Grimm, unpublished).

Chlide esterification is catalyzed by Chl synthase. Its activity was found in etioplast membranes. Substrates for a recombinant oat Chl synthase were Chl derivatives with bulky side chains at rings A and B, but not at ring E, and not pheophorbide (Pheide) *a* (Schmid *et al.*, 2001). Therefore, it was assumed that the single Chl *a′* in photosystem I is epimerized after phytylation and that pheophytin in photosystem II is inserted as Chl and subsequently dechelated.

The chlorophyll cycle

The formation of Chl *b*, i.e. the oxidation of the C7-methyl group to formyl preferentially occurs at the stage of Chlide *a* rather than Chl *a* and involves 7-hydroxy-Chl(ide) *a* as intermediate. Like Ptc52 and PAO (see below), Chl(ide) *a* oxygenase is a Rieske-type [2Fe–2S] cluster containing monooxygenase (Tanaka *et al.*, 1998). The enzyme was functionally expressed in *E. coli* and the formation for Chlide *b* and 7-hydroxy-Chlide *a* from Chlide *a* and Zn-containing derivatives was demonstrated. In contrast, neither Chl *a* nor Pchlide *a* were substrates for the recombinant enzyme (Oster *et al.*, 2000).

The inverse reduction of Chl *b* to Chl *a* also involves 7-hydroxy Chl(ide) (Ito and Tanaka, 1996). The first reduction step requires NADPH, whereas the second step depends on reduced ferredoxin (Fd) (Scheumann *et al.*, 1998), indicating that two enzymes, denominated Chl *b* reductase and hydroxychlorophyll *a* reductase are involved. Both reactions use esterified and non-esterified substrates. The interconversion of Chl(ide) *a* and Chl(ide) *b* has been termed 'Chl cycle' (Tanaka *et al.*, 1998) and it was proposed to be a regulatory mechanism which enables plants to react to changing light conditions. In senescent barley, Chl *b* reductase activity is closely connected with chlorophyllase (Chlase) in thylakoid membranes, indicating its involvement in Chl degradation (Scheumann *et al.*, 1999; see below).

Some aspects of pathway control

In the plastid-ribosome-deficient barley mutant, *albostrians*, MgCh and MTF activities were diminished and FeCh activity was strongly increased in white leaves, suggesting that loss of photosynthesis resulted in a preferential redirection of tetrapyrrolic metabolites into heme rather than into Chl biosynthesis (Yaronskaya *et al.*, 2003). These changes of enzyme activity reflect variations in Chl and heme demand.

The change of Proto allocation for Chl and heme synthesis under diurnal growth conditions or in the absence of photosynthetic capacities is at least partially explained with a parallel alteration in the expression of the respective nuclear genes. Plastid-derived signals mediate the physiological state of chloroplasts to the nucleus and, thus, contribute to the control of nuclear gene expression. The analysis of different experimental systems indicates the involvement of Mg–porphyrins in the communication between plastids and cytoplasm/nucleus. Incubation of *Chlamydomonas reinhardtii* cells with MgProto and MgProtoMe, but not Proto, Pchlide and Chlide, induced *HSP70* gene expression (Kropat *et al.*, 1997). In addition, the light-induced transcriptional activity of *HSP70* could be substituted by Mg–porphyrins (Kropat *et al.*, 2000).

In an *Arabidopsis* mutant screen employing norflurazon, an inhibitor of carotenoid synthesis, *genomes uncoupled* (*gun*) mutants were identified which exhibited a relatively independent *LHCB1* expression from impaired plastid development (Susek *et al.*, 1993). Two mutants, *gun2* and *gun3*, are affected in phytochromobiline synthesis. GUN4 interacts with MgCh and stimulates its activity. Interestingly, this protein binds both substrate and product of MgCh, i.e. Proto and MgProto (Larkin *et al.*, 2003). Recently, an orthologue of GUN4 from *Synechocystis* was identified that apparently plays a more pronounced role in tetrapyrrole biosynthesis, as a respective knockout mutant could not be generated. This protein was not only involved in Mg-chelation but also in Fe-chelation of Proto (Wilde *et al.*, 2004).

Another *Arabidopsis* mutant, *gun5*, is affected in the CHLH subunit of MgCh (Mochizuki *et al.*, 2001). Upon norflurazon treatment, the level of MgProto was significantly lower in *gun5* leaves than in wildtype and inversely correlated with *LHCB* expression (Strand *et al.*, 2003). These latter results are consistent with a function of MgProto as a plastidal signal molecule that above a certain threshold triggers the repression of *LHCB1* transcription (Strand *et al.*, 2003).

Pale green transgenic tobacco plants expressing antisense RNA for *CHLH* or *CHLI* showed reduced MgCh activity, and consequently reduced MgProto level. In response to low MgCh activity, the transcript levels for *HEMA*, *ALAD* and *LHCB1* were reduced. The loss of the former transcripts accounted for a reduced ALA synthesis rate. It was suggested that a plastidic control mechanism senses the levels of Mg–porphyrins and releases a transmembrane signal to the cytosol (Papenbrock *et al.*, 2000). The different findings of the regulatory impact of Mg–porphyrins on *LHCB* transcription may be due to different experimental systems used.

Chlorophyll breakdown

The fate of Chl during plant senescence has been enigmatic until quite recently (Hendry *et al.*, 1987). Only the first elucidation of the chemical structure of a nonfluorescent Chl catabolite (NCC) from barley (*Hv*-NCC-1) in 1991 shed light on the reactions that might occur during Chl catabolism. Now, 13 years later, the biochemistry of Chl breakdown is nearly fully solved and some of the enzymes have been identified at the molecular level.

Reactions on colored pigments

Chl breakdown starts with the removal of the hydrophobic phytol chain, followed by the release of the central Mg atom. These reactions are catalyzed by Chlase and Mg–dechelatase, respectively, and result in the formation of, respectively, Chlide and Pheide. Two independent groups succeeded in cloning Chlase (Jakob-Wilk *et al.*, 1999; Tsuchiya *et al.*, 1999). Although Chlase had been attributed to the envelope membrane (Matile *et al.*, 1997), the amino acid sequences predicted soluble proteins. Furthermore, the different sequences identified so far point to two groups of Chlases that are either located inside (e.g. AtCLH2) or outside (AtCLH1) plastids (Tsuchiya *et al.*, 1999). The different location has been explained by a second pathway of Chl breakdown occurring outside plastids, probably in the vacuole (Takamiya *et al.*, 2000). Site directed mutagenesis confirmed that the lipase motif with a conserved serine residue present in all Chlases is indispensable for *in vitro* activity (Tsuchiya *et al.*, 2003). Expression of Chlase genes is constitutive, but hormones known to accelerate leaf senescence or fruit ripening, methyl jasmonate and ethylene, cause a strong induction of At*Clh1* and the *Citrus* Chlase gene, respectively (Jakob-Wilk *et al.*, 1999; Tsuchiya *et al.*, 1999). Overexpression of At*Clh1* caused an increase in the Chlide to Chl ratio, but this had no phenotypic effect. AtCLH1 preferably acts on Chl *a*, thereby increasing the Chlide *a* to Chlide *b* ratio (Benedetti and Arruda, 2002).

Information on the second reaction, Mg-dechelation, is scarce, but the activity has been suggested to be associated with a low molecular weight compound termed Mg-chelating substance. *In vitro*, the activity is able to remove Mg from Chlide, but also from an artificial dephytylated substrate, chlorophyllin. Recently, a protein of 20 kDa has been purified from *Chenopodium album* that also exhibits Mg-releasing activity with chlorophyllin as substrate, but not with Chlide (Suzuki and Shioi, 2002).

In some species, pyro forms of Chl, in particular pyropheophorbide, have been described as Chl breakdown products. In higher plants, pyropheophorbide formation has been attributed to the activity of pheophorbidase which *in vitro* catalyzes the conversion of Pheide *a* to an intermediate, $C13^2$-carboxyl-pyropheophorbide *a*, followed by a nonenzymatic decarboxylation (Suzuki *et al.*, 2002). Pheophorbidase has been cloned recently (Y. Shioi, personal communication), but its possible cytosolic localization questions its role in Chl breakdown along the PAO/RCCR route (see below). PAO and RCCR are plastidal enzymes, i.e. if pheophorbidase would act upstream of PAO/RCCR, an unlikely transport of catabolites out and back into the plastid has to be postulated.

The loss of green color – PAO/RCCR

The early identification of Pheide *a* and fluorescent Chl catabolites (FCCs) as intermediates of Chl breakdown (Thomas *et al*., 1989; Vicentini *et al*., 1995) enabled the establishment of an *in vitro* assay in which Pheide *a* is converted to a primary FCC (pFCC) (Hörtensteiner *et al*., 1995). In pFCC, the porphyrin macrocycle of Pheide *a* had been oxygenolytically opened between pyrroles A and B (Figure 2). In addition, two of the remaining methine bridges (β and δ) are reduced. Further investigations demonstrated that the reaction from Pheide *a* to pFCC consists of (at least) two steps (Rodoni *et al*., 1997a). The first step is catalyzed by an inner envelope located enzyme, Pheide *a* oxygenase (PAO), and causes the macroring cleavage to produce a red colored intermediate (RCC) (Matile *et al*., 1999). In contrast to other enzymes of Chl catabolism that are all constitutively expressed, activity of PAO is restricted to senescent tissues. The specificity of PAO for Pheide *a* explains that NCCs identified so far are exclusively derived from Chl *a*. Thus, it has been proposed that Chl(ide) *b* is converted to Chl(ide) *a* by the reductive reaction(s) of the 'Chl cycle' before entering the catabolic pathway.

Exploiting the biochemical characteristics of PAO for a functional genomic approach, candidate genes for PAO in *Arabidopsis* were identified recently (Pružinská *et al*., 2003). One of them, *Accelerated cell death 1* (*Acd1*), encodes a protein (AtPAO) that could be expressed in *E. coli* as an active PAO. At*Pao* is slightly upregulated upon senescence induction, but at the same time PAO activity increases much more. This indicates that the observed senescence-specific activity of PAO may result from post-transcriptional regulation (Pružinská *et al*., 2003). AtPAO is the orthologue of lethal leaf spot 1 (LLS1) of maize (Gray *et al*., 2002) and is a member of a small family of structurally related but functionally distinct Rieske-type oxygenases (Gray *et al*., 2002; Gray *et al*., 2004). Rieske-type oxygenases are widely distributed in pro- and eukaryotes and in all cases ferredoxin supplies the electrons necessary to drive the redox cycle of the Rieske-center irons (Schmidt and Shaw, 2001).

The second part of the reaction from Pheide *a* to pFCC is catalyzed by RCC reductase (RCCR). Like PAO, RCCR depends on reduced Fd as the source of electrons to stereospecifically reduce the C20/C1 double bond of RCC. Thereby, two possible C1 stereoisomers of pFCC (Figure 2), pFCC-1 or pFCC-2, occur (Rodoni *et al*., 1997a). The source of RCCR determines which isomer is formed. Thus, e.g. RCCR of *Arabidopsis* (AtRCCR) produces pFCC-1, whereas pFCC-2 is formed with RCCR from tomato (Hörtensteiner *et al*., 2000). RCCR has been cloned from barley (Wüthrich *et al*., 2000) and its *Arabidopsis* homologue is identical to accelerated cell death 2 (ACD2) (Mach *et al*., 2001). RCCR is a novel protein that does not have homology to other reductases, but is distantly related to a family of Fd-dependent bilin reductases involved in the biosynthesis of phycobilins and phytochromobiline (Frankenberg *et al*., 2001). In contrast to other Fd-dependent enzymes, these bilin reductases and RCCR lack a metal or flavin cofactor. Instead, electron transfer is believed to occur directly from Fd to the respective substrates (Frankenberg and Lagarias, 2003). In this respect, RCCR may rather be active as a kind of 'chaperone' that enables the interaction between Fd and RCC, thereby controlling the regio- and stereoselective reduction. RCCR is located in the stroma and is constitutively expressed (Rodoni *et al*., 1997b; Wüthrich *et al*., 2000; Mach *et al*., 2001).

The final destination of Chl catabolites

By comparing the structure of pFCC with structures of known NCCs (Figure 2), additional (late) reactions could be predicted in Chl breakdown. A common modification relates to an isomerization involving pyrrole D and the γ-methine bridge. Recently, it has been shown that *in vitro* pFCC rapidly (with a half-life of 30 min) tautomerizes to the respective NCC at a pH of 4.9 (Oberhuber *et al*., 2003). The mechanism of tautomerization has been proposed to be a two-fold protonation/deprotonation reaction, leading to an intermediary NCC in which the methoxycarbonyl function at $C13^2$ and pyrrole D are *cis* to each other. With a half-life of 2 h, this instable NCC isomerizes to the final product, thereby establishing a *trans* arrangement of the bulky groups at $C13^2$ and C15 (Figure 2). Since FCCs rather than NCCs are the proposed substrates for vacuolar import (see below), these data suggest that, *in vivo,* FCC to NCC

conversion is nonenzymically catalyzed by the acidic vacuolar sap (Oberhuber *et al.*, 2003).

Consequently, further modifications of NCCs most likely occur at the level of FCCs before the Chl catabolites reach their final site of disposal in the vacuole. Except for a common hydroxylation of $C8^2$, these modifications are species-specific. The simultaneous occurrence of different NCCs (e.g. five NCCs in spinach; Berghold *et al.*, 2002) may depend on the rate of import of the respective FCCs into the vacuole. The transporter at the tonoplast is a primary active ATPase (Hinder *et al.*, 1996) and *in vitro* two members of the ATP binding cassette transporters of *Arabidopsis*, AtMRP2 and 3, have been shown to be capable of transporting Chl catabolites (Lu *et al.*, 1998; Tommasini *et al.*, 1998). In contrast to the vacuolar transport, the nature of the transporter at the chloroplast envelope remains elusive.

Recent reports indicate that NCCs may be further degraded. In barley, a degradation product of *Hv*-NCC-1 has been identified in which the formyl group attached to pyrrole B is absent (Losey and Engel, 2001). In addition, monopyrrolic degradation products of Chl have been identified in senescent barley leaves (Suzuki and Shioi, 1999).

Control of photoreactive intermediates in Chl breakdown

As outlined above, it is generally believed that Chl breakdown is completed with the disposal of tetrapyrrolic NCCs inside the vacuole. Thus, plants are not attuned to remobilize the nitrogen of Chl. Despite, Chl breakdown has a vital role during senescence. The remobilization of proteins, including Chl-binding proteins (Pružinská *et al.*, 2003), causes the release of potentially phototoxic Chl. To avoid this hazard, plants evolved a mechanism for the detoxification of Chl by a complete destruction of its π-electron system. Indeed, Chl breakdown exhibits similarities to the known three-step detoxification process of plants for xenobiotics or herbicides (Kreuz *et al.*, 1996), i.e. hydroxylation, conjugation and excretion. The importance of the Chl catabolic pathway for plant development and survival is clearly demonstrated by the analysis of mutants and antisense lines, in which absence of PAO or RCCR causes a lesion mimic phenotype (Greenberg and Ausubel, 1993; Greenberg *et al.*, 1994; Gray *et al.*, 2002; Spassieva and Hille, 2002; Tanaka *et al.*, 2003). In *lls1* and *acd1*, Pheide *a* accumulation positively correlates with the observed light-dependent lesion mimic phenotype indicating that Pheide *a* induces a cell death pathway, due to its photodynamic properties (Pružinská *et al.*, 2003; G. Tanner and S. Hörtensteiner, unpublished). Surprisingly, other mutants that have been shown to have reduced levels of PAO activity and accumulate Pheide *a*, such as *Festuca pratensis* Bf 993 and Gregor Mendel's green pea variety, exhibit a 'stay-green' phenotype, but do not show cell death symptoms (Vicentini *et al.*, 1995; Thomas *et al.*, 1996). In addition to Pheide *a*, these mutants accumulate Chlides, indicating that the genetic defect is distinct from *Pao*.

In analogy to *pao* mutants, the cell death phenotype observed in *acd2,* which is defective in RCCR (Mach *et al.*, 2001), is due to accumulation of RCC upon dark incubation (A. Pružinská and S. Hörtensteiner, unpublished). It is attractive to elucidate the interdependence of accumulating Chl catabolites and cell death-inducing mechanisms.

Conclusions and perspectives

Despite the recent research progress on Chl metabolism, many questions remain to be solved, for example the mutual control of Chl synthesis and breakdown as well as the fate of Chl during turnover in green leaves. The half-life of Chl has been estimated between 16 and 58 h (Hendry and Stobart, 1986) implying an ongoing Chl turnover, but NCCs have not been found in photosynthetically active, mature leaves (Matile *et al.*, 1999). Moreover, the phototoxicity of many porphyrinic or tetrapyrrolic Chl intermediates implies the requirement of sophisticated protection mechanisms during Chl biosynthesis and degradation. Thus, it is likely that individual reactions are collectively carried out in enzyme complexes without release of intermediates. It is tempting to speculate whether enzymes catalyzing consecutive reactions are assembled in high-molecular weight multimeric protein complexes, as proposed for MgCh and MTF, for GluTR and glutamate 1-semialdehyde aminotransferase, and for PAO and RCCR (Hinchigeri *et al.*, 1997;

Rodoni *et al.*, 1997a; Moser *et al.*, 2001). This supports the idea of 'metabolic channeling', a mechanism by which the release of potentially toxic intermediates is minimized. In addition, subcellular transport between different compartments requires carriers of Chl intermediates and catabolites. However, these transporters for membrane translocation are largely unknown. Last but not least, since intracellular signaling was attributed to Mg–porphyrins, it provokes the question to what extent Chl and its biosynthetic or catabolic intermediates may be additionally involved in the control of plastid development.

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