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Mechanism and Significance of Chlorophyll Breakdown

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Abstract Chlorophyll breakdown is the most obvious sign of leaf senescence and fruit ripening. A multistep pathway has been elucidated in recent years that can be divided into two major parts. In the first phase, which commonly is active in higher plants, chlorophyll is converted via several photoreactive intermediates to a primary colorless breakdown product within the chloroplast. The second part of chlorophyll breakdown takes place in the cytosol and the vacuole. During this phase, the primary colorless intermediate is modified in largely species-specific reactions to a number of similar, yet structurally different, linear tetrapyrrolic products that finally are stored within the vacuole of senescing cells. To date, most of the biochemical reactions of the first phase of chlorophyll breakdown have been elucidated and genes have been identified. By contrast, mechanisms of catabolite transport and modification during the second phase are largely unknown. This review summarizes the current knowledge on the biochemical reactions involved in chlorophyll breakdown, with a special focus on the second-phase reactions and the fate of by-products that are released from chlorophyll during its breakdown.

Keywords Chlorophyll breakdown · Chlorophyll catabolites · Detoxification · Nutrient remobilization · Senescence

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Introduction

Chlorophyll (Chl) breakdown is the hallmark of senescence because of its impact on the color of senescing organs: loss of green Chls unmasks yellow carotenoids and highlights the synthesis of red anthocyans. Why do plants degrade Chls? Chloroplasts have been shown to contain up to 75 % of the nitrogen content in photosynthetic tissues (Peoples and Dalling 1988; Makino and Osmond 1991). Rubisco, the most abundant protein of the chloroplast, accounts for 20–30 % of total leaf nitrogen, and protein complexes of the thylakoids such as photosystems (PS) constitute the second largest pool of chloroplast nitrogen (Hörtensteiner and Feller 2002; Feller and others 2008). During senescence and remobilization of chloroplast nutrients, Chls are uncoupled from Chl-binding proteins within the PS and become phototoxic. Therefore, coordinated Chl breakdown maintains cell viability during senescence and allows an efficient remobilization of nutrients to growing organs.

Most enzymes involved in Chl degradation are now characterized and form the so-called "PAO pathway" (Fig. 1). This pathway can be divided into two main parts. The first occurs within the chloroplast at the thylakoid membrane and involves phototoxic catabolites. The second part of the PAO pathway consists of modifications of colorless Chl catabolites and their translocation from the chloroplast to the vacuole. Recently, new insights have been obtained on the topology of Chl degradation within the chloroplast as well as on cytosolic modifications of colorless catabolites. The aim of this review is to provide an updated summary of the PAO pathway and to discuss its biological functions.

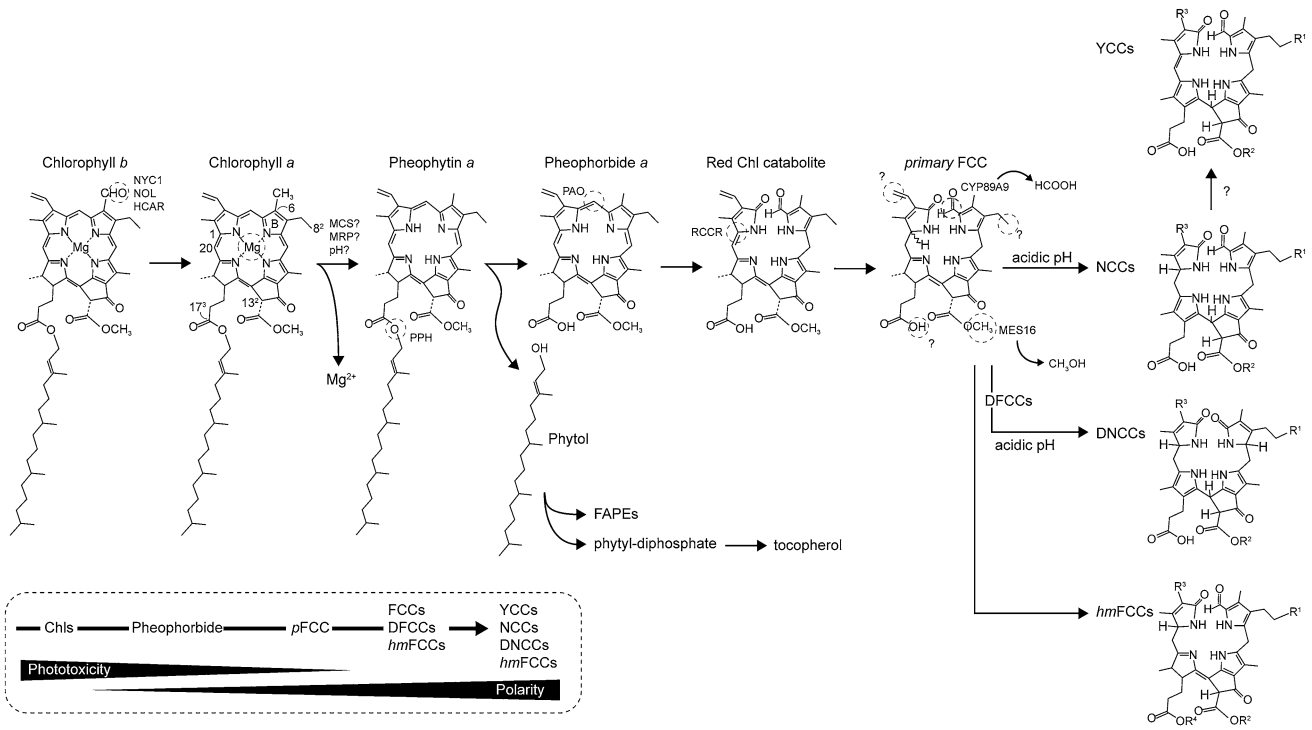

Fig. 1 Chl breakdown by the PAO pathway. Enzymes and their target sites (*dashed circles*) are indicated. Magnesium, phytol, formate, and methanol, the major by-products of the PAO pathway, are also depicted. Side chain residues (R^1–R^4) of the to-date characterized NCCs, DNCCs, and FCCs are listed in Table 1. *Inset* Chl breakdown leads to the formation of water-soluble nonphototoxic catabolites. Relevant pyrrole rings and carbon atoms are labeled in the structure of Chl *a*. For abbreviations, see text

The PAO Pathway

Detoxification of Colored Chlorophyll Catabolites in the Chloroplast

Conversion of Chl b to a

Chl *b* is converted to Chl *a* by the action of two enzymes, Chl *b* REDUCTASE (CBR) and 7-HYDROXYMETHYL Chl *a* REDUCTASE (HCAR, Fig. 1) (Tanaka and Tanaka 2011). Plants possess two isoforms of CBR called NON-YELLOW COLORING1 (NYC1) and NYC1-LIKE (NOL) (Kusaba and others 2007; Sato and others 2009). In silico hydrophobicity analysis of NYC1 and NOL protein sequences predicted NYC1 to be a membrane protein and NOL to be a soluble protein (Kusaba and others 2007). Furthermore, in vitro immunoprecipitation and localization experiments in rice (*Oryza sativa*) suggested that the two proteins could form heterodimers at the thylakoid membrane (Kusaba and others 2007; Sato and others 2009). Although only NOL activity has been demonstrated in vitro, the two enzymes are thought to catalyze the same reaction, that is, the reduction of Chl *b* to 7-hydroxymethyl Chl *a* (HMChl *a*). Interestingly, an *Arabidopsis* (*Arabidopsis thaliana*) *nyc1 nol* double mutant does not show an altered Chl *a/b* ratio during the unstressed vegetative phase, but displays a stay-green phenotype during leaf senescence and seed maturation due to the retention of Chl *b* (Horie and others 2009). Taken together, these data suggest that NYC1 and NOL do not participate actively in Chl *a/b* ratio regulation during vegetative growth but are required for developmental processes such as leaf senescence (Tanaka and Tanaka 2011). Finally, Chl *b* to *a* reduction appears to be crucial for light-harvesting complex (LHC) degradation during senescence because *nyc1 nol* mutants retain LHC proteins in particular (Kusaba and others 2007; Horie and others 2009).

Recently, the enzyme catalyzing the second step of Chl *b* to Chl *a* reduction, conversion of HMChl *a* to Chl *a*, was characterized in *Arabidopsis* (Meguro and others 2011). HCAR contains a flavin adenine dinucleotide and an iron–sulfur center as cofactors, and phylogenetic analysis revealed that HCAR evolved from divinyl chlorophyllide reductase of the Chl biosynthesis pathway (Meguro and others 2011). HCAR is able to catalyze the conversion of HMChl *a* to Chl *a* in vitro, and T-DNA insertion mutants for HCAR accumulate low levels of HMChl *a* during leaf senescence. Surprisingly, *hcar* mutants retain significant amounts of pheophorbide (Pheide) *a*, a downstream intermediate of the Chl degradation pathway. Pheide *a* OXYGENASE (PAO, the

enzyme catalyzing the degradation of Pheide; see below) protein levels were unchanged in *hcar* as compared to wild-type plants. These observations, together with the fact that Pheide *a* accumulates in a *hcar nyc1 nol* triple mutant, suggest that HMChl or some degradation products of HMChl could inhibit PAO activity by an unknown mechanism, resulting in the accumulation of Pheide *a* (Meguro and others 2011).

There is strong evidence that the first step of Chl *b* degradation is its conversion to Chl *a*. The main argument is that PAO is specific for the "*a*" form of Pheide (Hörtensteiner and others 1995; Pružinská and others 2003). In addition, *nyc1 nol* mutants are unable to degrade Chl *b* during leaf senescence. As mentioned above, *nyc1 nol* mutants retain large amounts of LHC protein, indicating that Chl *b* to *a* conversion is a prerequisite for both LHC and Chl *b* degradation. Finally, it has been shown that Chl *b* to *a* conversion must precede demetalation and dephytylation because HCAR is able to reduce HMChl *a* in vitro but not 7-hydroxymethyl pheophytin *a* or 7-hydroxymethyl Pheide *a* (Shimoda and others 2012). All together, these data indicate that Chl *b* to *a* conversion is necessary for Chl *b* and Chl *b*-containing complex (that is, LHC) degradation, and that Chl *b* to *a* conversion (but not demetalation or dephytylation) is the first step of Chl *b* degradation. Thus, Chl *a* degradation seems to be independent of Chl *b* to *a* reduction. In line with this, *Arabidopsis nyc1 nol* degrades Chl *a* and PS core complexes as wild-type (Horie and others 2009). Furthermore, *nyc1 cao* double mutants of rice, in which the entire Chl cycle (interconverting Chl *a* and Chl *b*) is abolished, solely produce Chl *a* during vegetative growth and are able to degrade it during leaf senescence (Kusaba and others 2007).

Interestingly, it has been demonstrated that overproduction of Chl *b* in *Arabidopsis* leads to the incorporation of Chl *b* into PS core complexes, which seems to partially inhibit their degradation during senescence (Hirashima and others 2006; Sakuraba and others 2010, 2012a; Shimoda and others 2012). These findings indicate that Chl *b* to *a* conversion has evolved specifically to act on LHC complexes. It is interesting to note that Chl *b* overproduction in *Arabidopsis* appears to also retard age-dependent senescence via transcriptional downregulation of senescence-associated genes (SAG) (Sakuraba and others 2012a). Incorporation of Chl *b* in PS core complexes, and their consequent increased stability, is thought to maintain active PS during the senescence phase. The increased photosynthetic capacity resulting from Chl *b* overproduction could modulate *SAG* expression through an unknown signaling pathway (Sakuraba and others 2012a).

Destabilization of Chl–Apoprotein Complexes by STAY-GREEN

Deletion of the chloroplast-localized protein STAY-GREEN (SGR), as the name implies, causes a stay-green phenotype (Hörtensteiner 2009). Conversely, many but not all mutations triggering a stay-green phenotype affect the *SGR* gene. Among the more than 14 stay-green mutants identified in natural populations, mutagenesis screens, or breeding programs of different plant species, eight are deficient of SGR (Hörtensteiner 2009; Schelbert and others 2009). Notably, presence or absence of a functional *SGR* gene in pea (*Pisum sativum*) determines the color of the cotyledons (green or yellow), originally described by Mendel (Mendel 1866; Armstead and others 2007). Furthermore, the commercial tomato (*Solanum lycopersicum*) variety "*green-flesh*" is also deficient in SGR (Barry and others 2008). The brown coloration of ripe *green-flesh* fruits is due to Chl retention and simultaneous carotenoid accumulation.

The exact role of the SGR protein, which does not contain any known domain, is still unclear (Hörtensteiner 2009). Suppression of *SGR* leads to a cosmetic stay-green phenotype (Jiang and others 2007; Park and others 2007; Ren and others 2007), that is, the photosynthesis capacity of *sgr* mutants during leaf senescence decreases as in corresponding wild types. However, several LHC and core subunits of PSI and II are partially retained (Jiang and others 2007; Park and others 2007; Aubry and others 2008). Together with the finding that SGR can interact with LHC proteins of PSII (Park and others 2007), these observations suggest that SGR participates in the destabilization of Chl–apoprotein complexes of PSII during senescence. Recently, this hypothesis has been corroborated by the finding that SGR plays a central role in recruiting Chl catabolic enzymes (CCE) at the thylakoid membrane (Sakuraba and others 2012b, 2013).

Interestingly, SGR seems to also have other functions not directly related to Chl degradation. SGR appears to be implicated in root nodule senescence (Zhou and others 2011). Root nodules of legume species such as *Medicago sativa* and *Medicago truncatula* are specialized organs hosting soil bacteria capable of reducing atmospheric nitrogen to ammonium. Nodule senescence stops nitrogen fixation and results in the loss of the symbiotic interaction. Silencing of *SGR* in *M. truncatula* was shown to affect nodule senescence and thus indicates that SGR could also have a function in nonphotosynthetic tissues (Zhou and others 2011). Recently, SGR has been shown to regulate lycopene and β-carotene biosynthesis in tomato fruits (Luo and others 2013). SGR directly interacts with PHYTOENE SYNTHASE 1, a carotenoid synthetic enzyme and thereby

inhibits its activity. These two studies clearly demonstrate that SGR, besides its requirement for Chl breakdown, is also involved in other biochemical processes.

Demetalation

With regard to the recent knowledge acquired about dephytylation of Chl during leaf senescence (see below), it is likely that demetalation precedes dephytylation and thus occurs on Chl *a* (Fig. 1) (Morita and others 2009; Schelbert and others 2009; Ren and others 2010; Shimoda and others 2012). To date, the mechanism involved in Chl demetalation is unknown (Hörtensteiner 2013). Several biochemical approaches described the involvement of either a heat-stable metal-chelating substance (MCS) or a metal-releasing protein (MRP) (Vicentini and others 1995; Shioi and others 1996a; Suzuki and Shioi 2002; Büchert and others 2011). All these studies used chlorophyllin (an artificial and soluble Chl derivative) as substrate, but not Chl (Hörtensteiner 2013). A recent proteomic study of plastoglobules in *Arabidopsis* annotated a possible MRP protein (Lundquist and others 2012). However, analysis of T-DNA insertion lines in this gene did not show any delay in Chl degradation during dark-induced senescence (Guyer and Hörtensteiner, unpublished data). Release of Mg^{2+} from Chl is known to occur at slightly acidic pH (Saga and Tamiaki 2012). Thus, it can be speculated that the decrease of photosynthesis during senescence lowers the stromal pH sufficiently to remove Mg^{2+} from the Chl macrocycle. Thereby, SGR could have a decisive role; during vegetative growth, the stromal pH also decreases at night, but absence of senescence-regulated SGR, which is thought to destabilize Chl–apoprotein complexes (Park and others 2007), could avoid massive demetalation of Chls before the onset of senescence.

Dephytylation

Already a century ago, Willstätter and Stoll (1911) described an enzyme, called CHLOROPHYLLASE (CLH), that was able to cleave the phytol tail of Chls thereby producing chlorophyllide. Later, Mayer (1930) found CLH activity in several plant species, and Holden (1961) partially purified CLHs from sugar beet (*Beta vulgaris*) and pea and further characterized their activity. To date, more than 250 studies have been performed on plant CLHs according to the Web of Knowledge database [search for titles with "chlorophyllase(s)" in them]. The majority of these studies describe the (partial) purification of CLHs from different plant species and the characterization of their in vitro activity. Cloning of CLH genes from plants such as *Chenopodium album*, *Arabidopsis*, and citrus (*Citrus sinensis*) revealed that CLHs contain a lipase motif. In addition, gene transcription of most CLHs is highly induced by ethylene and JA treatments, which are known to promote senescence (Jakob-Wilk and others 1999; Tsuchiya and others 1999). However, two CLHs (*Arabidopsis* *At*CLH2 and *C. album* *Ca*CLH) are not induced by phytohormones and show constitutively low levels of expression (Jakob-Wilk and others 1999; Tsuchiya and others 1999). Using biochemical and immunolocalization approaches, several studies could localize CLH to the chloroplast (Brandis and others 1996; Matile and others 1997; Harpaz-Saad and others 2007; Azoulay-Shemer and others 2011). In citrus, CLH appears to be post-translationally regulated by N- and C-terminal proteolysis within chloroplast membranes and mature CLH was shown to be more active than the CLH precursor (Harpaz-Saad and others 2007; Azoulay-Shemer and others 2011). Collectively considered, all these data suggest a major role for CLH in hydrolyzing the phytol chain of Chl. However, reverse genetic approaches of CLH-deficient plants did not corroborate this hypothesis (Benedetti and Arruda 2002; Schenk and others 2007). Although silencing of *Arabidopsis* *At*CLH1 (*Arabidopsis* has two CLH homologs) was believed to decrease chlorophyllide/Chl ratios in green leaves, analysis of T-DNA insertion mutant lines for *At*CLH1 (*clh1*) and *At*CLH2 (*clh2*) did not reveal any delay of Chl degradation during senescence in single as well as in double mutants (Benedetti and Arruda 2002; Schenk and others 2007). Decrease and increase of chlorophyllide/Chl ratios caused by, respectively, silencing and overexpression of CLH in *Arabidopsis* reported by Benedetti and Arruda (2002) could be experimental artifacts and may reflect the in vitro rather than the in vivo activity of CLH. Indeed, in this study, extraction of green pigments from plant tissue was performed in acetone at 4 °C during 12 h. However, CLH has been reported to be active in vitro under such conditions (Barrett and Jeffrey 1964). Lastly, the hypothesis that *Arabidopsis* CLHs are not involved in Chl degradation during age-dependent and dark-induced leaf senescence is corroborated by the finding that *At*CLHs are located in the cytosol of senescent cells (Schenk and others 2007).

The question of how the phytol group of Chl may be hydrolyzed during age-dependent leaf senescence remained unanswered until recently. Using an elegant *in silico* approach in *Arabidopsis*, Schelbert and others (2009) identified a chloroplast-targeted serine-type hydrolase, the mutation of which leads to a stay-green phenotype during leaf senescence. Surprisingly, this senescence-induced hydrolase is not active on Chl in vitro but was found to specifically hydrolyze the phytol chain of pheophytin (Phein) and to produce Pheide. The protein, termed Phein Pheide HYDROLASE (PPH), was shown to be indispensable for Chl degradation during leaf senescence in

Arabidopsis and rice (Morita and others 2009; Schelbert and others 2009; Ren and others 2010). *Arabidopsis pph* mutants are also affected in LHC and PS core subunit degradation, indicating that phytol cleavage, in addition to Chl *b*–*a* reduction (see above), is crucial for degradation of PS proteins during leaf senescence (Schelbert and others 2009).

Opening and Reduction of the Macrocycle

The light absorption capacity of tetrapyrroles and thus the potential phototoxicity of some Chl catabolites is due mostly to electron conjugation within the porphyrin ring (Hörtensteiner 2006; Scheer 2006; Hörtensteiner and Kräutler 2011). Opening of the macrocycle of Pheide *a* by PAO and the subsequent reduction of the conjugated C20/C1 double bond of the intermediate, red Chl catabolite (RCC), by RCC REDUCTASE (RCCR) are the two steps of the Chl degradation pathway that lead to the loss of Chl catabolite phototoxicity (Fig. 1) (Hörtensteiner 2006; Hörtensteiner and Kräutler 2011). Thus, *primary* fluorescent Chl catabolite (*p*FCC), the product of the consecutive PAO and RCCR activities, is considered as nonphototoxic (Hörtensteiner 2006, 2013; Hörtensteiner and Kräutler 2011).

The enzymatic activities responsible for the opening of the Pheide macrocycle and production of *p*FCC were originally detected in isolated intact gerontoplasts and were shown to be promoted by the supply of glucose-6-phosphate or ATP (Schellenberg and others 1990; Matile and others 1992). Later, *p*FCC production from Pheide was shown to be possible in vitro by using isolated thylakoid membranes and reduced ferredoxin (Schellenberg and others 1993; Ginsburg and others 1994). Partial purification of this activity from *Brassica napus* revealed its dependency on a stromal fraction, senescence inducibility, and specificity towards Pheide *a*, that is, Pheide *b* not being a substrate (Hörtensteiner and others 1995). Dependency of the activity on a stromal fraction was explained by the finding that the conversion of Pheide to *p*FCC was a two-step reaction performed by two enzymes, one localized in chloroplast membranes (PAO) and the other in the stroma (RCCR; Rodoni and others 1997). For a long time, PAO was considered to localize to the chloroplast envelope, but recent reconsideration of Chl catabolic enzyme localization revealed that PAO is instead inserted into the thylakoid membrane (Matile and others 1996; Pružinská and others 2003; Kleffmann and others 2004; Sakuraba and others 2012b). Further characterization of PAO has shown that the enzyme is a Fe-dependent monooxygenase belonging to the Rieske-type iron–sulfur oxygenase family (Hörtensteiner and others 1998; Gray and others 2002; Pružinská and others 2003). Interestingly, an additional factor called RCC FORMING FACTOR (RFF), indispensable for PAO/RCCR activity in vitro, has also been described (Pružinská and others 2005). RFF could be a reactive oxygen species (ROS)-scavenging protein such as a peroxidase that possibly is required to remove ROS that are likely produced as by-products of PAO activity (Aubry and Hörtensteiner, unpublished data).

Suppression of PAO in *Arabidopsis*, corn (*Zea mays*), rice, and tomato has been shown to induce premature cell death (Spassieva and Hille 2002; Pružinská and others 2003, 2005; Tanaka and others 2003; Tang and others 2011). Stay-green and cell-death phenotypes of *Arabidopsis pao1* mutants [originally identified as *accelerated cell death 1* (*acd1*; Greenberg and Ausubel 1993)] is due to the accumulation of Pheide *a* (Pružinská and others 2003; Tanaka and others 2003). Surprisingly, the cell death phenotype of *PAO* mutants is not strictly connected to light, but also occurs in the dark (Pružinská and others 2003, 2005; Hirashima and others 2009). Thus, rather than being solely phototoxic, Pheide has been speculated to act as a signaling molecule that may be exported from the chloroplast, as suggested for Mg-protoporphyrin IX, the first intermediate of the Chl branch of the tetrapyrrole biosynthesis pathway (Mochizuki and others 2001; Hirashima and others 2009). However, characterization of METHYL ESTERASE (MES) 16, the enzyme demethylating FCCs in the cytosol of *Arabidopsis* (see below), revealed that an export of Pheide from the chloroplasts of *pao1* is unlikely (Christ and others 2012). In vitro, MES16 can demethylate Pheide and convert it to pyroPheide, but pyroPheide was not detected in *pao1*. However, when MES16 was mistargeted to the chloroplast, 75 % of the Pheide accumulating in *pao1* was converted to pyroPheide (Schelbert and others 2009; Christ and others 2012). This finding indicates that Pheide is most probably not a chloroplast-to-nucleus retrograde signal itself; instead, it seems to trigger a signaling cascade involving other factors.

RCCR was originally cloned from barley (*Hordeum vulgare*) and *Arabidopsis* and was localized to the chloroplast (Wüthrich and others 2000). Reduction of RCC by RCCR occurs in a stereospecific fashion, which can be different between RCCR orthologs (Mühlecker and others 1997, 2000). For instance, *Arabidopsis* RCCR produces *p*FCC, whereas *Capsicum annuum* RCCR converts RCC into the C1 epimer of *p*FCC, *epi*-*p*FCC (Hörtensteiner and others 2000). Interestingly, the *Arabidopsis* RCCR stereospecificity can be manipulated by a Phe-to-Val exchange at residue 218 (Pružinská and others 2007). RCCR crystallization and site-directed mutagenesis confirmed that residue 218 together with Glu154 and Asp291 are located within the substrate-binding pocket of RCCR and are required for its activity (Sugishima and others 2009, 2010; Pattanayak and others 2012). In contrast to senescence-related

expression of *PAO*, Northern blot analysis revealed a constitutive expression of *RCCR* in leaves and roots (Wüthrich and others 2000). Furthermore, RCCR was shown to be targeted to the mitochondria, suggesting that RCCR could have other roles besides converting RCC to *p*FCC (Mach and others 2001). Loss of RCCR in *Arabidopsis* causes the *acd2* phenotype, which is characterized by the spontaneous spread of light-dependent cell death lesions during plant growth and development and by constitutive activation of defenses in the absence of environmental stress (Mach and others 2001; Yao and Greenberg 2006; Pružinská and others 2007). *acd2* accumulates RCC and RCC-like pigments in the vacuole, indicating that these tetrapyrroles can move within the cell (Pružinská and others 2007). RCC and RCC-like pigments are thought to act as signaling molecules and trigger the cell death observed in *acd2* (Mach and others 2001; Yao and Greenberg 2006; Pattanayak and others 2012). Part of the cascade leading to cell death in *acd2* is the loss of mitochondrial membrane potential and mitochondrial H_2O_2 production (Yao and Greenberg 2006). Specific targeting of RCCR to the mitochondria of *acd2* dramatically reduces RCC accumulation, cell death, and mitochondrial ROS production (Pattanayak and others 2012). This rescue effect is dependent on the activity of RCCR because a mitochondria-targeted Glu154Ala variant of RCCR did not complement the cell death phenotype of *acd2*. Collectively, these in vivo data on RCCR function(s) provide evidence that this enzyme is involved in protection against prodeath molecules (such as RCC) in both chloroplast and mitochondria. These prodeath molecules, substrates of RCCR, are mobile within cells and have a major effect on mitochondria (Pattanayak and others 2012).

Interaction of CCEs at the Thylakoid Membrane

Recently, advances have been made in understanding the topology of the first steps of Chl breakdown during leaf senescence (Sakuraba and others 2012b, 2013). Using co-immunoprecipitation and bimolecular fluorescence complementation approaches, it has been demonstrated that SGR, NYC1, NOL, HCAR, PPH, PAO, and RCCR interact at the thylakoid membrane (Fig. 2). These interactions are proposed to create an enzyme complex that mediates channeling of phototoxic catabolites. The observation that these interactions do not occur in a *sgr* mutant suggests the possibility that SGR recruits the other CCEs at the thylakoid membrane and acts as a hub. In the model proposed by Sakuraba and others (2012b), all these steps of Chl degradation occur at the thylakoid membrane and ultimately lead to the release of *p*FCC, the first nonphototoxic catabolite, into the stroma. SGR has been shown to specifically interact with LHCII but not with LHCI subunits,

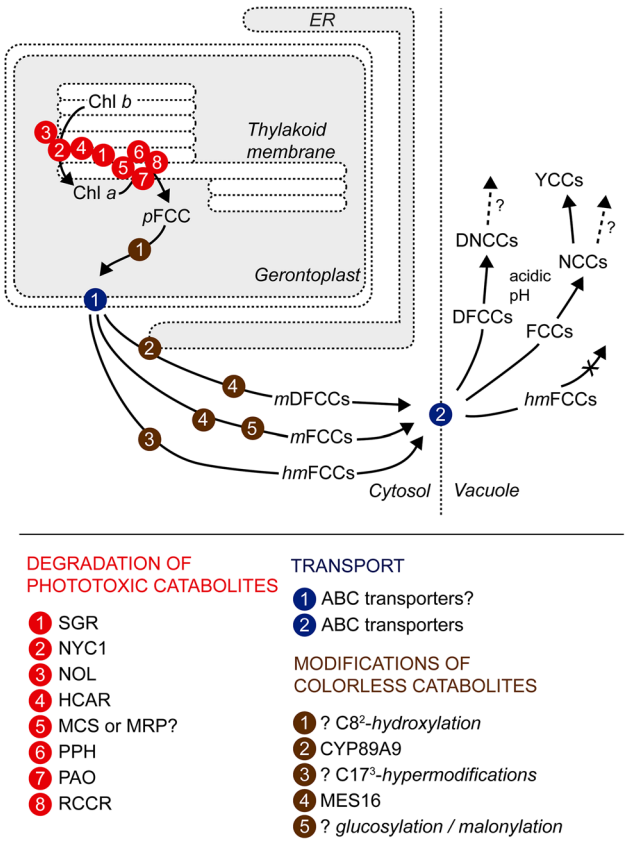

Fig. 2 Topology of Chl breakdown. Proteins involved in the degradation of phototoxic intermediates in the chloroplast are depicted in *red*, translocation mechanisms across the chloroplast envelope and the tonoplast in *blue*, and enzymes that modify FCC side chains in *brown*. See text for more information and abbreviations (Color figure online)

and to participate in the destabilization of the Chl–apoprotein complexes (Park and others 2007). Existence of the same or a similar complex for Chl degradation in PSI is highly probable but has not yet been demonstrated.

Modifications of Fluorescent Chlorophyll Catabolites

Diversity of Colorless Chlorophyll Catabolites

After its release into the chloroplast stroma, nonphototoxic and colorless *p*FCC is modified by different enzymes, leading to the production of *modified* FCCs (*m*FCCs). All colorless Chl catabolites are thought to derive from *p*FCC, to be modified in both the chloroplast and cytosol, and finally to be nonenzymatically isomerized and stored in the vacuole as nonfluorescent Chl catabolites (NCCs) or dioxobilin-type nonfluorescent Chl catabolites (DNCCs). Since the identification of the first NCC, *Hv*-NCC-1 in 1991 (Kräutler and others 1991), up to 40 different colorless Chl catabolites have been structurally characterized (Table 1) (Hörtensteiner 2013). These catabolites are

Table 1 List of FCCs, NCCs, and DNCCs identified from higher plants

Name	R^{1c}	R^{2c}	R^{3c}	R^{4c}	C1-epimer[d]	Source[e]	Reference
*p*FCCs							
*p*FCC	H	CH_3	Vinyl	H	1	E	Mühlecker and others (1997)
*epi-p*FCC	H	CH_3	Vinyl	H	*epi*	E	Mühlecker and others (2000)
*m*FCCs							
At-FCC-1[a]	OH	H	Vinyl	H	1	L	Pružinská and others (2005)
At-FCC-2[a]	H	H	Vinyl	H	1	L	Pružinská and others (2005)
*hm*FCCs							
Mc-FCC-49[b]	*O*-glucosyl	CH_3	Vinyl	Daucic acid	*epi*	F	Moser and others 2009
Mc-FCC-56[b]	OH	CH_3	Vinyl	Daucic acid	*epi*	F	Moser and others (2008b)
Ma-FCC-61[b]	OH	CH_3	Vinyl	Digalactosylglyceryl	*epi*	L	Banala and others (2010)
Ma-FCC-63/64[b]	OH	CH_3	Vinyl	Glucopyranosyl	*epi*	L	Vergeiner and others (2013)
Ma-FCC-69[b]	OH	CH_3	Vinyl	3,4-Dihydroxyphenyl-ethyl-glucopyranosyl	*epi*	L	Vergeiner and others (2013)
Sw-FCC-62[b]	OH	CH_3	Vinyl	Dihydroxyphenyl-ethylglucosyl	1	L	Kräutler and others (2010)
NCCs							
At-NCC-1[a]	*O*-glucosyl	H	Vinyl	H	1	L	Pružinská and others (2005)
At-NCC-2[a]	OH	H	Vinyl	H	1	L	Pružinská and others (2005)
At-NCC-3[a]	OH[f]	H	Vinyl	H	1	L	Pružinská and others (2005)
At-NCC-4[a]	*O*-glucosyl	CH_3	Vinyl	H	1	L	Pružinská and others (2005)
At-NCC-5[a]	H	H	Vinyl	H	1	L	Pružinská and others (2005)
Bn-NCC-1[a]	*O*-malonyl	H	Vinyl	H	1	L	Mühlecker and Kräutler (1996)
Bn-NCC-2[a]	*O*-glucosyl	H	Vinyl	H	1	L	Mühlecker and Kräutler (1996)
Bn-NCC-3[a]	OH	H	Vinyl	H	1	L	Mühlecker and Kräutler (1996)
Bn-NCC-4[a]	H	H	Vinyl	H	1	L	Pružinská and others (2005)
Cj-NCC-1[a]	OH	CH_3	Vinyl	H	*epi*	L	Curty and Engel (1996)
Cj-NCC-2[a]	H	CH_3	Vinyl	H	*epi*	L	Oberhuber and others (2003)
Hv-NCC-1[a]	OH	CH_3	Dihydroxyethyl	H	1	L	Kräutler and others (1991)
Lo-NCC-1[a]	OH	CH_3	Vinyl	H	nd	L	Iturraspe and others (1995)
Ls-NCC-1[a]	OH	CH_3	Vinyl	H	nd	L	Iturraspe and others (1995)
Ms-NCC-2[a]	OH	CH_3	Vinyl	H	*epi*	F	Müller and others (2007)
Nr-NCC-1[a]	*O*-glucosylmalonyl	CH_3	Vinyl	H	*epi*	L	Berghold and others (2004)
Nr-NCC-2[a]	*O*-glucosyl	CH_3	Vinyl	H	*epi*	L	Berghold and others (2004)
Pc-NCC-1[a]	*O*-glucosyl	CH_3	Vinyl	H	*epi*	F	Müller and others (2007)
Pc-NCC-2[a]	OH	CH_3	Vinyl	H	*epi*	F	Müller and others (2007)

Table 1 continued

Name	R^{1c}	R^{2c}	R^{3c}	R^{4c}	C1-epimer[d]	Source[e]	Reference
So-NCC-1[a]	OH	H	Dihydroxyethyl	H	*epi*	L	Berghold and others (2002)
So-NCC-2[a]	OH	CH_3	Dihydroxyethyl	H	*epi*	L	Oberhuber and others (2001)
So-NCC-3[a]	OH	H	Vinyl	H	*epi*	L	Berghold and others (2002)
So-NCC-4[a]	OH	CH_3	Vinyl	H	*epi*	L	Berghold and others (2002)
So-NCC-5[a]	H	CH_3	Vinyl	H	*epi*	L	Berghold and others (2002)
Sw-NCC-58[b]	OH	CH_3	Vinyl	H	1	L	Kräutler and others (2010)
Tc-NCC-1	*O*-glucosyl	CH_3	Dihydroxyethyl	H	*epi*	L	Scherl and others (2012)
Tc-NCC-2	*O*-glucosyl	CH_3	Vinyl	H	*epi*	L	Scherl and others (2012)
Zm-NCC-1[a]	*O*-glucosyl	CH_3	Dihydroxyethyl	H	*epi*	L	Berghold and others (2006)
Zm-NCC-2[a]	*O*-glucosyl	CH_3	Vinyl	H	*epi*	L	Berghold and others (2006)
DNCCs							
Hv-UCC-1[a,g,h]	OH	CH_3	Dihydroxyethyl	H	1	L	Losey and Engel (2001)
Ap-UCC-1[a,g,h]	OH	CH_3	Dihydroxyethyl	H	*epi*	L	Müller and others (2011)
At-NDCC-1	OH	H	Vinyl	H	*epi*	L	Christ and others (2013)
YCCs							
Cj-YCC-1	OH	CH_3	Vinyl	H	N/A	L	Moser and others (2008a)
Tc-YCC-1	*O*-glucosyl	CH_3	Dihydroxyethyl	H	N/A	L	Scherl and others (2012)

Adapted from Hörtensteiner (2013)

[a] A nomenclature for NCCs (and FCCs) has been defined (Ginsburg and Matile 1993) in which a prefix indicates the plant species and a numerical suffix indicates decreasing polarity in reversed-phase HPLC

[b] These catabolites are indexed according to their retention time in HPLC analysis. Ap *Acer platanoides*, At *Arabidopsis thaliana*, Bn *Brassica napus*, Cj *Cercidiphyllum japonicum*, Hv *Hordeum vulgare*, Lo *Liquidambar orientalis*, Ls *Liquidambar styraciflua*, Ma *Muca acuminate*, Mc *Musa cavendish*, Ms *Malus sylvestris*, Nr *Nicotiana rustica*, Pc *Pyrus communis*, So *Spinacia oleracea*, Sw *Spathiphyllum wallisii*, Tc *Tilia cordata*, Zm *Zea mays*

[c] R^1–R^4 indicate residues at C^3, $C8^2$, $C13^2$, and $C17^3$ side positions, respectively, of FCCs, NCCs, DNCCs, and YCCs as shown in Fig. 1

[d] C1 stereochemistry refers to the type of *p*FCC, that is, *p*FCC (1) or *epi*-*p*FCC (*epi*), formed in the respective species or genus; nd = not determined; N/A = not applicable

[e] Source of material used for catabolite isolation: *E* in vitro enzymatic PAO/RCCR assays, *F* fruits, *L* leaves

[f] In *At*-NCC-3, the site of hydroxylation is indicated to be C7 (rather than $C8^2$) (Müller and others 2006)

[g] *Hv*-UCC-1 and *Ap*-UCC-1 are indicated to be pseudo-enantiomers (Müller and others 2011)

[h] DNCCs have earlier been named urobilinogenoidic Chl catabolites (UCCs)

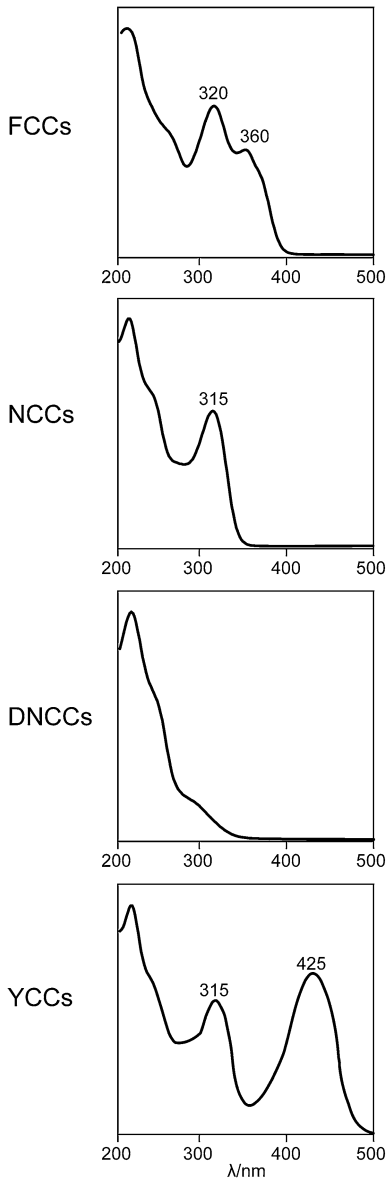

Fig. 3 UV/Vis spectra of FCCs, NCCs, DNCCs, and YCCs. The YCC spectrum is adapted from Moser and others (2008a)

divided into four classes (FCCs, NCCs, DNCCs, and YCCs; Fig. 1), depending on specific modifications at several side positions of the tetrapyrrole backbone that determine a distinct UV/Vis spectrum for each class of catabolite (Fig. 3). The recently identified yellow Chl catabolites (YCCs) have been speculated to originate from the oxidation of NCCs in the vacuole (Moser and others 2008a; Scherl and others 2012).

Demethylation

Demethylation of Chl catabolites at $C13^2$ is species-specific: demethylated Chl catabolites have so far been found only in *Arabidopsis*, rape (*B. napus*), and spinach (*Spinacia oleracea*) (Mühlecker and Kräutler 1996; Berghold and others 2002; Pružinská and others 2005; Hörtensteiner and Kräutler 2011). In a recent study, *Arabidopsis* MES16 was identified as the enzyme that catalyzes demethylation of Chl catabolites (Christ and others 2012). MES16 localizes to the cytosol and in vivo acts within the Chl degradation pathway on the level on FCCs but not on Pheide, as suggested in previous studies that proposed pheophorbidase, a homolog of MES16, to demethylate Pheide to pyroPheide in *Chenopodium album* and radish (*Raphanus sativus*) (Shioi and others 1996b; Suzuki and others 2002, 2006). Notably, demethylation of FCCs accelerates their isomerization in the vacuole. As a consequence, senescent leaves of *Arabidopsis mes16* mutants are fluorescent under UV light due to the vacuolar accumulation of methylated FCCs. Interestingly, *mes16* mutants are not affected in Chl *a* and *b* degradation and do not show any visible phenotype under controlled growth conditions.

Hydroxylation and Glucosylation

$C8^2$ hydroxylation of *p*FCC is the only side chain modification found in all species from which colorless Chl catabolites have been structurally characterized. The nature of the enzyme(s) responsible for hydroxylation is not known. However, isolation of gerontoplasts from barley revealed that not only *p*FCC, but also a second, more polar FCC is produced *in organello* (Matile and others 1992). The structure of this polar FCC has not yet been determined, but it was speculated to be $C8^2$ hydroxy-*p*FCC, and cytochrome P450 monooxygenases have been suggested as possible candidates for *p*FCC hydroxylation (Matile and others 1999). Although the majority of the 244 full-length P450 proteins that are encoded in the *Arabidopsis* genome are predicted to localize in the endoplasmic reticulum (ER), some P450 have been experimentally shown to be targeted to the chloroplast (Schuler and others 2006). However, dark incubation of detached *Arabidopsis* leaves in an atmosphere containing carbon monoxide (CO), known to be an inhibitor of cytochrome P450s, does not seem to prevent FCC hydroxylation (Christ and others 2013). Assuming that CO can diffuse into the chloroplast, FCC hydroxylation thus appears not to be mediated by a cytochrome P450 enzyme.

In some species such as *Arabidopsis*, tobacco (*Nicotiana rustica*), and rape, the $C8^2$ hydroxyl group of FCCs appears to be subsequently malonylated and/or glucosylated (Hörtensteiner 1998; Berghold and others 2004; Pružinská and others 2005). The molecular nature of respective activities remains unknown, although a malonyltransferase activity has been partially purified from *Brassica napus* (Hörtensteiner 1998). *Arabidopsis* UDP-DEPENDENT GLYCOSYLTRANSFERASEs (UGTs) are known to catalyze the

addition of a sugar group to hydroxyl groups of target molecules by formation of a glycosidic bond (Paquette and others 2003; Osmani and others 2009). Therefore, it can be imagined that one or several of the 120 cytosol-localized UGTs (Paquette and others 2003) are responsible for the addition of glucose to $C8^2$ hydroxy-FCCs in *Arabidopsis*.

Deformylation

Deformylated Chl catabolites have been found in barley, Norway maple (*Acer platanoides*), and *Arabidopsis* and are known as DNCCs (Losey and Engel 2001; Müller and others 2011; Christ and others 2013). NCCs and DNCCs differ at the C6 side group of pyrrole ring B, which is a formyl group in NCCs and an oxo group in DNCCs. DNCCs are the major Chl catabolites in *Arabidopsis*, accounting for more than 80 % of all final Chl catabolites (Christ and others 2013). Cytochrome P450 (CYP) CYP89A9 has recently been identified in *Arabidopsis* to be responsible for the oxidative deformylation of FCCs to dioxobilin-type FCCs (DFCCs), extending the wide substrate spectrum of CYPs (Bak and others 2011) to linear tetrapyrroles. *cyp89a9* mutants do not produce DNCCs but accumulate proportionally higher amounts of NCCs. CYP89A9, as most CYP proteins, likely localizes to the ER membrane. Interestingly, deformylation of FCCs by CYP89A9 must precede demethylation by MES16 because demethylated catabolites are not substrates of CYP89A9, suggesting a close interaction between the ER and the chloroplast envelope (Fig. 2).

Hypermodification

Hypermodified FCCs (*hm*FCCs) are *m*FCCs in which the C17 propionic acid chain is conjugated with different groups such as digalactosylglycerol or daucic acid (Moser and others 2008b, 2009; Banala and others 2010; Kräutler and others 2010; Vergeiner and others 2013). *hm*FCCs were shown to be persistent and to accumulate in senescent leaves, because the C17 modifications inhibit their isomerization to respective NCCs (Moser and others 2009; Vergeiner and others 2013). Although it remains to be experimentally proven, *hm*FCCs are, like most *m*FCCs, most probably imported into the vacuole. As a consequence of the accumulation of *hm*FCCs, ripe fruits and senescent leaves of some species, such as banana (*Musa acuminate* or *Musa cavendish*) and *Spathiphyllum wallisii*, fluoresce under UV light. Interestingly, in a yellow banana fruit, more intense fluorescence than in other parts of the peel is observed around necrotic spots that first appear around stomata (Moser and others 2009). Occurrence of these highly fluorescent rings is thought to result from the conversion of *m*FCCs to specific *hm*FCCs just prior to cell death. The mechanism that increases fluorescence surrounding the necrotic spots remains unknown. De novo synthesis of *hm*FCCs from Chl precursors is unlikely. However, it could be due to the degradation of the remaining Chl in the yellow peel of banana, although most Chl has already been broken down at this stage.

Isomerization of FCCs in the Vacuole

*m*FCCs have been described to be converted to their respective NCCs inside the vacuole by nonenzymatic isomerization. This significantly occurs only under acidic conditions such as found in the vacuole (pH 5–6; Figs. 1, 2) (Oberhuber and others 2003). Likewise, DFCCs produced by CYP89A9 in *Arabidopsis* through an oxidative deformylation of FCCs were also shown to be converted to respective DNCCs at pH 5 in vitro (Christ and others 2013). The rate of FCC-to-NCC isomerization increases with decreasing pH and also seems to be influenced by certain modifications of FCC side chains (Moser and others 2009; Christ and others 2012). Thus, *m*FCCs harboring an intact methyl group at $C13^2$ show slower isomerization in vitro when compared to demethylated FCCs (Christ and others 2012). Moreover, $C17^3$ modifications found in *hmFCC*s are thought to inhibit their conversion to *hm*NCCs (Moser and others 2009).

Transport of FCCs within the Cell

Chl degradation starts in the chloroplast and ends in the vacuole (Fig. 2). Therefore, two translocation systems have to transport Chl catabolites across the chloroplast envelope and the tonoplast. A single study on Chl catabolite transport across the plastid envelope showed that the release of FCCs from isolated barley gerontoplasts is enhanced by an external supply of ATP (Matile and others 1992). This observation has led to the hypothesis that transport of FCCs across the chloroplast envelope could be mediated by ATP BINDING CASSETTE (ABC) transporter(s) (Hörtensteiner 2006). Interestingly, suppression of ABCG2, a mammalian ABC-type transporter of the G subfamily, in mice triggers a porphyria-like phenotype (phototoxic ear lesions), which is due to the inability of the animals to detoxify food-derived Pheide and protoporphyrin IX circulating in the blood stream (Jonker and others 2002). The *Arabidopsis* genome encodes 130 ABC transporters, which are localized in membranes of most subcellular compartments (Kang and others 2011a). To date, screening single and multiple T-DNA insertion lines for ABCG2 homologs and other ABC transporters that are predicted to localize to the chloroplast envelope did not provide any evidence for their involvement in FCC export from the chloroplast (Aubry and others unpublished data). Most probably,

chloroplast export of FCCs and probably other linear tetrapyrroles such as phytochromobillin is performed by several transporters. This potential redundancy increases the difficulty of identifying these transporters in vivo.

Compared to transport across the chloroplast envelope, import of FCCs from the cytosol to the vacuole is better understood. Heterologous expression in yeast of *Arabidopsis* *At*ABCC2 and *At*ABCC3, two members of the C subfamily of ABC transporters, revealed them to be capable of importing NCCs in vitro (Lu and others 1998; Tommasini and others 1998). Although these results have been obtained using NCCs rather than FCCs as substrate, this type of transporter is most probably involved in Chl catabolite transport across the tonoplast in vivo. However, as for the export of FCCs from the chloroplast, redundancy could render their reverse genetic identification difficult.

Storage of Tetrapyrrole Catabolites or Further Degradation?

Monopyrrolic catabolites of Chl have been shown to accumulate during leaf senescence in barley and radish (Suzuki and Shioi 1999; Suzuki and others 1999). This finding is consistent with the observation that DNCCs and NCCs accumulating in senescent leaves of barley represent only a minor fraction of the total amount of Chl that has been degraded (Kräutler and others 1991; Losey and Engel 2001; Das and Hörtensteiner, unpublished data). By contrast, in *Arabidopsis*, quantification of DNCCs and NCCs accumulating in yellow leaves revealed that their amount reflects the Chl content of green leaves, indicating that DNCCs and NCCs are not further fragmented (Christ and others 2013). The same observation was made in *Cercidiphyllum japonicum* (Curty and Engel 1996). Together, these studies imply that the fate of NCCs/DNCCs in the vacuole (storage or further fragmentation) differs between plant species. However, it cannot be excluded that the inconsistency between amounts of colorless catabolites and degraded Chl in some species is due to a second and entirely different pathway for Chl breakdown that may be independent of PAO activity.

By-Products of Chlorophyll Breakdown

During leaf senescence, 1 mol of degraded Chl leads to the production of 1 mol of colorless catabolites. Within the chloroplast this leads to the release of magnesium and phytol as by-products (Fig. 1). Magnesium is thought to be reallocated to growing tissues together with other metal ions set free during the degradation of thylakoid components. Two main routes have been described for the fate of phytol. Free phytol can be phosphorylated to phytol diphosphate through the sequential action of two kinases and is subsequently employed for tocopherol synthesis (Ischebeck and others 2006; Valentin and others 2006). Recently, the characterization of the two acyltransferases PHYTYL ESTER SYNTHASE 1 and 2 provided evidence for an alternative fate of free phytol, that is, through fatty acid phytyl ester (FAPE) synthesis in plastoglobules (Lippold and others 2012).

FCC modifications also lead to the formation of by-products. Even though it has not been shown experimentally, Chl catabolite demethylation (through MES16) and oxidative deformylation (through CYP89A9) are thought to produce methanol and formate, respectively (Fig. 1). In *Arabidopsis*, the amount of Chl catabolites produced during leaf senescence is about 1 μmol g^{-1} fresh weight. Under the assumption that 100 % of the Chl catabolites are demethylated and deformylated, Chl breakdown is responsible for the formation of 1 μmol each of methanol and formate per gram fresh weight, amounts that are not negligible. Methanol is known to be produced in leaves by processes such as pectin and lignin degradation and to be metabolized by C1 metabolism via production of formate, or to exit the leaf via the stomata (Fall and Benson 1996; Igamberdiev and others 1999; Gout and others 2000). Although stomata are thought to remain open during the late stages of senescence (Zhang and Gan 2012), it can still be imagined that Chl-derived methanol may partly accumulate within the leaves and have physiological effects. Indeed, in rice, methanol formation during leaf senescence has been connected to an increase in tryptophan biosynthesis that involves the transcription factor WRKY14 (Kang and others 2011b). Tryptophan biosynthesis has been shown to promote serotonin production, which in turn delays leaf senescence (Kang and others 2009). Furthermore, exogenous application of methanol modulates the expression of hundreds of genes involved in multiple detoxification and signaling pathways (Downie and others 2004). Although a regulatory role of methanol during senescence in *Arabidopsis* has not been established, it may be interesting to investigate the contribution of Chl catabolite demethylation to the total production of methanol during leaf senescence. However, the possibility that FCC demethylation may indirectly regulate gene expression is rather unlikely because *mes16* mutants do not show any accelerated or delayed leaf senescence phenotype.

In leaves, formate is known to be the by-product of photorespiration and fermentation pathways, and possibly the product of direct CO_2 reduction in chloroplasts (Igamberdiev and others 1999). As mentioned above, formate could also be formed from methanol generated by pectin and lignin degradation, and potentially also by FCC demethylation. In theory, FCC-to-DFCC conversion by CYP89A9 should also contribute to the formation of formate in senescent leaves. Because formate is less volatile

than methanol, it has to be metabolized within senescing leaves. Two routes for formate utilization have been described in plants (Igamberdiev and others 1999). The first one is mediated by FORMATE DEHYDROGENASE (FDH), which converts formate to CO_2 (Li and others 2000; Olson and others 2000). Although overexpression of FDH in *Arabidopsis* has been shown to increase tolerance to exogenous application of formate, the role of FDH in leaves remains unknown (Li and others 2002). Transcriptome coexpression analysis using ATTED-II (Obayashi and others 2009) reveals that *Arabidopsis* FDH is coexpressed with CCEs, indicating that the enzyme could have a role during leaf senescence. To the best of our knowledge, accumulation of formate in *fdh* mutants has never been reported in the literature. Furthermore, *Arabidopsis fdh* mutants do not show any accumulation of formate during dark-induced leaf senescence (Christ and Hörtensteiner, unpublished data). The nonaccumulation of formate in *fdh* mutants could be the result of a compensation effect of a second route of formate utilization. Indeed, formate can be condensed with tetrahydrofolate (THF) to produce formyl-THF by the action of 10-FORMYL-THF SYNTHETASE (THFS) (Igamberdiev and others 1999). Formyl-THF is then further used for serine and methionine biosynthesis. In *Arabidopsis*, THFS is encoded by a single-copy gene but has not been analyzed thus far.

Significance of Chlorophyll Breakdown

Detoxification and Nutrient Remobilization

After the onset of senescence, leaves undergo complex changes that should be seen as "transdifferentiation" rather than as "deterioration" processes (Thomas and Howarth 2000). Recycling and detoxification of (macro-)molecules are indeed the consequences of structural and biochemical changes occurring during leaf senescence. Disintegration of cellular components is mediated through fine-tuned catabolic as well as anabolic steps that reduce the deleterious effects of the intermediates of degradation and maximize nutrient remobilization (for example, nitrogen, phosphorus, sulfur, minerals, metals ions, and carbon skeletons). Chl breakdown is a direct prerequisite for the remobilization of chloroplast lipids, proteins, and metals. This fact is highlighted by the effect of the suppression of enzymes involved in Chl breakdown, such as PAO or RCCR (see above), which leads to the accumulation of toxic Chl intermediates and, thus, to early cell death phenotypes. Although it has not yet been precisely characterized, these mutants also seem to be affected in their ability to remobilize nutrients and consequently show a low germination rate. In addition, it has been demonstrated in

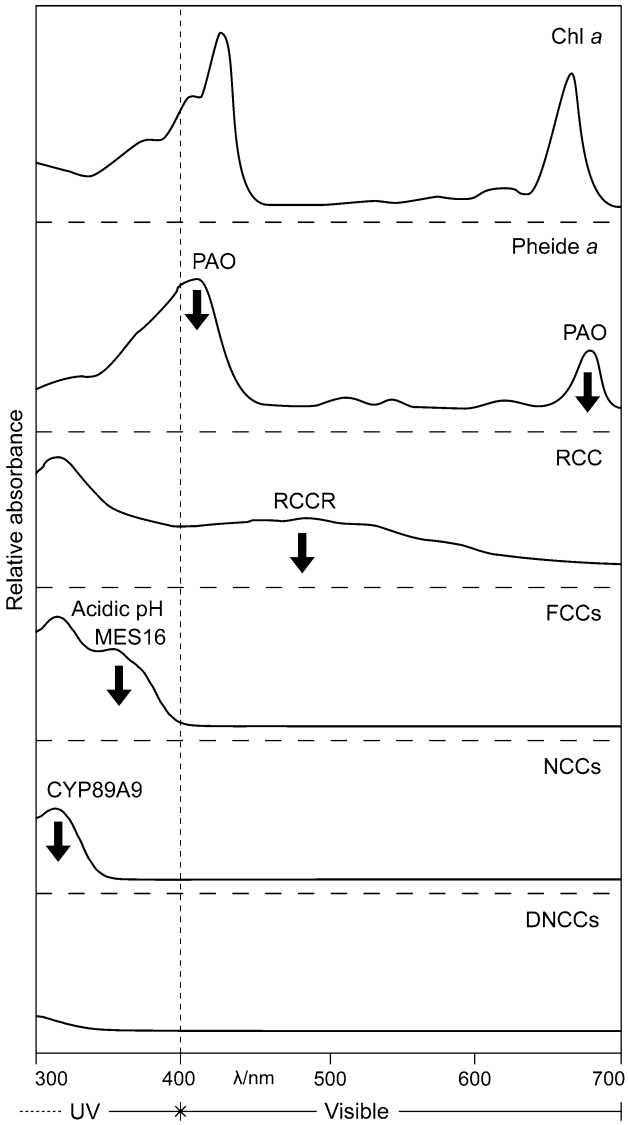

Fig. 4 Schematic representation of the loss of absorption capacity of different Chl catabolites during Chl breakdown. The enzymes with a direct influence on the UV/Vis spectra of the Chl catabolites are shown. The UV/Vis spectra are schematically drawn. Note that relative absorptions cannot be compared between different catabolites

Arabidopsis that the suppression of Chl *b* to *a* conversion, which leads to Chl retention in cotyledons during seed drying, dramatically decreases seed germination capacity (Nakajima and others 2012).

Protection Against Pathogens

Several lines of evidence suggest that (partial) degradation of Chl is involved in the response of plants against pathogens. SGR appears to be necessary for the development of chlorosis upon infection of *Arabidopsis* with *Pseudomonas syringae* pv *tomato* (Mecey and others 2011). Similarly, levels of SGR in silencing or

overexpression lines of *Arabidopsis* correlate with the severity of the hypersensitive response triggered by *P. syringae* infection (Mur and others 2010). Moreover, *At*CLH1 was proposed to be involved in pathogen responses (Kariola and others 2005). Silencing of *At*CLH1 was shown to alter resistance or susceptibility of plants toward two different types of necrotrophic pathogens, *Erwinia carotovora* and *Alternaria brassicicola*. Absence of CLH1, which is thought to degrade Chl in damaged tissue, was connected with ROS production during necrotrophic pathogen attack, which in turn would activate and inactivate salicylic acid-dependent and jasmonic acid-dependent responses, respectively (Kariola and others 2005). However, this model for CLH function during a pathogen attack is based on indirect observations and has to be considered with caution. Together, these studies suggest that Chl breakdown enzymes and catabolites could play an important role during pathogen infection.

Putative Role(s) of FCC Modifications

Retention of FCCs in *mes16* mutants and lack of DNCC formation in *cyp89a9* mutants does not seem to affect plant growth and/or leaf senescence, that is, no phenotype was observed in *mes16*, *cyp89a9*, and *mes16 cyp89a9* mutants during vegetative growth, and Chl *a* and *b* degradation during leaf senescence was indistinguishable from that of wild-type plants (Christ and others 2012, 2013). Thus, the role(s) of FCC modification remain(s) unclear. One hypothesis is that FCC modification could participate in the detoxification of Chl catabolites. The first steps of degradation produce phototoxic catabolites and FCC modifications could lead to a further decrease of the light absorption capacities of the catabolites (Fig. 4). Indeed, demethylation of FCCs increases the rate of isomerization to nonfluorescent catabolites and, thus, facilitates the loss of their 360-nm absorption peak. Furthermore, DFCC formation by CYP89A9 results in the loss of the C5-formyl group of FCCs and, consequently, of the 320-nm absorption peak. One reason for the absence of any phenotype in *mes16* and *cyp89a9* mutants could be that in controlled experimental facilities these mutants are grown under UV-limited conditions. It can be speculated that during senescence under sunlight conditions, plants could be affected if colorless Chl catabolites that absorb light between 300 and 380 nm are retained. This hypothesis could be tested by growing the mutants under natural or artificial UV-B–containing light conditions.

Besides reducing the light absorption capacity of colorless Chl catabolites, modifications of FCC side chains increase their polarity (Fig. 1). This observation corroborates the idea that FCC modification directly contributes to Chl catabolite detoxification. Indeed, sequential hydroxylation and glucosylation steps are known to participate in the detoxification of various molecules such as xenobiotics by increasing their polarity (Pedras and others 2001; Dosnon-Olette and others 2011). Therefore, an increase in the solubility of the catabolites likely facilitates the relocation of FCCs from the chloroplast to the vacuole. Identification of the enzyme(s) responsible for FCC hydroxylation, together with the knowledge on FCC demethylation and oxidative deformylation could help testing if FCC modification indeed has a physiological role. It would be of interest to study leaf senescence in *Arabidopsis* plants that are deficient in all FCC-modifying activities and would thus accumulate only *p*FCC and *p*NCC during Chl breakdown.

Why do FCC modifications occur in a species-specific manner (see Table 1)? One hypothesis is that FCCs could have ecological functions. On one hand, plants such as *Arabidopsis* appear to avoid FCC accumulation. On the other hand, permanent FCC accumulation has been shown to occur in leaves and fruits of banana and other related taxa (Moser and others 2009; Kräutler and others 2010; Hörtensteiner and Kräutler 2011; Vergeiner and others 2013). Furthermore, while humans are not overly sensitive to blue light between 400 and 500 nm, other animals, such as insects, are known to possess blue photoreceptors with a maximal sensitivity around 450 nm (Briscoe and Chittka 2001). It is therefore reasonable to assume that some insects are able to detect the blue fluorescence emitted by FCCs around 450 nm. In the case of plant species such as banana that naturally retain FCCs, these fluorescent catabolites may play a role in beneficial interactions with insects, such as pollination, or they may be a signal of fruit ripening for bigger animals contributing to seed dispersal. Other plant species may benefit from the further conversion of FCCs into NCCs/DNCCs because herbivores might be able to link FCC fluorescence of senescent leaves with reduced plant fitness at this late developmental stage. Thus, by facilitating FCC-to-NCC isomerization, MES16 could, for instance, help avoid attraction of herbivores by the plant during nutrient relocation and seed maturation. *Arabidopsis* mutants that accumulate different relative FCC amounts during senescence (*mes16*, *mes16 cyp89a9*; Christ and others 2012, 2013) could be used in future experiments to investigate whether insects can indeed detect FCC fluorescence.

Future Perspectives

Delaying the onset of leaf senescence has been described as a good strategy for increasing crop productivity (Thomas and Howarth 2000). Indeed, the record yield for corn was obtained with functional stay-green mutants. However,

suppression of Chl degradation leads to only cosmetic stay-green and/or cell-death phenotypes, which do not increase plant fitness but rather accelerate cell death and decrease seed germination. Nonetheless, understanding Chl breakdown during leaf senescence, fruit ripening, and other developmental processes or stress responses is not only important to increasing our fundamental knowledge, it can also improve post-harvest storage. Indeed, loss of the green color due to senescence in vegetables such as broccoli (*Brassica oleracea*) decreases their commercial value. Besides open questions about FCC modifications and transport, several other aspects of Chl breakdown by the PAO pathway need further investigation. For instance, although SGR and CCEs have been shown to interact with LHCII subunits and SGR has been implicated in the destabilization of Chl–apoprotein complexes during senescence, its mechanism of action remains to be defined. Furthermore, it is not known whether the same mechanism is involved in the degradation of Chl from PSI. It cannot be excluded that PSI degradation (partly) differs from that of PSII. Recent studies showing that SGR has other functions besides Chl breakdown could be helpful to better understand the role(s) of this protein. The mechanism involved in Chl demetalation also remains unknown. Whether it involves a MRP, a MCS, and/or simple changes in the pH of the chloroplast stroma still needs to be demonstrated. The in vivo role of CLHs, proteins that are highly active on Chl in vitro, is still unclear. A collective consideration of key studies on CLHs points to their involvement during stress responses against various biotic and abiotic stresses and not during age-dependent leaf senescence.

Chl degradation is a highly controlled process. This is seen in protein–protein interactions during the first steps of the pathway that allow metabolic channeling of phototoxic catabolites (Sakuraba and others 2012b, 2013), and by the high coregulation of Chl catabolic gene expression. However, the mechanism(s) of transcriptional regulation of Chl breakdown remain(s) unknown. Interestingly, overexpression of a single CCE results in an increased rate of degradation in the whole pathway (Sakuraba and others 2012b). Finally, another interesting research area for Chl breakdown is to learn how cell death is triggered by the accumulation of Chl catabolites in mutants such as *acd1* and *acd2*. Interestingly, cell death in either mutant is not mediated through a singlet oxygen signaling pathway involving EXECUTER proteins (Wagner and others 2004) and therefore seems to involve different, unknown mechanisms (Pattanayak and others 2012; S. Hörtensteiner, unpublished data).

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