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## Review

# Chlorophyll breakdown in higher plants

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### ABSTRACT

Chlorophyll breakdown is an important catabolic process of leaf senescence and fruit ripening. Structure elucidation of colorless linear tetrapyrroles as (final) breakdown products of chlorophyll was crucial for the recent delineation of a chlorophyll breakdown pathway which is highly conserved in land plants. Pheophorbide *a* oxygenase is the key enzyme responsible for opening of the chlorin macrocycle of pheophorbide *a* characteristic to all further breakdown products. Degradation of chlorophyll was rationalized by the need of a senescing cell to detoxify the potentially phototoxic pigment, yet recent investigations in leaves and fruits indicate that chlorophyll catabolites could have physiological roles. This review updates structural information of chlorophyll catabolites and the biochemical reactions involved in their formation, and discusses the significance of chlorophyll breakdown. This article is part of a Special Issue entitled: Regulation of Electron Transport in Chloroplasts.

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## 1. Introduction

Chlorophyll (Chl), the most abundant pigment on earth, is a key component of photosynthesis required for the absorption of sunlight. However, because of its light-absorbing properties Chl is a dangerous molecule and a potential cellular phototoxin. This is seen in situations where the photosynthetic apparatus of plants is overexcited, for example in high light conditions. Absorbed energy can then be transferred to oxygen, resulting in the production of reactive oxygen species (ROS). Likewise, inhibition of Chl biosynthesis or degradation can lead to ROS production and cell death. Because of this, metabolism of Chl is highly regulated during plant development. Whereas biochemistry and regulation of Chl biosynthesis are intensively studied, Chl degradation massively occurring during leaf senescence or fruit ripening, but also as a response to many biotic and abiotic stresses, is less well understood. For many years, Chl degradation was considered a biological enigma. Only the identification and structure determination of several key Chl catabolites as natural breakdown products allowed the stepwise elucidation of a Chl degradation pathway which is common to higher plants. The pathway can be divided into two parts: (i) early reactions with colored pigments as substrates, ending with the synthesis of a 'primary' colorless, blue-fluorescing breakdown product, termed *p*FCC, and (ii) late, *p*FCC-modifying reactions, typically ending with the non-enzymatic

isomerization of modified FCCs to their respective NCCs inside the vacuole. Transfer of catabolites from senescent chloroplasts to the vacuole is mediated by primary activated transport processes.

This review summarizes recent advances in the elucidation of chlorophyll catabolites from both leaves and fruits, outlines the biochemical reactions of the pathway including genes and catabolic mutants, and finally discusses chlorophyll breakdown in the context of evolution and stress response.

## 2. Chlorophyll catabolites in leaves and fruits

Breakdown of Chl has been a longstanding enigma and Chls seemed to disappear without leaving a visible trace [1,2]. The resulting gap in our knowledge was largely due to a lack of success in the identification of genuine 'non-green' Chl catabolites [3]. In the last 20 years, the intricate structures of key Chl catabolites were elucidated and have provided an unflinching basis for the molecular delineation of the breakdown path [4]. Following the identification of the nonfluorescent Chl catabolite *Hv*-NCC-1 by its structure determination in 1991 [5], the characterization of Pheide *a* [6] and of an intermediary 'primary' fluorescent Chl catabolite (*p*FCC) were further crucial steps [7]. They laid out a structural groundwork that pointed to a red Chl catabolite (RCC) [7,8] as so far elusive product of an oxidative ring opening enzyme, now known as Pheide *a* oxygenase (PAO) and representing the key element of the so called PAO pathway [9].

### 2.1. Early green catabolites with intact chlorin macrocycle

The structures of all tetrapyrrolic Chl catabolites from higher plants (except for one NCC from senescent leaves of *Arabidopsis*

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(*Arabidopsis thaliana*), see below [10] indicated a direct structural correlation with Chl *a*. However, no analogous catabolite carrying a formyl group, as displayed in Chl *b*, could be identified [11]. The original puzzle of the absence of Chl *b* derived catabolites was solved, when the enzymatic reduction of Chl *b* to Chl *a* was recognized to be a crucial early step in Chl breakdown (reviewed in [12,13]) (see Fig. 1). In senescent leaves a subsequent demetallation of Chl *a* to pheophytin (Phein) *a* now appears to precede the dephytylation to the (still green) catabolite pheophorbide (Pheide) *a* [14]. In fruit, evidence for the reverse order has been provided, according to which dephytylation of Chl *a* by chlorophyllase is first and gives chlorophyllide (Chlide) *a* [15], followed by demetallation by a magnesium dechelatease and a small molecular weight cofactor to give Pheide *a*, as well [16] (see below).

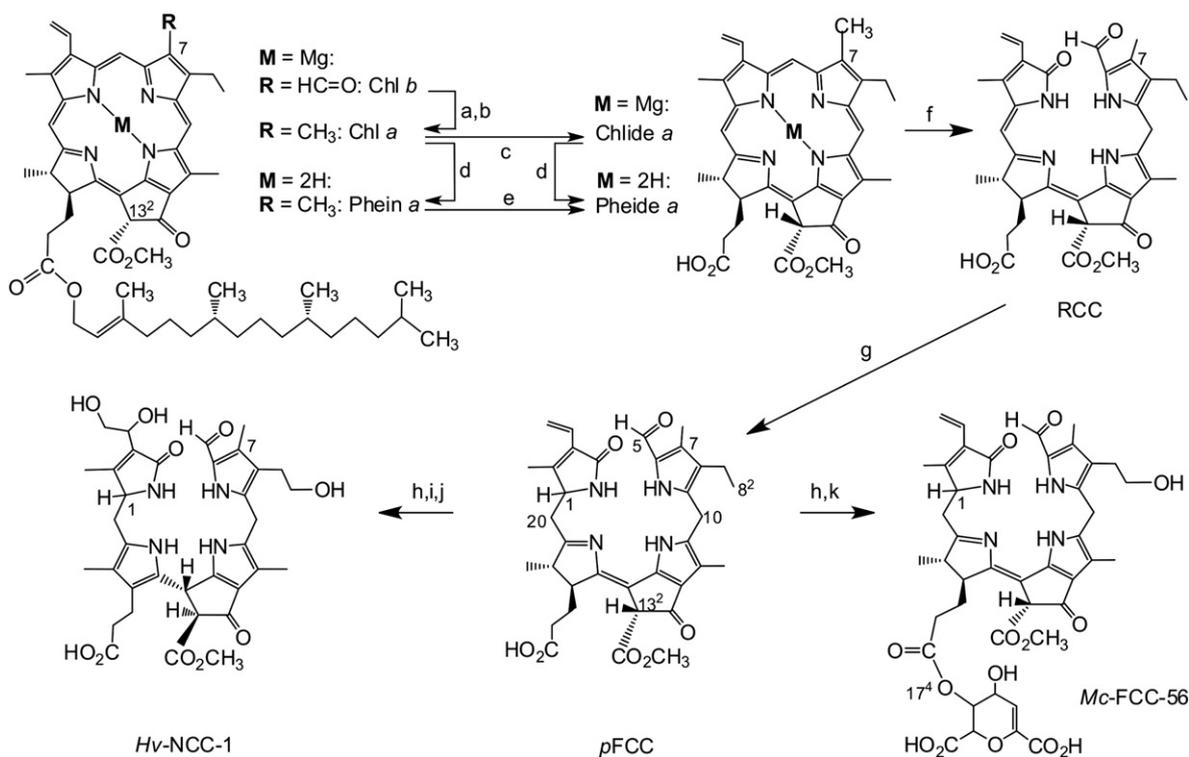
## 2.2. Elusive red linear tetrapyrroles from oxygenolytic opening of the chlorin macrocycle

The futile historic search for colored, non-green Chl catabolites was later rationalized by the efficient breakdown of Chls to colorless catabolites, which then accumulated in senescent plant tissue [17]. Structural identification of a nonpolar fluorescent Chl catabolite (FCC) as 'primary' FCC (*p*FCC) provided a crucial new structural pillar [7] at the interface between Pheide *a*, the established 'last' green catabolite in senescent leaves [6], and the various colorless NCCs [18]. Indeed, a molecule of *p*FCC turned out to contain 2 oxygen atoms and 4 hydrogen atoms more than a molecule of Pheide *a* [7]. Thus, to achieve the formation of *p*FCC from Pheide *a*, more than one enzyme was likely to be involved. *p*FCC was suggested to arise from an enzyme-catalyzed stereo-selective reduction of a then elusive red Chl

catabolite (RCC) (see below) [7]. RCC, in turn, would then be the product of an oxygenase that opened the macrocycle of Pheide *a*. In fact, related tetrapyrrolic compounds were known as Chl breakdown products excreted by the green alga *Auxenochlorella protothecoides* [19]. However, RCC itself only became available by partial synthesis from Pheide *a* via the Cd-complex of the methyl ester of Pheide *a* [8]. Samples of synthetic RCC were then used to trace and to identify this red linear tetrapyrrole as a Chl breakdown intermediate [20]. As presumed, RCC proved to be the direct precursor of *p*FCC by being the substrate of a reductase, now known as RCC reductase (RCCR) [20,21]. The failure to observe RCC in senescent leaves can now be rationalized by a lack of its accumulation due to 'metabolic channeling' resulting from a close interaction of PAO and RCCR [9] (see below). Incorporation of one oxygen atom from the obligate co-substrate, molecular oxygen, at the formyl carbon of RCC was deduced in the (labeled) reduction product, *p*FCC, by mass spectrometric analysis; this indicated PAO to be a monooxygenase [22], similar to the role of the elusive related enzyme in the green alga *A. protothecoides* [23].

## 2.3. Colorless fluorescent chlorophyll catabolites

During very active stages of senescence minute quantities of colorless and blue-fluorescent compounds were observed as presumed breakdown intermediates [18]. One of these fluorescing compounds, provisionally named *Bn*-FCC-2, was isolated from a preparation containing enzymes of senescent leaves of oilseed rape and Pheide *a* as substrate. Spectroscopic analysis of *Bn*-FCC-2 revealed its structure as the *p*FCC [7,17]. Soon after, from a related preparation using an extract of bell pepper (*Capsicum annuum*) *Ca*-FCC-2 was obtained and was characterized as the C-1 epimer of *p*FCC, i.e. as *epi*-



**Fig. 1.** Representative structural outline of major catabolites delineating the main paths of chlorophyll breakdown in higher plants [4,11]: Chls are degraded in the chloroplast by enzyme-catalyzed processes via pheophorbide (Pheide) *a* and the red chlorophyll catabolite (RCC) to give primary fluorescent chlorophyll catabolites (*p*FCC, or its C1-epimer, *epi*-*p*FCC). The relevant enzymes involved in this part are: a), Chl *b* reductase; b), 7-hydroxymethyl Chl reductase; c), chlorophyllase (CLH); d), magnesium dechelatease; e), pheophytinase (PPH); f), Pheide *a* oxygenase (PAO); g), RCC reductase (RCCR). *p*FCCs are modified further by unidentified hydroxylating enzymes (h, i). When carrying a free propionic acid group, FCCs are transported into the vacuole, where they are suggested to isomerize by a spontaneous, acid catalyzed reaction (j) to the corresponding nonfluorescent chlorophyll catabolites (NCCs), such as *Hv*-NCC-1 (the main tetrapyrrolic catabolite found in senescent leaves of barley, *Hordeum vulgare*). Else, they are esterified by unknown enzymes at the propionic acid group (k) to give 'persistent' hypermodified FCCs, such as *Mc*-FCC-56 (the main FCC in peels of ripe bananas, *Musa acuminata*, cavendish cultivar). Relevant atom numbering is specified.

pFCC [24]. This work established the presence of two stereo-divergent lines of RCCRs relevant for the formation of the pFCCs [21]. This insight has become of further interest in the context of questions on the evolution of higher plants (see below) [25]. On the other hand, the stereochemistry at a particular carbon of a degradation product that resulted from a 'detoxification process', as then assumed, appeared of little further relevance [24].

Interestingly, RCCRs do not contain a typical cofactor [21]. The enzymatic reduction of RCC to a pFCC by RCCRs thus posed an intriguing mechanistic question. Investigations of the electrochemical reduction of RCC [26] and of its methyl ester [27] provided first evidence for the ease of reduction of RCC. In addition, they indicated the reduction catalyzed by RCCR to be achievable via a radical pathway with intermediate one electron reduction and protonation steps [27]. Such a pathway was suggested for several of the related bilin reductases [28] as well, and now appears to be established also for RCCR (see below).

In the time since these early experiments pFCC has been identified in senescent leaves of *A. thaliana*, as were two modified At-FCCs, which were (tentatively) characterized as 13<sup>4</sup>-desmethyl-pFCC and 8<sup>2</sup>-hydroxy-13<sup>4</sup>-desmethyl-pFCC (see Table 1) [29].

In all of these FCCs a free propionic acid group would be available. This was recognized as a crucial structural element in FCCs, as it catalyzed the rapid (non-enzymatic) isomerization to the corresponding NCCs [26,30] and implemented the common stereochemistry of NCCs at C-15 with high stereo-selectivity, as was deduced from their circular dichroism spectra [26]. According to these findings, a spontaneous FCC-to-NCC isomerization occurs, that is acid catalyzed and specifically prone to taking place, once FCCs are imported into the vacuole [4,30].

The surprising discovery of the accumulation of 'hypermodified' FCCs (hFCCs) in ripening bananas (*Musa acuminata*, Cavendish cultivar) indicated a new role of Chl catabolites [31]: hFCCs were a group of unprecedented FCC-esters (see Table 1) and their accumulation in the peels of ripening bananas was rationalized by the corresponding deactivation of the natural acid-induced (FCC-to-NCC) isomerization [31], which was shown earlier to depend upon a free propionic acid function [30]. The accumulation of hFCCs was taken to indicate further physiological roles of these Chl catabolites, e.g. in producing a visible optical effect in the ripening fruit [31]. In the meantime, related 'persistent' hFCCs were also found to accumulate in senescent leaves of banana plants [32] and of the peace lily (*Spathiphyllum wallisii*), a tropical evergreen [33]. Interestingly, the

modifications at C-17<sup>3</sup> include unusual moieties, such as daucic acid in Mc-FCC-56 [34] and different C6-linked substituted pyranose units [32,33] (Table 1).

#### 2.4. Colorless nonfluorescent chlorophyll catabolites

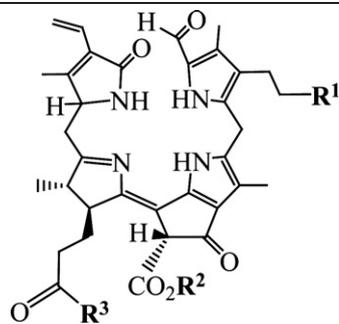
The structural characterization, and identification as a natural Chl breakdown product, of an easily decomposing colorless compound from senescent barley leaves (*Hordeum vulgare*), then called 'rusty pigment 14' (now named *Hv-NCC-1*) [5], provided research in the area of Chl breakdown in higher plants with the long-needed structural basis [18]. The polar NCC *Hv-NCC-1* was indicated by its structure to arise by an oxygenolytic ring opening at the  $\alpha$ -meso-position of the chlorin macrocycle [5,35], rather than at the  $\delta$ -position, as assumed earlier [1], and to have a close relationship with Chl *a* (rather than Chl *b*). *Hv-NCC-1* is thus a linear tetrapyrrole (a formyl-bilanone), which is colorless, as the three remaining meso-positions are saturated. This pioneering study with barley was followed by the identification of related polar NCCs from senescent leaves of oilseed rape (*Brassica napus*) [36,37], the deciduous trees *Liquidambar styraciflua* (sweet gum) [38] and *Cercidiphyllum japonicum* [39], spinach (*Spinacia oleracea*) [40,41], tobacco (*Nicotiana rustica*) [42], Arabidopsis [29], maize (*Zea mays*) [43], and the tropical evergreen *Spathiphyllum wallisii* [33] (see Table 2). This work firmly established the tetrapyrrolic NCCs as the accumulating Chl breakdown products in senescent leaves [4,11,17,18]. With the exception of an NCC from Arabidopsis, called *At-NCC-3* [10], all NCCs identified to date carried a methyl group at the C-7-position [11] a characteristic substituent at this position in Chl *a* [44]. In contrast, *At-NCC-3* displayed a hydroxymethyl group at this position (see general NCC formula in Table 2; in *At-NCC-3*: CH<sub>2</sub>OH at position 7; R<sup>1</sup>, H; R<sup>2</sup>, CH<sub>3</sub>; R<sup>3</sup>, OH), suggesting a route of its formation that would be slightly divergent from that of the other known NCCs [10]. In addition, in all natural NCCs a methoxycarbonyl group was attached at C-13<sup>2</sup>, as is characteristic of the Chls, or a carboxyl function, and a free propionic acid function was present at C-17 [11].

In the context of senescence-related Chl breakdown these findings appear to exclude the relevance e.g. of direct 13<sup>2</sup>-hydroxylation of the Chls or of their green catabolic intermediates (as suggested earlier [45]), or else of an enzymatic ester hydrolysis followed by decarboxylation ('decarboxymethylation') that would provide 13<sup>2</sup>-decarboxymethyl Pheide *a* (or pyropheophorbide *a*) as an early breakdown intermediate [46]. All major NCCs from senescent leaves carry a hydroxyl group at the

**Table 1**

List of modified FCCs from *Arabidopsis thaliana* (*At*), and of 'hypermodified' fluorescent chlorophyll catabolites (hFCCs) that accumulate in peels of ripening banana fruits (*Musa cavendish*, *Mc*), and in senescent leaves of bananas (*Musa acuminata*, *Ma*) and of the peace lily (*Spathiphyllum wallisii*, *Sw*). FCCs are named for their plant source and are listed here with reference to their common general formula and their differing modifications R<sup>1</sup>, R<sup>2</sup> and R<sup>3</sup>.

Compound	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	Ref.
<i>At</i> -FCC-1	OH	H	OH	[29]
<i>At</i> -FCC-2	H	H	OH	[29]
<i>Mc</i> -FCC-46 <sup>a</sup>	O-Glc <sup>b</sup>	CH <sub>3</sub>	DA <sup>c</sup>	[34]
<i>Mc</i> -FCC-49 <sup>a</sup>	O-Glc <sup>b</sup>	CH <sub>3</sub>	DA <sup>c</sup>	[34]
<i>Mc</i> -FCC-53 <sup>a</sup>	OH	CH <sub>3</sub>	DA <sup>c</sup>	[31]
<i>Mc</i> -FCC-56 <sup>a</sup>	OH	CH <sub>3</sub>	DA <sup>c</sup>	[31]
<i>Ma</i> -FCC-61 <sup>a</sup>	OH	CH <sub>3</sub>	DGG <sup>d</sup>	[32]
<i>Sw</i> -FCC-62 <sup>a</sup>	OH	CH <sub>3</sub>	DHP <sup>e</sup>	[33]



<sup>a</sup> hFCCs are indexed according to their retention time during HPLC analysis [31].

<sup>b</sup> Glc,  $\beta$ -glucopyranosyl.

<sup>c</sup> DA, daucic acid.

<sup>d</sup> DGG, digalactosylglyceryl.

<sup>e</sup> DHPG, dihydroxyphenylethyl- $\beta$ -glucopyranosyl.

**Table 2**  
List of nonfluorescent chlorophyll catabolites (NCCs) from higher plants. NCCs are named for their plant source and numbered according to their relative polarity (for details see text). NCCs are listed here with reference to their common general formula and with specification of their differing modifications R<sup>1</sup>, R<sup>2</sup> and R<sup>3</sup>.

Compound <sup>a</sup>	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	References
Hv-NCC-1	OH	CH <sub>3</sub>	CH(OH)-CH <sub>2</sub> OH	[5,35]
Cj-NCC-1/So-NCC-4/Pc-NCC-2/Ms-NCC-2	OH	CH <sub>3</sub>	CH CH <sub>2</sub>	[30,39,41,48]
Cj-NCC-2/So-NCC-5	H	CH <sub>3</sub>	CH CH <sub>2</sub>	[30,41]
Bn-NCC-1	O-Mal <sup>b</sup>	H	CH CH <sub>2</sub>	[36,37]
Bn-NCC-2/At-NCC-1	O-(6'-O-Mal)Glc <sup>b,c</sup>	H	CH CH <sub>2</sub>	[29,37]
Bn-NCC-3/At-NCC-2	OH	H	CH CH <sub>2</sub>	[29,37]
Bn-NCC-4/At-NCC-5	H	H	CH CH <sub>2</sub>	[29]
At-NCC-4	O-Glc <sup>c</sup>	CH <sub>3</sub>	CH CH <sub>2</sub>	[29]
So-NCC-1	OH	H	CH(OH)-CH <sub>2</sub> OH	[41]
So-NCC-2	OH	CH <sub>3</sub>	CH(OH)-CH <sub>2</sub> OH	[41]
So-NCC-3	OH	H	CH CH <sub>2</sub>	[41]
Nr-NCC-1	O-(6'-O-Mal)Glc <sup>b,c</sup>	CH <sub>3</sub>	CH CH <sub>2</sub>	[42]
Nr-NCC-2/Zm-NCC-2/Pc-NCC-1	O-Glc <sup>c</sup>	CH <sub>3</sub>	CH CH <sub>2</sub>	[42,43,48]
Zm-NCC-1	O-Glc <sup>c</sup>	CH <sub>3</sub>	CH(OH)-CH <sub>2</sub> OH	[43]
Sw-NCC-58 <sup>d</sup>	OH	CH <sub>3</sub>	CH CH <sub>2</sub>	[33]

<sup>a</sup> Plant species abbreviations: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Cj, *Cercidiphyllum japonicum*; Hv, *Hordeum vulgare*; Ms, *Malus sylvestris*; Nr, *Nicotiana rustica*; Pc, *Pyrus communis*; So, *Spinacia oleracea*; Sw, *Spathiphyllum wallisii*; Zm, *Zea mays*.

<sup>b</sup> Mal, malonyl.

<sup>c</sup> Glc, β-glucopyranosyl.

<sup>d</sup> This NCC is indexed according to its retention time during HPLC analysis and has been deduced to be the C-1-epimer of Cj-NCC-1 [33].

saturated side chain extending from C-8 [11]. Based on the hypothesis that the NCCs are the direct products of a non-enzymatic isomerization of the correspondingly functionalized FCCs, the functionalizations found in natural NCCs are believed to be introduced at the level of the FCCs [4]. In particular the ubiquitous 8<sup>2</sup>-hydroxyl group of nearly all natural NCCs indicates a C-8<sup>2</sup>-hydroxylation of FCCs by a still elusive oxygenase as a general and early (possibly first) modification step of the

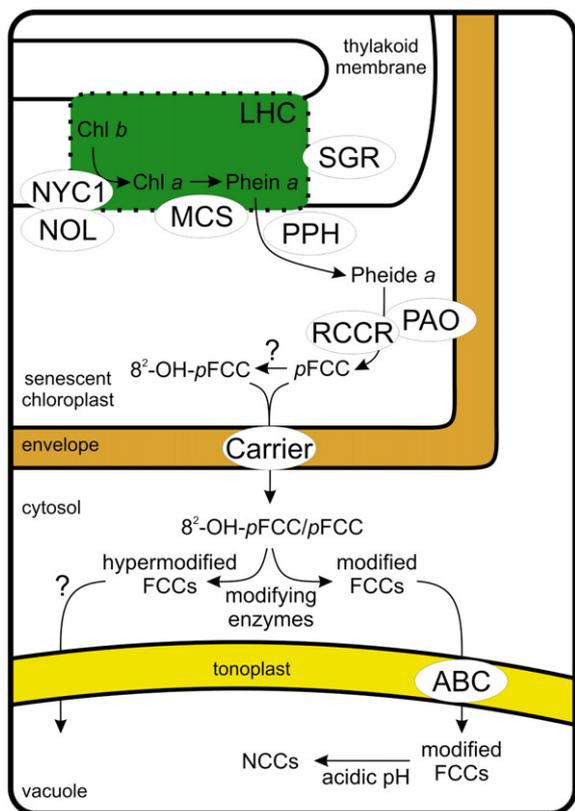
pFCCs [33]. Likewise, hydrolysis of the methoxycarbonyl group at the 13<sup>2</sup>-position of a pFCC or of its 8<sup>2</sup>-hydroxylation product is inferred to be an early functionalization in some higher plants, as deduced from the structures of NCCs in senescent *Arabidopsis* or oilseed rape leaves [4].

Attention has recently turned to the identification of tetrapyrrolic Chl catabolites in fruit [47]. In the peels of ripened apples and pears two NCCs were identified (see Table 2) that were identical to the NCCs found in senescing leaves of the corresponding fruit trees, in apparent support of the existence of a common path of Chl breakdown in ripening fruit and in senescing leaves [48]. However, this view has been weakened by more recent studies of the Chl catabolites in the peels of ripening bananas, where not only a series of NCCs were identified, but unusual 'persistent' FCCs, as well (see above) [31,34]. These investigations on the structures of FCCs and NCCs revealed a split of the path of Chl breakdown in higher plants (Fig. 2): They were only compatible with a basically common early part resulting in the formation of short-lived pFCCs and suggest the further transformations of the pFCCs to natural modified FCCs or natural NCCs to follow two divergent lines of downstream catabolic processing [33].

Natural NCCs were characterized as very active antioxidants [48]. Indeed, their oxidation to colored compounds occurs readily [49] and explains the original naming for the NCCs as 'rusty pigments' [50]. Some of these oxidation products are also found in fresh extracts of senescent leaves, e.g. of *C. japonicum*, in which a yellow Chl catabolite (YCC) was identified [49] as an oxidation product of the corresponding natural NCC.

### 3. Biochemistry of breakdown

The identification and structure elucidation of FCCs and NCCs from many species (Tables 1 and 2) indicated the existence of a basic common Chl breakdown pathway in higher plants (Fig. 1) [11]. Nowadays we call this the PAO pathway, because PAO is responsible for the characteristic macrocycle ring opening reaction seen in the downstream FCCs and NCCs [22]. The analysis of mutants that are affected in Chl breakdown (see below) and cell fractionation studies of senescent leaf tissue [51] allowed the identification of additional Chl catabolic intermediates, such as Pheide *a* and pFCC. This together with the biochemical characterization and isolation of several Chl catabolic enzymes paved the way for their recent identification at the gene level and the analysis of mutants deficient in these genes (see below). The following sections give an update on the biochemical reactions of the PAO pathway.



**Fig. 2.** Topographical model of Chl breakdown in a senescent leaf cell. The model incorporates the current knowledge about Chl catabolites and Chl catabolic reactions and shows their presumed subcellular localizations. Putative steps are labeled with question marks. Note that for NYC1/NOL and PAO/RCCR physical interaction has been demonstrated. The pathway is split at the late reactions, thereby either producing hypermodified FCCs or (ultimately) NCCs. For abbreviations see the text.

### 3.1. Reactions on chlorophylls and chlorin type pigments

#### 3.1.1. Chl *b* to Chl *a* conversion

The intriguing fact that all FCCs and except one all of the NCCs identified so far in higher plants are derived from Chl *a* indicated that reduction of Chl *b* to Chl *a* is a prerequisite for breakdown. This view is supported by senescence studies in barley in the presence of D<sub>2</sub>O [52], which caused specific partial labeling of the C-7 methyl group of *Hv*-NCC-1, indicating it to be derived in part from Chl *b*. Furthermore, PAO specifically cleaves Pheide *a* and is inhibited by Pheide *b* [6]. Thus, *b* to *a* conversion is required to allow degradation via PAO. A cycle of reactions, termed Chl cycle, has been described which interconvert Chl(ide) *a* and *b* via C-7 hydroxymethyl Chl(ide) [12]. The two successive oxygenation reactions from Chl(ide) *a* to Chl(ide) *b* are catalyzed by Chl(ide) *a* oxygenase, a Rieske-type oxygenase. Chl(ide) *a* oxygenase has recently been shown to be regulated at the protein level via a feedback mechanism through Chl *b*, which involves the chloroplast Clp protease system [53]. In contrast, the two steps of Chl(ide) *b* to Chl(ide) *a* conversion are catalyzed by two different enzymes, NADPH-dependent Chl(ide) *b* reductase and ferredoxin-dependent hydroxymethyl Chl(ide) reductase [54]. The involvement of Chl(ide) *b* reductase in Chl breakdown was substantiated by an increase in its activity during senescence [55]. Recently, Chl(ide) *b* reductase could be attributed to two short-chain dehydrogenase/reductase proteins from rice, termed NON-YELLOW COLORING1 (NYC1) and NYC1-LIKE (NOL) [56,57]. *nyc1* and *nol* mutants exhibited a stay-green phenotype and pigment analysis in the mutants demonstrated that particularly Chl *b* degradation was affected during senescence. Although, Chl(ide) *b* reductase activity could so far only be demonstrated for NOL, the mutant phenotypes supported the notion that both proteins catalyze the same reaction. In addition, the phenotypes of *nyc1* and *nol* were not additive as revealed in a *nyc1 nol* double mutant and immunoprecipitation analysis showed the interaction between NYC1 and NOL. Collectively these data suggested that the two proteins act in a heteromeric Chl(ide) *b* reductase enzyme complex [57]. NYC1 and NOL orthologs are also present in Arabidopsis [58], but it remains to be demonstrated whether the Arabidopsis proteins also physically interact. Both Arabidopsis and rice NYC1 contain three transmembrane domains and localize to thylakoid membranes [56] and also rice NOL co-purifies with thylakoid membranes [57]. This is consistent with the detection of Chl(ide) *b* reductase activity in thylakoid membranes of senescent chloroplasts [55] and indicates that Chl *b* to Chl *a* conversion is an early (or the initial) step of Chl breakdown (see below). Recombinant Arabidopsis NOL was able to reduce different potential chlorin substrates, but when isolated trimeric light harvesting complex II (LHCII) was employed as substrate only C-7 hydroxymethyl Chl was formed [58], implying that Chl *b* rather than Chlide *b* is the in vivo substrate for reduction.

#### 3.1.2. Phytol removal

The identification of Pheide *a* as an intermediate of Chl breakdown [6,59] indicated that removal of the central magnesium atom and of the hydrophobic phytol side chain precede the chlorin ring opening reaction by PAO. For many years, phytol hydrolysis catalyzed by chlorophyllase (CLH) was favored to occur before Mg removal, i.e. yielding Chlide as an intermediate [60–62], even though isolated reports had suggested an inverse order of reactions, i.e. Mg-removal occurring before dephytylation with Phein as an intermediate [15,63]. Cloning of CLH genes from different species and their biochemical characterization after heterologous expression [64–69] seemed to further support the involvement of CLHs in the pathway. Yet, cloning of CLH proteins also revealed surprises. Thus, the cloned CLHs were soluble proteins although the activity had been indicated to be

chloroplast-membrane localized [70]. In addition, not all deduced proteins of CLH genes were predicted to localize within the chloroplast [71]. Although CLHs preferentially hydrolyze Chl *a* other chlorins such as Chl *b* and Pheins are accepted as substrates as well. Recombinant wheat CLH accepted even lipophilic esters, which are unrelated to Chl. Collectively, these data challenged the view of CLHs being active during leaf senescence in vivo and two recent studies addressed this in Arabidopsis. Arabidopsis contains two CLHs, CLH1 and CLH2, but absence of either or both proteins in respective mutants as well as RNAi-silencing of CLH1 had only a marginal effect on leaf senescence. Hence, it was concluded that CLHs are dispensable for Chl breakdown during senescence [72,73]. By contrast, several investigations in different species support a function for CLHs. For example, antisense suppression of CLH1 of broccoli (*Brassica oleracea*) in transgenic plants delayed the rate of postharvest Chl breakdown [74]. Furthermore, Citrus CLH was convincingly shown to be active in Chl breakdown during Citrus fruit ripening, and, after heterologous over-expression in squash leaves and tobacco cells, to also promote Chl breakdown in these leaf tissues [75,76].

A functional genomics screen in Arabidopsis for alternative phytol-cleaving esterases uncovered pheophytinase (PPH), a 50 kDa protein located in the chloroplast stroma [14]. Recombinant Arabidopsis PPH exhibited phytol-cleavage activity, which was specific for Phein. Thereby, both Phein *a* and Phein *b* were accepted as substrates, but the enzyme did not dephytylate Chl. This intriguing specificity paralleled the fact that Arabidopsis PPH mutants accumulated significant amounts of Phein during senescence, pointing to an in vivo relevance of this enzyme. In an independent study [77], the same gene, termed *CRN1* (*Co-regulated with NYE1*), was identified by a reverse genetics approach because of its high co-regulation with NONYELLOWING1 (*NYE1*), a protein supposedly involved in regulating Chl breakdown (see below). *NON-YELLOW COLORING3* (*NYC3*), a rice (*Oryza sativa*) gene identified in a mutagenesis screen for retention of greenness during senescence, was shown to be defective in the closest homolog of PPH/*CRN1* in rice [78]. The biochemical role of the identified proteins has not been addressed in the latter two studies.

Both Arabidopsis PPH/*CRN1* and rice *NYC3* are serine-type hydrolases that contain a novel consensus domain which is similar to, but distinct from known lipase motifs. A mutation of the active site serine residue of PPH to alanine prevented complementation of the stay-green phenotype of the *pph-1* mutant, thereby confirming the in vivo hydrolytic activity of PPH [14].

In summary, PPH/*CRN1*/*NYC3* represents an alternative dephytylating activity. The stay-green phenotype of respective mutants points to an in vivo involvement of these hydrolases in Chl breakdown. It can be concluded that at least in rice and Arabidopsis leaf senescence, dechelation precedes dephytylation, and CLHs are probably not active. Yet for other systems, in particular fruit ripening (see below), involvement of CLHs cannot be ruled out.

#### 3.1.3. Magnesium dechelation

Two types of activities able to catalyze the release of the central Mg atom of Chl have been described in the literature. Different heat stable low-molecular weight compounds, termed metal chelating substance (MCS), isolated from *Chenopodium album* (<400 Da) [79] and strawberry (2180 Da) [80] have been shown to catalyze Mg dechelation in vitro. On the other hand, Mg-releasing proteins (MRP) have been described as well [16,81]. Differences between these two activities were shown to reside in their substrate specificity [16,82]. Thus, Mg-dechelatase was active with an artificial Chl substrate, chlorophyllin, but not with the natural substrate, Chlide. In contrast MCS acted on both, indicating that in vivo Mg removal from Chlide most likely is due to the activity of different low molecular weight compounds. The possibility of MCS representing a cofactor of MRP that might easily dissociate from the protein, has been ruled out [83]. Yet, elucidating the molecular nature of MCS is a prerequisite to

understand the mechanism of Mg-removal during Chl breakdown. In addition, based on the identification of PPH and the likely possibility that Mg is released from Chl not Chlide, re-examination of the activities of MCS and MRP using Chl as substrate is demanded.

### 3.2. Oxidative ring opening as key reaction

#### 3.2.1. Pheophorbide *a* oxygenase

Pheide *a*, the last chlorin-type pigment in Chl breakdown, is oxygenolytically opened by PAO to yield enzyme-bound RCC. PAO is a monooxygenase, which regioselectively attaches an oxygen atom at the C-5 position of Pheide *a* [22]. PAO is a Rieske-type iron-sulfur oxygenase [29,59] and in *Arabidopsis* belongs to a five-membered family of Rieske oxygenases [84]. Like other Rieske-type oxygenases, PAO receives electrons from reduced ferredoxin. PAO contains two C-terminally-located transmembrane domains, which were considered to anchor it to the chloroplast envelope [85]. PAO exhibits an intriguing specificity for Pheide *a*, and Pheide *b* inhibits the enzyme in a competitive manner [6].

#### 3.2.2. Red chlorophyll catabolite reductase

Attempts to biochemically characterize PAO activity in isolated chloroplast membranes were unsuccessful. Only after the addition of stromal proteins, i.e. a second enzyme fraction, PAO activity could be determined through the production of what is now known as pFCC [6]. The active component of the stroma was identified as RCC reductase (RCCR) and RCC was shown to be an intermediate of the reaction that was not released [20]. Subsequently, physical interaction of PAO and RCCR was demonstrated in a two hybrid screen, indicating that pFCC formation from Pheide *a* proceeds via metabolic channeling of the intermediary RCC [86]. RCCR is distantly related to a family of ferredoxin-dependent bilin reductases. These include different bilin reductases from algae and cyanobacteria required for phycobilin synthesis, such as phycocyanobilin:ferredoxin oxidoreductase (PcyA) and phycoerythrobilin synthase (PebS), and phytochromobilin synthase (HY2) of higher plants, catalyzing the final step in phytochrome chromophore synthesis. Like these reductases, RCCR requires ferredoxin as reductant and a radical mechanism as proposed for PcyA and HY2 [87,88] is likely to also occur in RCCR [27]. The recent elucidation of the crystal structure of *Arabidopsis* RCCR at 2.4 Å [89] uncovered a high degree of similarity to the structure of PcyA [90] and PebS [91], confirming the catalytic and mechanistic similarity of these reductases. RCCR regio- and stereoselectively reduces the C-20/C-1 double bond of RCC, yielding two possible C-1-stereoisomers, pFCC and *epi*-pFCC [24]. The source of RCCR defines which isomer is formed and it was shown that mutation of phenylalanine<sub>218</sub> to valine in *Arabidopsis* RCCR changed the stereospecificity of the pFCC-producing *Arabidopsis* RCCR to *epi*-pFCC production [86]. Interestingly, phenylalanine<sub>218</sub> locates within the putative substrate binding pocket in the crystal structure of RCCR [89].

### 3.3. Later transformations of tetrapyrrolic catabolites

The diversity of FCCs and NCCs identified from senescing leaves and ripening fruits of different species demonstrates that after the (common) formation of pFCC in the early pathway described above, additional reactions occur. Overall four peripheral positions are functionalized, i.e. C-3 vinyl, C-8 ethyl, C-13<sup>2</sup> carboxymethyl and C-17 propionyl (Table 2). In addition, FCCs with a free C-17 propionyl group finally isomerize to their respective NCCs. Some of the reactions, i.e. C-8<sup>2</sup> hydroxylation and FCC-to-NCC isomerization are commonly found in all species, whereas others occur species-specifically. The mechanisms involved in these reactions are largely unknown, and only the following reactions have been investigated in recent years.

#### 3.3.1. Demethylation at C-13<sup>2</sup>

An enzyme able to catalyze the hydrolysis of the C-13<sup>2</sup> carboxymethylester of Pheide has been identified in Chenopodiaceae and Brassicaceae species and was termed pheophorbidease (PPD) [46]. In vitro, the product of this reaction, C-13<sup>2</sup>-carboxyl pyropheophorbide, spontaneously decarboxylated to pyropheophorbide. Pyropheophorbide has been identified as a product of Chl breakdown mainly in algae [92], but also during leaf and postharvest senescence [93–95]. The distribution of PPD activities seems consistent with the presence of NCCs containing a free carboxyl group at C-13<sup>2</sup> in different plant species [96]. Yet, it remains arguable whether their occurrence can be attributed to PPD activity, because the presumed localization of PPD in the cytosol [97] does not match the occurrence and further metabolism of its substrate, Pheide *a*, inside the plastid [9] (see Fig. 2). Recently, PPD was purified from radish (*Raphanus sativus*) and the gene cloned [97]. PPD encodes a serine-type esterase and is highly homologous to members of the *Arabidopsis* methyl esterase (AtMES) family. Some AtMES proteins have been shown to catalyze methyl ester hydrolysis of different plant hormones. Thereby, the closest homolog of PPD in *Arabidopsis*, AtMES16, hydrolyzed methyl esters of indole acetic acid and jasmonic acid [98].

#### 3.3.2. Malonylation reactions at C-8

Malonyltransferase reactions have been described, which are able to transfer a malonyl moiety from malonyl-coenzyme A to NCCs, possessing either a free hydroxyl group at C-8<sup>2</sup>, such as oilseed rape *Bn*-NCC-3 [99] or a C-8<sup>2</sup>-glucose unit as tobacco *Nr*-NCC-2 [42] (Table 2). The activity isolated from oilseed rape was shown to be specific for Chl catabolites, yet, FCCs rather than NCCs are the in vivo substrates for the reaction, because of the presumed cytosolic localization of the malonyltransferase activity [99]. The molecular nature of the activity remains unknown.

#### 3.3.3. FCC-to-NCC isomerization

In most plant species analyzed so far, NCCs are the predominating final catabolites and FCCs are generally low in abundance [29,37]. This is explained by an import of (functionalized) FCCs into the vacuole (see below) followed by an acid-catalyzed isomerization [26,30] (see above). Conjugation of the C-17 side chain with different moieties (see Table 1) seems to be responsible for the persistence of hypermodified FCCs in banana (see above), but enzyme activities involved in the formation of these C-17<sup>3</sup> esters are not yet known.

#### 3.3.4. Are NCCs further degraded?

Although in some cases, like cotyledons of oilseed rape or leaves of the deciduous tree *C. japonicum*, amounts of NCCs account for almost all metabolized Chl, several lines of evidence indicate that NCCs could be further degraded in senescent leaves. Thus, a urobilinogenoid derivative of *Hv*-NCC-1 [100] and monopyrrolic fragmentation products of Chl were identified in senescent barley leaves [101]. Furthermore, a yellow-colored Chl catabolite (YCC) was isolated from freshly harvested senescent *C. japonicum* leaves [49]. It remains to be shown, whether these catabolites are the products of unspecific oxidation events after tissue disintegration or are produced by specific enzymes as part of a defined pathway.

### 3.4. Subcellular localization of chlorophyll breakdown and chlorophyll catabolite transport

#### 3.4.1. Localization of catabolic enzymes

Breakdown of Chl starts in senescing chloroplasts and ends with the deposition of NCC-type catabolites inside the vacuole. In contrast, the final localization of hypermodified FCCs is as yet unclear. Most enzymes involved in Chl-to-pFCC conversion have been localized to chloroplasts using fluorescent protein fusion analysis [14,21,56,102] and several enzymes were identified in chloroplast proteomics studies [103].

Furthermore, isolated senescent chloroplasts were shown to be competent in pFCC formation and release [51]. In contrast, pFCC modifying activities — as far as they are known — seem to reside in the cytosol [97,99]. The subcellular localization of the C-8<sup>2</sup>-hydroxylating activity is unknown, but may be in the chloroplast [104]. These data imply that all reactions required to produce the first colorless breakdown product, pFCC, reside in plastids, whereas further metabolism occurs outside the plastid. The proposed localization of different CLHs outside the chloroplast challenged this view in the past and additional, extraplasmidial, Chl-catabolizing pathways have been proposed [71]. This idea was supported by the identification of different types of chloroplast-derived vesicles, which seem to participate in the catabolism of chloroplast components, and by the demonstration that autophagic processes play a role in chloroplast degradation during senescence [105–108]. Whether such extraplasmidial degradation processes also involve Chl breakdown is unclear to date. The identification of PPH as the (main) dephytylating activity in senescing leaves together with the phenotypes of Chl catabolic mutants (see below) suggests that the bulk of Chl is degraded inside intact plastids. Extraplastidial Chl breakdown may — if occurring at all — only provide a minor contribution.

Sub-chloroplast localization of Chl catabolic enzymes has been inferred from different experimental approaches, but the results are partially conflicting. Thus, proteomics studies for example indicate localization of NOL in the chloroplast envelope [103], but NCY1, which physically interacts with NOL [57], resides in the thylakoid membrane. In addition, Chl *b* reductase activity has been attributed to thylakoids [55]. Likewise, RCCR was shown to localize to the stroma of chloroplasts [103,109,110], yet RCCR and PAO physically interact during catalysis [20,86], implying that RCCR could rather be located at the envelope, the proposed site of PAO [59,103]. Finally, PPH was localized to the stroma as well [14], but inferred from its activity on Phein, which likely is still attached to Chl-binding proteins, localization to thylakoid membranes could be expected. It is worth mentioning here that NOL was recently shown to catalyze Chl *b* reduction with isolated trimeric LHCII used as substrate. Upon this conversion, pigments were released from the apoprotein, indicating that all downstream steps of Chl breakdown, including dechelation and dephytylation could occur outside the thylakoid membrane [58]. Further work is required to fully elucidate the spatial arrangement of the Chl catabolic machinery.

#### 3.4.2. Chlorophyll catabolite transport

On their way from the plastid to the vacuole, catabolites need to pass the chloroplast envelope and the tonoplast. In both cases, active transport processes have been demonstrated to be involved and FCCs are believed to be the transported substrates [51,111], but the identity of respective transporters is unclear. Transport across the tonoplast is catalyzed by a primary active mechanism, and members of the ABCG subfamily of ABC (ATP-binding cassette) transporters were implicated in transport [112,113]. Yet to date proof for in vivo participation of these transporters is largely missing [114]. Likewise, the molecular nature of transporters at the chloroplast envelope is unknown.

#### 3.5. Chlorophyll breakdown during fruit ripening

Besides leaf senescence, fruit ripening is a further developmental process in many plant species that involves massive degradation of Chl. During the transition of chloroplasts to chromoplasts thylakoid membranes disappear like in leaf chloroplast senescence. PAO and RCCR were found to be active in chromoplast membranes isolated from tomato and bell pepper fruits [115,116], indicating operation of the PAO pathway in fruit ripening. The recent identification of NCCs as Chl catabolites in ripening apples and pears appeared to confirm this view [48]. However, the complex picture of FCCs and NCCs found in ripening bananas indicated a modification of the pathway at steps downstream of pFCC [31,34,47]. Analysis of several non-ripening mutants from different species, indicates that the

same gene, SGR, is affected, which is also required for initiating Chl breakdown during leaf senescence (see below). Identification of PPH as the major dephytylating enzyme during leaf senescence [14] points to possible differences in the early steps of the pathway, because contrary to leaves CLH was shown to be involved in Chl breakdown during ethylene-induced *Citrus* fruit ripening. Thus, by in situ immunofluorescence *Citrus* CLH was localized to the plastid and occurrence of CLH negatively correlated with Chl levels [75,76]. It remains to be shown whether PPH also might have a role during fruit ripening.

### 4. Chlorophyll catabolic mutants

#### 4.1. Stay-green mutants

##### 4.1.1. The stay-green protein

Analysis of stay-green mutants was crucial for elucidating the PAO pathway of Chl breakdown. Thus, the first breakdown products of chl, i.e. rust-colored oxidation products of NCCs, were identified because these compounds did not accumulate in a non-senescent, monogenic mutant, Bf993, of meadow fescue (*Festuca pratensis*) [117]. Despite the retention of permanent greenness, Bf993 senesced like wild type plants. Thus, loss of Chl and photosynthetic capacity, occurring simultaneously in wild type, was uncoupled in the mutant. Such mutants, classified as non-functional (or type C) stay-green mutants, are distinguished from functional mutants, in which senescence initiation (type A) or senescence progression (type B) are delayed [118]. The gene defective in Bf993 was recently identified [119,120] and orthologous genes were shown to be affected in a wide range of similar stay-green mutants of different species [121,122]. These include Gregor Mendel's *I* mutant of pea, *Arabidopsis nonyellowing1*, different alleles of rice *stay-green* and fruit ripening mutants of tomato (*green flesh*) and bell pepper (*chlorophyll retainer*).

STAY-GREEN (SGR) proteins of different species are highly homologous, but they do not contain any known domain that would indicate a possible function. SGRs are targeted to the chloroplast [102,123,124] and rice SGR was shown to interact with LHCII but not with LHCI [102]. This is in agreement with a particular high retention of LHCII subunits in SGR mutants of different species [125,126]. It was assumed that the function of SGR could be in destabilizing Chl-protein complexes as a prerequisite for the subsequent degradation of both apoprotein and Chl [102,121]. Noteworthy, Bf993 was shown to accumulate N-terminally truncated fragments of LHCII subunits, indicating that in the absence of Chl degrading activity, only the stroma-facing N-terminal part is accessible to proteolytic digest, whereas the membrane-embedded core part is resistant to degradation [118]. Interestingly, LHCII-binding to SGR also occurred in a valine<sub>99</sub> point mutation of rice SGR, which causes the stay-green phenotype, suggesting that this mutation might affect an (unknown) enzyme activity of SGR or affect binding of other factors required for Chl-apoprotein degradation [102].

##### 4.1.2. Defects in other genes causing a stay-green phenotype

Besides SGR-defective mutants, stay-green phenotypes have been described in *Arabidopsis* and rice mutants affected in three further genes of Chl breakdown, i.e. *NCY1*, *NOL* and *PPH/NYC3/CRN1* [14,56–58,77,78]. Like SGR mutants, they belong to the type C category and in all these mutants a particular high retention of LHCII subunits is evident as well. Thus, several activities acting on Chl seem to be required simultaneously to allow efficient degradation of Chl-binding proteins during senescence, pointing to the likelihood that Chl degradation is a prerequisite for LHC protein degradation. It is tempting to speculate that SGR might recruit different degradation enzymes to LHC complexes within the thylakoid membrane, possibly in the form of a high molecular weight complex. This would allow

concerted action on chl, thereby enabling control over the release of potentially phototoxic breakdown intermediates. Yet, except a physical interaction shown between NCY1 and NOL [57] and PAO and RCCR [86] formation of such Chl-degradation complexes has not been demonstrated.

#### 4.2. Cell death mutants

In contrast to stay-green mutants, deficiency of PAO or RCCR results in the formation of so-called lesion mimic phenotypes. Lesion mimic mutants were assumed to have specific defects in cell death signaling and/or defense pathways [127]. Similar to some cases of mutants defective in Chl biosynthetic steps, such as *les22* and *lin2* [128,129], cell death in PAO and RCCR mutants is triggered by the accumulation of excessive amounts of the Chl catabolic intermediates Pheide *a* and RCCs, respectively [29,59,86,130]. Cell death execution required light indicating that the accumulating tetrapyrroles act as photo-sensitizers causing the production of reactive oxygen species (ROS) and ultimately cell death [86,131,132]. In contrast, recent analysis of a PAO antisense line in Arabidopsis indicated light-independence [133]. Thus, it remains to be shown whether cell death in PAO mutants occurs through direct toxicity of the accumulating catabolic intermediates and/or as a consequence of cell death signaling. In case of the protochlorophyllide *a*-accumulating Arabidopsis *flu* mutant [134], cell death was proven to be triggered by singlet oxygen-mediated retrograde signaling involving EXECUTER1 and EXECUTER2 activity, rather than through direct phototoxicity of protochlorophyllide *a* [135,136]. In this respect it is interesting to note that in PAO mutants the level of SGR gene expression is reduced [102] indicating the activity of a retrograde signaling pathway from the plastid to the nucleus, whose function might be to limit further Chl breakdown if the pathway is impaired. Possible components of such a signaling pathway are unknown. Furthermore, in the case of *acd2*, which is deficient in RCCR, cell death was shown to occur, independent of Chl breakdown, as a function of mitochondria where RCCR partially localizes upon *Pseudomonas syringae* infection [137]. This indicated that RCCR in addition to its role in Chl breakdown [86] could have a function in cell death signaling involving mitochondria.

A likely difference between stay-green and cell death phenotypes in Chl breakdown mutants is the site of accumulation of catabolic intermediates. Thus, RCCs found in *acd2* are soluble and accumulate in the vacuole [86]. Likewise, Pheide *a* in PAO mutants most likely accumulates outside the thylakoid membranes [29], because PAO has been shown to localize to the chloroplast envelope [103]. This infers the requirement of a catabolite (Pheide *a*?) shuttle system from thylakoid membranes to the envelope. Members of the family of water soluble Chl proteins had been proposed to be involved [138], but this has experimentally not been confirmed. In contrast to lesion mimic mutants, Chl pigments remain attached to their apoproteins in stay-green mutants [14,58,102]. This might shield their photo-sensitizing properties even during the senescence-related decline of photosynthetic activity in the mutants, but the mechanism of Chl energy dissipation under these conditions remains to be elucidated.

### 5. Significance of chlorophyll breakdown

#### 5.1. Nitrogen remobilization

Nutrient mobilization is an intrinsic feature of leaf senescence. Nitrogen in particular is efficiently remobilized to seeds and/or storage organs (mainly in the form of amino acids), because nitrogen is often a limiting factor for plant growth and substantial energy input is required for de novo incorporation of inorganic nitrogen into organic nitrogen-containing compounds [139]. The photosynthetic

apparatus is the major source of nitrogen and degradation of soluble as well as membrane-bound photosynthetic protein constituents is a hallmark of leaf senescence. It had been argued for a long time, that Chl breakdown also aims in recycling of pyrrole-bound nitrogen during senescence [1]. Yet this view seems wrong given the fact that in many plant species NCCs were shown to represent the final products of Chl breakdown [4]. Nevertheless, the requirement for a pathway degrading Chl to colorless products is rationalized by the need to detoxify Chl to sustain viability of the senescing cell in order to allow for the degradation of Chl-binding proteins [9,132], which account for some 20% of total nitrogen in mesophyll cells [140]. This is supported by the high retention of LHC subunits in mutants that are deficient in different Chl catabolic enzymes [14,56,102,125,126], i.e. apoprotein degradation requires the simultaneous degradation of Chl. The proteases responsible for degradation of LHC subunits during senescence are largely unknown, but members of the families of Lon, Clp and FtsH proteases have been implicated [141,142].

#### 5.2. Evolutionary aspects of chlorophyll breakdown

With the exception of the green alga *A. protothecoides*, which upon nitrogen-starvation was shown to excrete RCC-like pigments into the medium [19], Chl breakdown has been characterized more or less exclusively in Angiosperms. Cloning of Chl catabolic genes from higher plants allowed searching for the presence of homologs/orthologs in the genomes of both higher and lower plant species as well as in cyanobacteria. All genes identified so far, i.e. *NYC1*, *NOL*, *SGR*, *PPH*, *CLH*, *PAO* and *RCCR* are widely distributed in Embryophytes, and it is commonly accepted that the PAO pathway of Chl breakdown is highly conserved [9]. This is supported by the occurrence of NCCs in a wide variety of higher plant species [4].

The situation is more unclear in the case of algae and cyanobacteria [143]. The RCC-like linear tetrapyrroles identified in *Auxenochlorella* were shown to be formed by a monooxygenase type reaction mechanism [23] identical to the PAO reaction of higher plants [22], indicating that a structurally similar enzyme could be responsible for the respective reaction in algae. Indeed, the genome of *Chlamydomonas reinhardtii* encodes at least three proteins with high homology to PAO and also *Synechocystis* sp. PCC 6803 (*slr1747*) and other cyanobacteria contain PAO-like genes [84,143]. In contrast, RCCR homologs are absent from eukaryotic algal genomes, but are present in cyanobacteria, with the closest homolog being *all5024* from *Nostoc* sp. PCC 7120 [86]. The relation of RCCR to phycobillin-forming reductases [144] may indicate that it is the evolutionary precursor of the latter. Whereas PPH homologs are found in all eukaryotic photosynthetic species, they are absent from cyanobacteria [14]. It remains to be shown if algal and bacterial homologs of Chl catabolic genes indeed encode catalytically active Chl breakdown enzymes.

#### 5.3. Chlorophyll breakdown and its relevance to stress response

Many biotic and abiotic stresses cause leaf chlorosis, which results from a loss of Chl. In agreement with this is the observation from a large collection of microarray studies [145] that expression of many Chl catabolic genes is regulated in response to different biotic and abiotic stresses. Thus, e.g. *PAO*, *SGR* and *PPH* expression is highly up-regulated under drought and osmotic stress, and in response to challenge by several pathogens, including *P. syringae*. Furthermore, *PAO* expression is wound-inducible [146]. The interaction between *P. syringae* and Arabidopsis is a model for plant pathogenesis and a relation between Chl breakdown and pathogenesis has been established through different observations. Thus, the lesion mimic mutants *acd1* and *acd2* affected in PAO and RCCR, respectively, were originally identified because of their accelerated cell death (*acd*) phenotype in response to *P. syringae* infection [147,148]. In addition,

Chl breakdown, i.e. yellowing or bleaching, occurs as part of cell death both during a compatible interaction, i.e. causing disease, and during an incompatible interaction, i.e. resulting in localized programmed cell death and disease resistance (hypersensitive response; HR). Chl bleaching as a symptom of pathogen infection has been exploited for the development of a chemical screen in *Arabidopsis* for resistance to *P. syringae* [149]. Furthermore, execution of the HR in the *P. syringae*-*Arabidopsis* system has been shown to be linked to light. Thus, plant defense capabilities following an incompatible *P. syringae* infection are increased when grown in light compared to dark, but local resistance occurred independent of known photoreceptors and was clock-independent [150]. Among other mechanisms, imbalances in Chl metabolism were considered in triggering pathogen defense through ROS signaling. In this scenario, ROS are produced by light excitation of photosensitive Chl intermediates, like shown in several cases of lesion mimic mutants [59,129,131,134]. Indeed, increased and decreased expression, respectively, of *Arabidopsis* SGR accelerated and suppressed HR-related cell death, and Pheide *a* accumulation was shown to play a crucial role in ROS-mediated establishment of the HR [151]. This indicates that the light-dependency of the HR in *Arabidopsis* elicited by some strains of *P. syringae* is at least partially associated with Chl breakdown. Yet, whether Chl catabolites are directly involved in the execution of cell death after pathogen attack and/or in the establishment of plant pathogen defenses remains to be shown. Surprisingly, RCCR/ACD2-overexpressing lines showed neither detectable reduction of HR, nor increased resistance to a virulent *P. syringae* strain [131]. Light-dependence for the establishment of an HR has been demonstrated also in other instances of plant–pathogen and plant–herbivore interactions [152], indicating that targeting Chl breakdown could be a common plant defense strategy against pathogens. *Arabidopsis* CLH1, which, besides other stimuli, is induced upon infection with different necrotrophic pathogens, was considered to prevent Chl-derived ROS accumulation in damaged tissue [153]. Modulation of *CLH1* expression demonstrated a link of Chl breakdown to salicylic acid- and jasmonic acid-dependent resistance pathways through the control of ROS signaling, but the exact function of CLH1 in this process, together with the possible involvement of respective Chl catabolites remains unknown.

## 6. Conclusion and outlook

Compared with our ignorance as to the fate of Chl some 20 years ago, the to-date knowledge on this important catabolic pathway has advanced in different aspects. First of all, a variety of FCC- and NCC-type catabolites of Chl accumulating in senescent leaves and ripening fruits have been structurally elucidated. In addition, the enzymes producing the main intermediary catabolites are largely known and genes encoding several of the Chl catabolic enzymes have been cloned. A simple chemical reaction accounts for the formation of the ‘final’ colorless catabolites. The ubiquitous occurrence of FCCs and NCCs and the fact that the PAO pathway of Chl breakdown is evolutionary highly conserved in higher plants, indicate that other activities suggested to be active in Chl breakdown, such as peroxidative or photooxidative activities [2], are irrelevant *in vivo*. Such activities may become relevant under non-physiological conditions, such as after tissue death or during post-harvest [9]. Interestingly, in rare cases have di- and tetrapyrrolic Chl degradation products been identified that are not derived from PAO activity, because they harbor an intact  $\alpha$ -methine bridge [154,155]. The mechanisms that account for their occurrence remain elusive. Originally the PAO pathway exhibited one straight series of reactions leading to the (final) deposition of NCCs inside the vacuole and the pathways function was limited to its role in detoxifying Chl. Yet, recent advances in the structure elucidation of hFCCs isolated from fruits and leaves indicate a split of the pathway in the late steps.

Furthermore several reports hint to potential physiological roles for Chl catabolites, for example as sources for internal signals [151], as antioxidants [48] and as pigments that contribute to the optical appearance of fruits [33] and to the fall colors of deciduous trees [49]. In summary, our picture of the PAO pathway as a sole Chl-detoxifying pathway might be too simple. Future research on the pathway and on potential physiological roles of Chl catabolites is clearly demanded.

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