Minireview

Chlorophyll breakdown in oilseed rape

Stefan Hörtensteiner[1],* & Bernhard Kräutler[2]
[1]*Institute of Plant Biology, University of Zürich, CH-8008 Zürich, Switzerland;* [2]*Institute of Organic Chemistry, University of Innsbruck, A-6020 Innsbruck, Austria;* *Author for correspondence*

Abstract

Chlorophyll catabolism accompanying leaf senescence is one of the most spectacular natural phenomena. Despite this fact, the metabolism of chlorophyll has been largely neglegted until recently. Oilseed rape has been used extensively as a model plant for the recent elucidating of structures of chlorophyll catabolites and for investigation of the enzymic reactions of the chlorophyll breakdown pathway. The key reaction which causes loss of green color is catalyzed in a two-step reaction by pheophorbide a oxygenase and red chlorophyll catabolite reductase. In this Minireview, we summarize the actual knowledge about catabolites and enzymes of chlorophyll catabolism in oilseed rape and discuss the significance of this pathway in respect to chlorophyll degradation during *Brassica napus* seed development.

Abbreviations: Chl – chlorophyll; Chlide – chlorophyllide; FCC – fluorescent chlorophyll catabolite; NCC – nonfluorescent chlorophyll catabolite; PaO – pheophorbide *a* oxygenase; Pheide – pheophorbide; pFCC – primary fluorescent chlorophyll catabolite; RCC – red chlorophyll catabolite; RCCR – red chlorophyll catabolite reductase

Introduction

The disappearance of chlorophyll (Chl) and the emergence of autumnal colors belong to the most manifest and fascinating natural phenomena. In spite of its high visibility, biochemical information on the breakdown of Chl in plants has been very scarce until recently (Brown et al. 1991). Only in the last decade, some of the mysteries of Chl catabolism in higher plants were solved (Kräutler et al. 1991; Matile et al. 1996; Kräutler and Matile 1999).

Seasonal appearances and disappearances of the green pigments probably are the most visual sign of life on earth. An estimated amount of more than 10^9 tons of Chl are biosynthesized and degraded every year on earth (Brown et al. 1991). Considerable knowledge has accumulated concerning the biosynthesis of the Chls (Von Wettstein et al. 1995), in contrast to the lack of information concerning the fate of the green plant pigments (Brown et al. 1991). Still recently the Chls were suspected to disappear 'without leaving a

trace' (Matile 1987). As we know now, the major Chl catabolites are colorless, in contrast to what was generally expected. This was the main reason also, why they had remained undetected. By analogy to heme breakdown in animals, an oxygenolytic opening of the porphinoid macrocycle of the Chls was commonly considered as the key step in Chl breakdown (Brown et al. 1991). Based on experiences on the reactivity of chlorins towards electrophilic agents (Woodward and Skaric 1961; Brown et al. 1980, 1991), it was assumed, that opening of the macroring would occur at the 'western' δ-meso position (next to the peripherically reduced ring D).

The discovery of chlorophyllase and the enzymic hydrolysis of Chl to chlorophyllide (Chlide) and phytol (see Figure 1) by A. Stoll (Willstätter and Stoll 1913) provided an early (isolated) contribution to our knowledge on Chl breakdown. The lipophilic phytol anchor is crucial for the insertion of the green pigment–protein complexes into the thylakoid membranes of chloroplasts. The loss of phytol sets the stage
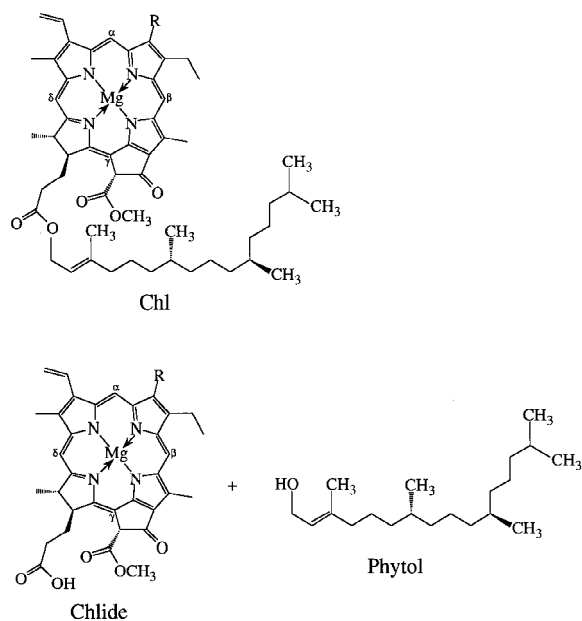
Figure 1. Structural formulae; top: Chl a (**1a**, R=CH₃), Chl b (**1b**, R= CH=O); bottom: Chlide a (**2a**, R=CH₃), Chlide b (**2b**, R= CH=O) and phytol.

for further enzymic degradation of both Chlide and the apoproteins (Matile 1992; Kräutler and Matile 1999; Matile et al. 1999).

Nongreen Chl catabolites were first discovered in extracts of senescent leaves of a nonyellowing genotype of the grass *Festuca pratensis* (Matile et al. 1987). Pink and rust-colored compounds (termed 'rusty pigments') appeared on the plates as chemical degradation products from the colorless catabolites. Similar compounds were found in primary leaves of barley (Bortlik et al. 1990; Peisker et al. 1990), when forced to degreen in permanent darkness. The structure of a predominant compound, of 'rusty pigment 14' (**3**), was determined by a combination of modern spectroscopic methods and unambiguously identified **3** as a colorless catabolite of Chl a (**1a**) (Kräutler et al. 1991, 1992). This work revealed the first structure of a nongreen Chl catabolite from plants and gave clues as to the major structural changes occurring in the degradation of Chl during plant senescence (Kräutler et al. 1991). It indicated, among other things, an oxygenolytic opening of the porphinoid macroring to have occurred, not at the δ-position, but rather at the 'northern' α-meso position.
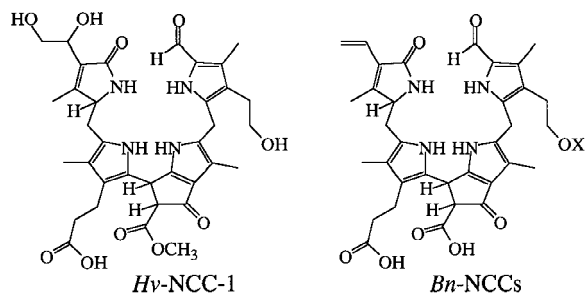

Figure 2. Constitutional formulae of nonfluorescent Chl catabolites (NCCs) from degreened plants. Left: *Hv*-NCC-1 (**3**) from barley; Right: *Bn*-NCC-1 (**4**, X=C(O)CH₂CO₂H), *Bn*-NCC-2 (**5**, X=β-glucopyranosyl) and *Bn*-NCC-3 (**6**, X=H), all from oilseed rape.

Structures of Chl catabolites from the cotyledons of oilseed rape

In recent years, oilseed rape has been used extensively for investigations of the biochemistry of Chl breakdown. This model plant is attractive for several reasons. It is closely related to *Arabidopsis thaliana* which is largely unsuitable for biochemical investigations and, in addition, oilseed rape is a major crop and a highly manipulable organism.

Colorless and nonfluorescent Chl catabolites (NCCs)

The cotyledons of the dicot oilseed rape (canola, *Brassica napus*) were found to be a rich source for colorless catabolites, when allowed to senesce in permanent darkness and also under natural growth conditions. Under these conditions in degreened cotyledons of oilseed rape, three colorless and nonfluorescent Chl-catabolites could be detected (termed *Bn*-NCCs) (Ginsburg and Matile 1993). These three *Bn*-NCCs (*Bn*-NCC-1 (**4**), *Bn*-NCC-2 (**5**), *Bn*-NCC-3 (**6**)) were found to account for practically all of the Chl broken down in the cotyledons of oilseed rape. They were isolated for structural analysis and by thorough spectroscopic analyzes (fast atom bombardment mass spectrometry and nuclear magnetic resonance spectroscopy) they were shown to all have the same basic structure, as *Hv*-NCC-1 (**3**) from barley (Mühlecker et al. 1993; Mühlecker and Kräutler 1996). The *Bn*-NCCs differ from *Hv*-NCC-1 only by the peripheral (re)functionalization. Most notably, the *Bn*-NCCs proved to be linear tetrapyrroles which were again derived from Chl a (**1a**) by an oxygenolytic ring opening at the α-meso position (see Figure 2) (Mühlecker et al. 1993; Mühlecker and Kräutler 1996).

In the meantime, compounds with spectral characteristics similar to those of the NCCs from barley and from canola were discovered in the autumn leaves of sweet gum (*Liquidambar styraciflua*) (Iturraspe et al. 1995) and *Cercidiphyllum japonicum* (Curty and Engel 1996). Thus, all NCCs isolated so far from a variety of degreened plants represent linear tetrapyrroles of uniform basic build-up (Figure 2) and relate to Chl *a* (**1a**) rather than to Chl *b* (**1b**) (Matile and Kräutler 1995). In addition, these structures (of the catabolites **3–6**) contradicted the relevance (suspected earlier) (Schoch et al. 1984; Brown et al. 1991) of enzymic transformations at the substituted cyclopentanone unit of the Chls, except for that of an enzymic hydrolysis of the methyl ester function (Shioi et al. 1996b). This latter hydrolysis produces β-keto carboxylic acids, known to be prone to decarboxylation. Accordingly, the decarboxylation products (pyropheophorbides), that were identified occasionally (Brown et al. 1991; Shioi et al. 1996b), may arise from nonenzymic transformations (Mühlecker and Kräutler 1996).

Fluorescent Chl catabolites (FCCc)

Analysis of extracts of senescent cotyledons of *B. napus*, when rates of Chl breakdown were high, revealed the intermediary occurence of tiny amounts of fluorescent compounds. provisionally named 'fluorescing Chl catabolites' (FCCs), because ^{14}C-labeling identified them as porphyrin derivatives (Matile et al. 1992; Ginsburg et al. 1994). As none of these compounds accumulated *in vivo*, they were considered to represent early or even primary products of porphyrin cleavage. An *in vitro* system, based on senescent cotyledons of oilseed rape, was established for the purpose of the preparation of the 'primary' FCC, in a quantity sufficient for structural analysis (Ginsburg et al. 1994; Hörtensteiner et al. 1995). An extract of the chloroplast membranes from senescent cotyledons of oilseed rape containing the enzymic oxygenating activity was used for the conversion of pheophorbide *a* (**7a**, Pheide *a*) into the major (less polar) FCC, originally named *Bn*-FCC-2 (**8**). From about 2 mg of Pheide *a* (**7a**) about 0.1 mg of *Bn*-FCC-2 were produced *in vitro*, so that the constitution of **8** could again be elucidated by the help of modern spectroscopy (Mühlecker et al. 1997). The structure of *Bn*-FCC-2 (**8**) indicated it to be derived rather directly from **7a**, formally by addition of one molequivalent of dioxygen and two molequivalents of dihydrogen. The structure suppor-
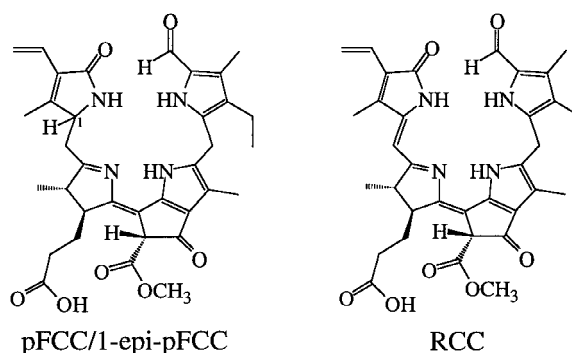

Figure 3. Left: Common constitutional formula of the fluorescing Chl catabolites pFCC (**8**) and 1-epi-pFCC (**9**): right: structural formula of RCC (**10**).

ted the view that **8** could be considered the 'primary' FCC (and therefore now is called pFCC) (Mühlecker et al. 1997). In the meantime, another primary FCC (**9**) was isolated from sweet pepper (*Capsicum annuum*) in an amount sufficient for analysis of its structure. The two catabolites (**8** and **9**) could be shown to have the same constitution, i.e. to be stereoisomers of each other, that differ only in the absolute configuration at C(1), the chiral center newly introduced by a reduction step (Mühlecker et al. 2000) (i.e. **9** = 1-epi-pFCC, Figure 3).

Another important information on the pathway of Chl breakdown came about by the finding that Pheide *a* (**7a**), but not Pheide *b* (**7b**), accumulated in the absence of molecular oxygen (Vicentini et al. 1995), hinting at the involvement of molecular oxygen and **7a** as common substrates in an oxidative enzymic step during Chl breakdown. In line with this, the putative oxygenase, turned out to be remarkably specific for **7a**, with **7b** as competitive inhibitor (Hörtensteiner et al. 1995). These findings suggested that Chl *b* (**1b**) might be reduced to Chl *a* (**1a**) in the course of degreening and indeed, in the meantime, the existence of a Chl *b* to Chl *a* conversion has been demonstrated (see below) (Ito et al. 1996; Scheumann et al. 1996).

Accordingly, Pheide *a* (**7a**) appeared to be the last intermediate with an intact chlorin macrocycle in senescence-induced Chl breakdown in oilseed rape. Between **7a** and the *Bn*-NCCs there still existed a large gap, for which the fluorescing catabolite pFCC (**8**) provided a first structural link (Mühlecker et al. 1997): it indicated the oxygenolytic opening of the macrocycle of **7a** and the saturation of two of its three other meso-positions to precede the other

(re)functionalization reactions (minimally) involved in the path from the Chls towards the NCCs.

A red tetrapyrrolic catabolite as elusive intermediate in Chl breakdown

Considering the structure of pFCC (**8**) and the other findings outlined (Vicentini et al. 1995; Mühlecker et al. 1997), it appeared likely that the oxygenolytic cleavage of the ring would occur first and would be followed by a reduction step, leading to saturation of the 'western' δ-meso position. Accordingly, the red tetrapyrrole (**10**, RCC) appeared likely as a direct precursor of **8** and, therefore, as a putative intermediate in Chl breakdown (Mühlecker et al. 1997). Therefore, **10** would be similar (but not identical) to the red bilinones which had been found to be excreted as final degradation product of the Chls in the green alga *Chlorella protothecoides* (Oshio and Hase 1969; Engel et al. 1991). Fortunately, by partial degradation of **1a** in a sequence of five chemical steps and having as the key step an photooxygenolytic opening of the porphinoid macrocycle of a cadmium pheophorbidate the red tetrapyrrole **10** could be prepared (Kräutler et al. 1997). The red compound **10** was then available for tracing experiments, in which **10** could be shown to be identical with RCC, obtained from **7a** by enzymic oxygenolysis. This was demonstrated by the incubation of thoroughly washed membrane extracts from chloroplasts of degreened cotyledons of canola and under suitable *in vitro* conditions, from **7a** the elusive RCC could be produced in traces, identified with the synthetic **10** by HPLC (Rodoni et al. 1997a). In addition, incubation of chemically prepared **10** with a preparation of stroma proteins from chloroplasts resulted in the formation of three FCCs (two of which had identical chromatographic characteristics as **8** and **9**), provided that reduced ferredoxin was furnished under anaerobic conditions (Rodoni et al. 1997b).

During senescence in degreened plants, RCC is formed in trace amounts only and in an enzyme bound state. Formally, RCC arises from Pheide a by addition of one equivalent each of dioxygen and dihydrogen. Reduction of RCC (by addition, formally, of one equivalent of dihydrogen) converts it into either one of the stereoisomeric pFCCs (Mühlecker et al. 1997; Rodoni et al. 1997b). Accordingly, the enzyme in the stroma fraction is a reductase, which converts RCC into pFCC and therefore was named RCC reductase (RCCR).

The enzymic activity in washed chloroplast membranes from senescent cotyledons converts Pheide a (**7a**) in traces into RCC (**10**), an effectively irreversible oxygenolytic cleavage of the porphinoid macroring involving molecular oxygen and apparently inhibited by the oxygenation product (Rodoni et al. 1997a). The minimal requirements of this transformation could be achieved either by a monooxygenase or by the joint action of a dioxygenase and a reductase. As is delineated in more detail below, a single enzyme is indicated to achieve the conversion of Pheide a (**7a**) into (a bound form of) RCC (**10**), an oxygenase termed pheophorbide a oxygenase (PaO) (Rodoni et al. 1997a).

The Chl catabolic pathway

Work on Chl catabolism in rape and other species, such as barley or *F. pratensis* (for recent reviews see Hörtensteiner 1999; Kräutler and Matile 1999; Matile et al. 1999), has lead to the establishment of a Chl catabolic pathway as depicted in Figure 5. In the following paragraphs, individual reactions are outlined in detail with keeping a focus on data available from canola.

Chlorophyllase

Chlorophyllase, first described by Stoll (Willstätter and Stoll 1913), catalyzes the hydrolysis of Chl (**1**) to Chlide (**2**) and phytol. Phytol remains located within chloroplasts, largely in esterified form (Peisker et al. 1989). Chlorophyllase activity has been demonstrated in a number of different species and its properties are rather unusual. Thus, activity is latent and *in vitro* can only be assessed in the presence of high concentrations of solvents or detergents (Holden 1961; Trebitsh et al. 1993). The enzyme was found to be located at the inner envelope membrane of chloroplasts which may explain this structural latency (Matile et al. 1997). Interestingly, dephytylating activity can be demonstrated at all stages of leaf development implying that during Chl breakdown, a mechanism comes into action which establishes a physical contact between the enzyme and its substrate, Chl (Matile et al. 1996). Chlorophyllase has been demonstrated to be modulated by factors affecting senescence and Chl breakdown, such as ethylene (Trebitsh et al. 1993).

Chlorophyllase has been purified from several species, such as *Citrus* (Trebitsh et al. 1993) and *Chlorella regularis* (Nishiyama et al. 1994) and

cDNAs confering chlorophyllase activity, when over-expressed in *E. coli*, have been isolated from *Citrus* (Jakob-Wilk et al. 1999), *Chenopodium album* and *Arabidopsis thaliana* (Tsuchiya et al. 1999).

Mg-dechelation

The identification of Mg-free forms of Chl accumulating during senescence in a wide range of species (e.g. Schoch et al. 1981; Shimokawa et al. 1990) have suggested the presence of an enzyme catalyzing the respective dechelation step. Using senescent rape cotyledons or isolated chloroplasts, considerable quantities of Pheide *a* (**7a**) accumulated, when oxidative cleavage of the porphyrin of Chl was inhibited by iron chelating substances. In contrast, Pheide *a* did not accumulate in normal yellowing leaf material (Langmeier et al. 1993). mg dechelatase removes the central Mg^{2+} of Chlide (**2**) in exchange with 2 H^+. Attempts to purify this enzyme have produced surprising results: Activity appears to be associated with a substance of low molecular weight which is heat stable (Shioi et al. 1996a).

The key reaction is catalyzed by PaO and RCCR

The third step in the Chl catabolic pathway is most significant for the yellowing process during senescence because it is responsible for the loss of green color. This is achieved by oxygenolytic opening of the porphyrin macrocycle of Pheide *a* (**7a**). Two enzymes have been shown to be necessary for the formation of the first accessible cleavage product, pFCC (**8**), PaO and RCCR (see Figure 5). PaO which is located at the inner envelope membrane of chloroplasts (Matile and Schellenberg 1996) is exclusively present in senescent leaves and absent from presenescent tissue (Hörtensteiner et al. 1995). In contrast, activity of RCCR has been demonstrated at all stages of leaf development and also in roots (Rodoni et al. 1997a). The requirement of two protein components was rationalized by the finding that Pheide *a* to pFCC transformation occurs in a two step reaction: RCC (**10**), the primary product of oxygenolytic Pheide *a* (**7a**) cleavage by PaO is subsequently reduced to pFCC (**8**) by RCCR (Rodoni et al. 1997a). RCC (**10**) appears not to be released from PaO, but is directly reduced to pFCC (**8**) by RCCR, suggesting a close physical contact between the two protein components during catalysis and metabolic channeling of the red intermediate. Both partial reactions require reduced ferredoxin as the source of electrons, whereby ferredoxin is kept in the reduced
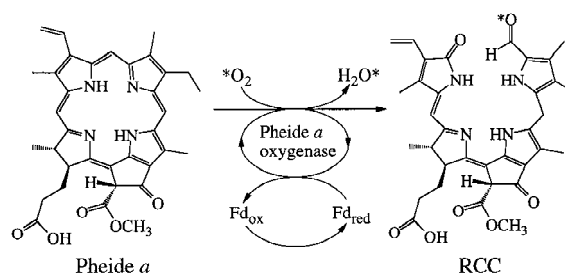

Figure 4. Cleavage of Pheide *a* (**7a**) by PaO to give a bound form of the red catabolite RCC (**10**).

state either by Photosystem I or the pentose phosphate cycle.

The properties of PaO of canola were investigated mainly in assays together with RCCR and Pheide *a* as substrate. Iron chelation and reconstitution experiments established that PaO is a non-heme iron containing oxygenase (Hörtensteiner et al. 1995). It is specific for Pheide *a* with Pheide *b* (**7b**) as a competitive inhibitor. This substrate specificity appears to be responsible for the presence in higher plants of final catabolites, NCCs, exclusively derived from Chl (Pheide) *a*. Before entering the catabolic pathway, Chl *b* (**1b**) must be converted to the *a* form (**1a**), most probably through a Chl *b* reducing mechanism which has recently been described in cucumber and barley (Ito et al. 1996; Scheumann et al. 1999).

In the presence of $^{18}O_2$, a mixture of partially purified rape PaO and RCCR converted Pheide *a* (**7a**) into an ^{18}O-labeled sample of pFCC (^{18}O-**8**), that contained one ^{18}O-atom per molecule of catabolite, as determined by fast atom mass spectrometry (Hörtensteiner et al. 1998b). From mass spectral analysis of fragment ions of ^{18}O-**8**, the isotopic label could be assigned to the formyl group (Figure 4). These results indicate incorporation of one oxygen atom from molecular oxygen at the α-meso position of **7a** in the course of the oxygenolytic cleavage of the macrocycle.

Accordingly, the key ring cleavage step of Chl degradation in senescent plants is catalyzed by a monooxygenase (Figure 5) (Hörtensteiner et al. 1998b). One of the two oxygen atoms introduced in the ring cleavage reaction is derived from O_2, the other from another source, most likely from water. Over all, the transformation of Pheide *a* (**7a**) into RCC (**10**) corresponds to a remarkable structural change, as ring opening at the newly oxygenated sites with formation of two carbonyl functions and the saturation of the 'eastern' β-meso position accompany the incorporation of the two oxygen atoms. Sufficient ex-
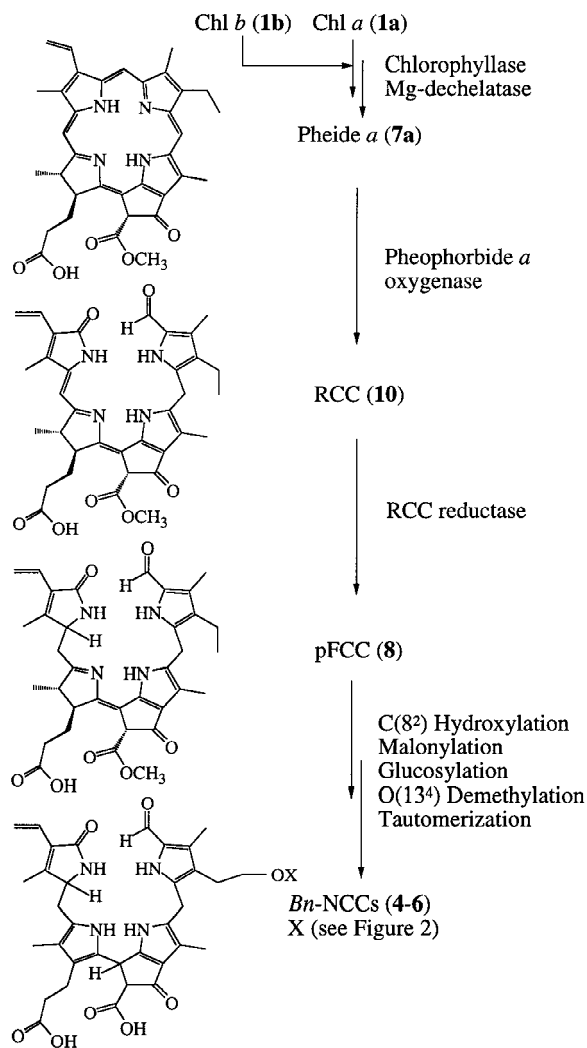
Figure 5. Structural outline of the pathway of Chl breakdown in senescent cotyledons of oilseed rape.

perimental data are not available yet to help resolve the mechanistic questions concerning the hypothetical isomerization of the primary enzymic oxygenation product to the ring-opened enzyme-bound form of RCC. The formation of the related red bilinone in the green alga *C. protothecoides* has been suggested to result from hydration of an epoxide intermediate and subsequent rearrangement (Curty et al. 1995; Curty and Engel 1997).

RCCR has been partially purified from barley (Rodoni et al. 1997b; Wüthrich et al. 2000) and a cDNA encoding RCCR was subsequently cloned from *A. thaliana* (Wüthrich et al. 2000). Employing the native barley protein, the properties of RCCR were ana-

lyzed using chemically synthesized RCC (Kräutler et al. 1997) as substrate. Under these conditions, RCCR activity was sensitive to oxygen suggesting that the interaction of PaO and RCCR is a prerequisite for RCCR action. Three different FCCs were formed from RCC (**10**), two of which were identified as pFCC (**8**) and its C(1) epimer, 1-epi-pFCC (**9**). The same principal results were obtained with rape and *A. thaliana* using crude protein extracts. In contrast, with recombinant *A. thaliana* RCCR heterologously expressed in *Escherichia coli*, the third most polar FCC did not accumulate suggesting that its occurence was due to FCC modifiying activities present in the respective enzyme preparations (Wüthrich et al. 2000).

When RCCR was assayed together with PaO and Pheide *a* as substrate, only one of the two epimeric pFCCs (**8** or **9**) accumulated, depending on the nature of RCCR. Obviously, interaction with RCC (**10**) bound to PaO causes site-specific reduction by RCCR, a feature which is lost with 'free' RCC. A major survey of RCCRs from more than 60 species revealed this stereospecificity towards reduction at C(1). Thereby, all investigated genera and species within a plant family exhibited the same specificity (Hörtensteiner et al. 2000).

Reactions from pFCC to NCCs

From the chemical structures of pFCC (**8**) (Mühlecker et al. 1997) and the final *Bn*-NCCs (**4–6**) (Mühlecker et al. 1993; Mühlecker and Kräutler 1996), additional reactions occuring in the Chl catabolic pathway of oilseed rape could be postulated. These are (in an as yet unknown sequence) hydroxylation at $C(8^2)$ and subsequent conjugation with malonic acid or glucose and hydrolysis of the $C(13^2)$ carboxymethyl ester. These transformations introduce polar groups into the tetrapyrrole. Finally, FCCs are tautomerized to NCCs.

There are no biochemical data available for the hydroxylation of the C(8) ethyl side chain, but the presence of a respective activity has been postulated (Hörtensteiner 1999). Indirect evidence is given by radiolabeling of a polar *Bn*-FCC derived from Chl (Ginsburg and Matile 1993) which can be malonylated in assays of NCC malonyltransferase (see below) and, hence, most likely represents the $C(8^2)$ hydroxylated form of pFCC (S. Hörtensteiner, unpublished). The nature of the respective activity could be a cytochrome P450 monooxygenase which have been shown to catalyze a number of different hydroxylation reactions (Schuler 1996).

In *Bn*-NCC-1 (**4**), the major NCC of rape, the C(8^2) hydroxyl group is esterified with malonic acid. Malonyltransferase activity has been shown to be present in protein extracts from rape (Hörtensteiner 1998). *In vitro*, malonyl coenzyme A served as malonyl donor and different NCCs from several species were accepted as a substrate, provided they contained a free C(8^2) hydroxyl group. The activity was rather specific for NCCs as other malonyl acceptors, such as 1-aminocyclopropane-1-carboxylic acid, did not inhibit NCC transmalonylation. Glucosyltransferase activity, necessary for the formation of *Bn*-NCC-2 (**5**) has not been identified yet.

Compared to NCCs from other higher plant species structurally described so far (Kräutler et al. 1991; Iturraspe et al. 1995; Curty and Engel 1996), *Bn*-NCCs are unique in respect to the presence of a free *β*-keto carboxylic acid group in their cylopentanone moiety (Mühlecker et al. 1993; Mühlecker and Kräutler 1996).The instability of this functional group towards (nonenzymic) decarboxylation may be the cause for the appearance of pyroforms of Chl catabolites that have been inferred as true Chl catabolites in several instances (Shimokawa et al. 1990; Engel et al. 1991, 1996). Likewise, recent investigations in *Chenopodium album* suggest that the demethylation of Pheide is catalyzed by an enzyme, tentatively named pheophorbidase, whereas the subsequent decarboxylation step occurs nonenzymically (Shioi et al. 1996b). In oilseed rape, hydrolysis of pFCC (**8**) to the respective demethylated tetrapyrrole has been shown to be catalyzed by a soluble enzyme (Hörtensteiner et al. 1998a). The identity of the product was confirmed by mass spectroscopy (S. Hörtensteiner and B. Kräutler, unpublished). Only FCCs but no NCCs served as substrates to the reaction (S. Hörtensteiner, unpublished) indicating that demethylation occurs prior to the tautomerization of FCCs to NCCs.

The three NCCs of oilseed rape (**4-6**) are localized in the vacuoles of senescent cotyledons (Hinder et al. 1996) and a primary active transport system has been shown to catalyze vacuolar import of *Bn*-NCC-1 (**4**) (Lu et al. 1998; Tommasini et al. 1998). Due to limited amounts of material available, respective uptake experiments have not been performed with pFCC (**8**), but inhibitor studies of *Bn*-NCC-1 (**4**) using an FCC from barley indicate that FCCs may be the prefered substrates for vacuolar transport (Hinder et al. 1996). These data together with preliminary experiments of a nonenzymic, acid-catalyzed conversion of pFCC (**8**) into an NCC (S. Hörtensteiner, unpublished) indicate

that *in vivo* the modifications of pFCC, as reflected in the structures of the final NCCs, occur on the level of FCCs. After import of FCCs into the vacuole, tautomerization to NCCs is triggered by the acidic milieu of the vacuolar sap.

Compartmentation and regulation

Chl bound to apoproteins in the thylakoid membrane is degraded to final NCCs which are deposited in the vacuoles of senescent leaf tissues. Thus, the Chl catabolic pathway extends over several subcellular compartments and includes transport processes at the chloroplast envelope and the tonoplast. The first four enzymes, i.e. chlorophyllase, dechelatase, PaO and RCCR, have been shown to be located inside senescing chloroplasts (gerontoplasts) in all species investigated so far, including oilseed rape. Of the later rape-specific reactions, most probably demethylation, malonyltransfer and the hypothetical glucosyltransfer take place in the cytoplasm. The location of the hypothetical C(8^2) hydroxylase is unclear. Data from export studies using intact barley gerontoplasts suggest that the major exported FCC is not pFCC (**8**) but an as yet unknown more polar FCC (Matile et al. 1992). The export required the hydrolysis of ATP for energization, but the nature of the transport protein has to be established.

Chl breakdown is a highly regulated process which requires cytoplasmic protein synthesis (Matile 1992). Of the catabolic enzymes investigated so far only PaO appeared to be exclusively present during Chl breakdown, hence, it is senescence-specific (Schellenberg et al. 1993; Ginsburg et al. 1994; Hörtensteiner et al. 1995). In this respect, it is worth mentioning that in most cases of stay-green phenotypes from different species biochemically analyzed so far, the defect could be attributed to a reduction or absence of PaO activity (Vicentini et al. 1995; Thomas et al. 1996).

Significance of Chl breakdown in oilseed rape

Detoxification of Chl

Like other porphyrins, Chl is a photodynamically active and, therefore, potentially cell toxic compound. During senescence, Chl is liberated due to remobilization of the thylakoidal apoproteins accounting for some 30% of the total plastid nitrogen pool (Thomas

1997). Thus, plants have evolved a mechanism which is able to safely dispose of this hazard. The pathway of Chl catabolism largely resembles the process of detoxification of xenobiotics and herbicides, including hydroxylation, modification reactions and, finally, deposition within a metabolically inactive cellular compartment, the vacuole (Kreuz et al. 1996). In this respect, the breakdown of Chl can be considered as an inevitable metabolic process accompanying the remobilization of nutrients during plant senescence.

The 'green seed problem'

Chl degradation is not only an integral part of leaf senescence or fruit ripening, but in several species, such as oilseed rape, also occurs in maturing seeds. The cotyledons of developing canola embryos are rich in Chl up to the mid-phase of maturation. Thereafter, rapid Chl degradation is responsible for a complete loss of green pigments before the end of the desiccation phase is reached (Green et al. 1998). During this phase, a short sublethal freezing stress which often occurs in areas with a limited growth season causes retention of Chl in mature oilrape seeds. The Chl content of canola oil is known to have an important impact on the quality, since it affects stability, odor and flavor (Levadoux et al. 1987). Consequently, the market value of Chl-contaminated rape oil is markedly lowered, because costly processes are needed for the removal of Chl. The underlying biochemistry of this 'green seed problem' has been investigated extensively (Johnson-Flanagan and Thiagarajah 1990; Johnson-Flanagan and Spencer 1994) and inhibition of Chl catabolism has been attributed to accelerated dessication of the seeds (Green et al. 1998). However, it remains to be shown which of the Chl catabolic enzymes is (are) affected. The accumulation in maturing seeds of Chlides and Pheides when degreening is inhibited by sublethal freezing (Johnson-Flanagan and McLachlan 1990) indicates that the subsequent porphyrin macrocycle cleavage by PaO could be blocked, as is the case in other instances of stay-green genotypes (Vicentini et al. 1995; Thomas et al. 1996).

Acknowledgements

References

Bortlik K-H, Peisker C and Matile P (1990) A novel type of chlorophyll catabolite in senescent barley leaves. J Plant Physiol 136: 161–165

Brown SB, Houghton JD and Hendry GAF (1991) Chlorophyll breakdown. In: Scheer H (ed) Chlorophylls, pp 465–489. CRC Press, Boca Raton, Florida

Brown SB, Smith KM, Bisset GMF and Troxler RF (1980) Mechanism of photooxidation of bacteriochlorophyll *c* derivatives. A possible model for natural chlorophyll breakdown. J Biol Chem 255: 8063–8068

Curty C and Engel N (1996) Detection, isolation and structure elucidation of a chlorophyll *a* catabolite from autumnal senescent leaves of *Cercidiphyllum japonicum*. Phytochemistry 42: 1531–1536

Curty C and Engel N (1997) Chlorophyll catabolism: high stereoselectivity in the last step of the primary ring cleaving process. Plant Physiol Biochem 35: 707–711

Curty C, Engel N and Gossauer A (1995) Evidence for a monooxygenase-catalyzed primary process in the catabolism of chlorophyll. FEBS Lett 364: 41–44

Engel N, Curty C and Gossauer A (1996) Chlorophyll catabolism in *Chlorella protothecoides*. 8. Facts and artefacts. Plant Physiol Biochem 34: 77–83

Engel N, Jenny TA, Mooser V and Gossauer A (1991) Chlorophyll catabolism in *Chlorella protothecoides*. Isolation and structure elucidation of a red bilin derivative. FEBS Lett 293: 131–133

Ginsburg S and Matile P (1993) Identification of catabolites of chlorophyll porphyrin in senescent rape cotyledons. Plant Physiol 102: 521–527

Ginsburg S, Schellenberg M and Matile P (1994) Cleavage of chlorophyll-porphyrin. Requirement for reduced ferredoxin and oxygen. Plant Physiol 105: 545–554

Green BR, Singh S, Babic I, Bladen C and Johnson-Flanagan AM (1998) Relationship of chlorophyll, seed moisture and ABA levels in the maturing *Brassica napus* seed and effect of a mild freezing stress. Physiol Plant 104: 125–133

Hinder B, Schellenberg M, Rodoni S, Ginsburg S, Vogt E, Martinoia E, Matile P and Hörtensteiner S (1996) How plants dispose of chlorophyll catabolites. Directly energized uptake of tetrapyrrolic breakdown products into isolated vacuoles. J Biol Chem 271: 27233–27236

Holden M (1961) The breakdown of chlorophyll by chlorophyllase. Biochem J 78: 359–364

Hörtensteiner S (1998) NCC malonyltransferase catalyses the final step of chlorophyll breakdown in rape (*Brassica napus*). Pytochemistry 49: 953–956

Hörtensteiner S (1999) Chlorophyll breakdown in higher plants and algae. Cell Mol Life Sci 56: 330–347

Hörtensteiner S, Vicentini F and Matile P (1995) Chlorophyll breakdown in senescent cotyledons of rape, *Brassica napus* L.: Enzymatic cleavage of phaeophorbide *a in vitro*. New Phytol 129: 237–246

Hörtensteiner S, Wüthrich KL and Matile P (1998a) New aspects on the catabolic pathway of chlorophyll in senescent rape cotyledons. J Exp Bot 49 (Suppl 65)

Hörtensteiner S, Wüthrich KL, Matile P, Ongania K-H and Kräutler B (1998b) The key step in chlorophyll breakdown in higher

plants. Cleavage of pheophorbide *a* macrocycle by a monooxygenase. J Biol Chem 273: 15335–15339

Hörtensteiner S, Rodoni S, Schellenberg M, Vicentini F, Nandi OI, Qiu Y-L and Matile P (2000) Evolution of chlorophyll degradation: The significance of RCC reductase. Plant Biol 2: 63–67

Ito H, Ohysuka T and Tanaka A (1996) Conversion of chlorophyll *b* to chlorophyll *a via* 7-hydroxymethyl chlorophyll. J Biol Chem 271: 1475–1479

Iturraspe J, Moyano N and Frydman B (1995) A new 5-formylbilinone as the major chlorophyll *a* catabolite in tree senescent leaves. J Org Chem 60: 6664–6665

Jacob-Wilk D, Holland D, Goldschmidt EE, Riov J and Eyal Y (1999) Chlorophyll breakdown by chlorophyllase: Isolation and functional expression of the *Chlase1* gene from ethylene-treated *Citrus* fruit and its regulation during development. Plant J 20: 653–661

Johnson-Flanagan AM and McLachlan G (1990) The role of chlorophyllase in degreening canola (*Brassica napus*) seeds and its activity by sublethal freezing. Physiol Plant 80: 460–466

Johnson-Flanagan AM and Spencer MS (1994) Ethylene production during degreening of maturing seeds of mustard and canola. Plant Physiol 106: 601–606

Johnson-Flanagan AM and Thiagarajah MR (1990) Degreening in canola (*Brassica napus* cv. Westar) embryos under optimum conditions. J Plant Physiol 136: 180–186

Kräutler B and Matile P (1999) Solving the riddle of chlorophyll breakdown. Acc Chem Res 32: 35–43

Kräutler B, Jaun B, Amrein W, Bortlik K, Schellenberg M and Matile P (1992) Breakdown of chlorophyll: Constitution of a secoporphinoid chlorophyll catabolite isolated from senescent barley leaves. Plant Physiol Biochem 30: 333–346

Kräutler B, Jaun B, Bortlik K-H, Schellenberg M and Matile P (1991) On the enigma of chlorophyll degradation: The constitution of a secoporphinoid catabolite. Angew Chem Int Ed Engl 30: 1315–1318

Kräutler B, Mühlecker W, Anderl M and Gerlach B (1997) Breakdown of chlorophyll: partial synthesis of a putative intermediary catabolite. Helv Chim Acta 80: 1355–1362

Kreuz K, Tommasini R and Martinoia E (1996) Old enzymes for a new job. Herbicide detoxification in plants. Plant Physiol 111: 349–353

Langmeier M, Ginsburg S and Matile P (1993) Chlorophyll breakdown in senescent leaves: Demonstration of Mg-dechelatase activity. Physiol Plant 89: 347–353

Levadoux WL, Kalmokoff ML, Pickard MD and Groot-Wassink JWD (1987) Pigment removal from canola oil using chlorophyllase. J Am Oil Chem Soc 64: 139–144

Lu Y-P, Li Z-S, Drozdowicz Y-M, Hörtensteiner S, Martinoia E and Rea PA (1998) AtMRP2, an *Arabidopsis* ATP binding cassette transporter able to transport glutathione S-conjugates and chlorophyll catabolites: Functional comparisons with AtMRP1. Plant Cell 10: 267–282

Matile P (1987) Seneszenz bei Pflanzen und ihre Bedeutung für den Stickstoffhaushalt. Chimia 41: 376–381

Matile P (1992) Chloroplast senescence. In: Baker NR and Thomas H (eds) Crop Photosynthesis: Spatial and Temporal Determinants, pp 413–440. Elsevier Science Publishers, Amsterdam

Matile P and Kräutler B (1995) Wie und warum bauen Pflanzen das Chlorophyll *ab*. Chem unserer Zeit 29: 298–306

Matile P and Schellenberg M (1996) The cleavage of pheophorbide *a* is located in the envelope of barley gerontoplasts. Plant Physiol Biochem 34: 55–59

Matile P, Ginsburg S, Schellenberg M and Thomas H (1987) Catabolites of chlorophyll in senescent leaves. J Plant Physiol 129: 219–228

Matile P, Schellenberg M and Peisker C (1992) Production and release of a chlorophyll catabolite in isolated senescent chloroplasts. Planta 187: 230–235

Matile P, Hörtensteiner S, Thomas H and Kräutler B (1996) Chlorophyll breakdown in senescent leaves. Plant Physiol 112: 1403–1409

Matile P, Schellenberg M and Vicentini F (1997) Localization of chlorophyllase in the chloroplast envelope. Planta 201: 96–99

Matile P, Hörtensteiner S and Thomas H (1999) Chlorophyll degradation. Annu Rev Plant Physiol Plant Mol Biol 50: 67–95

Mühlecker W and Kräutler B (1996) Breakdown of chlorophyll: Constitution of nonfluorescing chlorophyll-catabolites from senescent cotyledons of the dicot rape. Plant Physiol Biochem 34: 61–75

Mühlecker W, Kräutler B, Ginsburg S and Matile P (1993) Breakdown of chlorophyll: The constitution of a secoporphinoid chlorophyll catabolite from senescent rape leaves. Helv Chim Acta 76: 2976–2980

Mühlecker W, Ongania K-H, Kräutler B, Matile P and Hörtensteiner S (1997) Tracking down chlorophyll breakdown in plants: elucidation of the constitution of a 'fluorescent' chlorophyll catabolite. Angew Chem Int Ed Engl 36: 401–404

Mühlecker W, Kräutler B, Moser D, Matile P and Hörtensteiner S (2000) Breakdown of chlorophyll: A fluorescent chlorophyll catabolite from sweet pepper (*Capsicum annuum*). Helv Chim Acta 83: 278–286

Nishiyama Y, Kitamura M, Tamura S and Watanabe T (1994) Purification and substrate specificity of chlorophyllase from *Chlorella regularis*. Chem Lett 69–72

Oshio Y and Hase E (1969) Studies on red pigments excreted by cells of *Chlorella protothecoides* during the process of bleaching induced by glucose or acetate. I. Chemical properties of the red pigments. Plant Cell Physiol 10: 41–49

Peisker C, Düggelin T, Rentsch D and Matile P (1989) Phytol and the breakdown of chlorophyll in senescent leaves. J Plant Physiol 135: 428–432

Peisker C, Thomas H, Keller F and Matile P (1990) Radiolabelling of chlorophyll for studies on catabolism. J Plant Physiol 136: 544–549

Rodoni S, Mühlecker W, Anderl M, Kräutler B, Moser D, Thomas H, Matile P and Hörtensteiner S (1997a) Chlorophyll breakdown in senescent chloroplasts. Cleavage of pheophorbide *a* in two enzymic steps. Plant Physiol 115: 669–676

Rodoni S, Vicentini F, Schellenberg M, Matile P and Hörtensteiner S (1997b) Partial purification and characterization of red chlorophyll catabolite reductase, a stroma protein involved in chlorophyll breakdown. Plant Physiol 115: 677–682

Schellenberg M, Matile P and Thomas H (1993) Production of a presumptive chlorophyll catabolite *in vitro*: Requirement for reduced ferredoxin. Planta 191: 417–420

Scheumann V, Ito H, Tanaka A, Schoch S and Rüdiger W (1996) Substrate specificity of chlorophyll(ide) *b* reductase in etioplasts of barley (*Hordeum vulgare*). Eur J Biochem 242: 163–170

Scheumann V, Schoch S and Rüdiger W (1999) Chlorophyll *b* reduction during senescence of barley seedlings. Planta 209: 364–370

Schoch S, Scheer H, Schiff JA, Rüdiger W and Siegelman HW (1981) Pyropheophytin *a* accompanies pheophytin *a* in darkened light grown cells of *Euglena*. Z Naturforsch 36c: 827–833

146

Schoch S, Rüdiger W, Lüthy B and Matile P (1984) 13^2-hydroxychlorophyll *a*, the first product of the reaction of chlorophyll-oxidase. J Plant Physiol 115: 85–89

Schuler MA (1996) Plant cytochrome P450 monooxygenases. Critical Rev Plant Sci 15: 235–284

Shimokawa K, Hashizume A and Shioi Y (1990) Pyropheophorbide *a*, a catabolite of ethylene-induced chlorophyll *a* degradation. Phytochemistry 29: 2105–2106

Shioi Y, Tomita N, Tsuchiya T and Takamiya K (1996a) Conversion of chlorophyllide to pheophorbide by Mg-dechelating substance in extracts of *Chenopodium album*. Plant Physiol Biochem 34: 41–47

Shioi Y, Watanabe K and Takamiya K (1996b) Enzymatic conversion of pheophorbide *a* to a precursor of pyropheophorbide *a* in leaves of *Chenopodium album*. Plant Cell Physiol 37: 1143–1149

Thomas H (1997) Chlorophyll: A symptom and a regulator of plastid development. New Phytol 136: 163–181

Thomas H, Schellenberg M, Vicentini F and Matile P (1996) Gregor Mendel's green and yellow pea seeds. Bot Acta 109: 3–4

Tommasini R, Vogt E, Fromenteau M, Hörtensteiner S, Matile P, Amrhein N and Martinoia E (1998) An ABC transporter of *Arabidopsis thaliana* has both glutathione-conjugate and chlorophyll catabolite transport activity. Plant J 13: 773–780

Trebitsh T, Goldschmidt EE and Riov J (1993) Ethylene induces *de novo* synthesis of chlorophyllase, a chlorophyll degrading enzyme, in *Citrus* fruit peel. Proc Natl Acad Sci USA 90: 9441–9445

Tsuchiya T, Ohta H, Okawa K, Iwamatsu A, Shimada H, Masuda T and Takamiya K (1999) Cloning of chlorophyllase, the key enzyme in chlorophyll degradation: Finding of a lipase motif and the induction by methyl jasmonate. Proc Natl Acad Sci 96: 15362–15367

Vicentini F, Hörtensteiner S, Schellenberg M, Thomas H and Matile P (1995) Chlorophyll breakdown in senescent leaves: Identification of the biochemical lesion in a *stay-green* genotype of *Festuca pratensis* Huds. New Phytol 129: 247–252

von Wettstein D, Gough S and Kannangara CG (1995) Chlorophyll biosynthesis. Plant Cell 7: 1039–1057

Willstätter R and Stoll A (1913) Die Wirkungen der Chlorophyllase. In: Willstätter R and Stoll A (eds) Untersuchungen über Chlorophyll, pp 172–187. Verlag Julius Springer, Berlin

Woodward RB and Skaric V (1961) A new aspect of the chemistry of chlorins. J Am Chem Soc 83: 4676–4678

Wüthrich KL, Bovet L, Hunziker PE, Donnison IS and Hörtensteiner S (2000) Molecular cloning, functional expression and characterisation of RCC reductase involved in chlorophyll catabolism. Plant J 21: 189–198