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# Update on the biochemistry of chlorophyll breakdown

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**Abstract** In land plants, chlorophyll is broken down to colorless linear tetrapyrroles in a highly conserved multi-step pathway. The pathway is termed the 'PAO pathway', because the opening of the chlorine macrocycle present in chlorophyll catalyzed by pheophorbide *a* oxygenase (PAO), the key enzyme of the pathway, provides the characteristic structural basis found in all further downstream chlorophyll breakdown products. To date, most of the biochemical steps of the PAO pathway have been elucidated and genes encoding many of the chlorophyll catabolic enzymes been identified. This review summarizes the current knowledge on the biochemistry of the PAO pathway and provides insight into recent progress made in the field that indicates that the pathway is more complex than thought in the past.

**Keywords** Chlorophyll catabolites · Chlorophyll breakdown · Detoxification · Fruit ripening · Leaf senescence

## Introduction

Chlorophyll (Chl), the most abundant pigment on Earth, is essential for light absorption during photosynthesis. However, when the photosynthetic apparatus is overexcited, for example under light stress conditions, Chl can act as a photosensitizer, that can cause cell damage and death (Apel and Hirt 2004). Likewise, defects in Chl biosynthesis and degradation result in cytotoxic effects, which are caused by the accumulation of respective photodynamic metabolic intermediates (Mochizuki et al. 2010; Pružinská et al. 2003). Therefore, tight regulation mechanisms, well known in the case of Chl biosynthesis (Tanaka and Tanaka 2006, 2007), are required to prevent these toxic effects. It is evidenced that Chl breakdown is regulated at different levels to limit the accumulation of photodynamic breakdown intermediates (Park et al. 2007; Hörtensteiner 2006; Sakuraba et al. 2012).

Chl breakdown does not only occur during leaf senescence and fruit ripening, but also at steady state, during post-harvest and in response to biotic and abiotic stresses, Chl is turned over or degraded, at least to some extent. However, it remains unknown whether the mechanism of Chl breakdown under all these conditions is the same. To date the best characterized mechanism is the 'PAO pathway' of Chl breakdown. It is named after pheophorbide *a* oxygenase (PAO), which accounts for the open-tetrapyrrolic backbone structure of different types of Chl catabolites found in senescent leaves and fruits (Hörtensteiner and Kräutler 2011). Thus, the PAO pathway is active during leaf senescence and in ripening fruits. It can be divided into two parts, (1) reactions on colored intermediates that end in the formation of a primary fluorescent Chl catabolite (*p*FCC) and (2) *p*FCC-modifying reactions, that typically end with the isomerization of modified FCCs (*m*FCCs) to respective nonfluorescent Chl catabolites (NCCs). These two parts of the pathway are also spatially separated in the cell: formation of *p*FCC occurs in plastids whereas subsequent modification and isomerization are localized in cytosol and vacuole, respectively.

This review summarizes the biochemistry of the PAO pathway of Chl breakdown. Some of the newly identified catabolites such as hypermodified FCCs (*h*FCCs) (Hörtensteiner and Kräutler 2011) and urobilinogenoidic Chl

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catabolites (UCCs) (Müller et al. 2011), point to divergent paths of the PAO pathway that might exist in some plant species.

## NCCs, the final chlorophyll breakdown products

The pioneering identification and structure determination of the first (final) Chl breakdown product, a NCC from barley, named *Hv*-NCC-1, in 1991 (Kräutler et al. 1991) marked a milestone in the elucidation of Chl breakdown, which until then was described as 'biological enigma' (Hendry et al. 1987; Brown et al. 1991). To date more than 15 structures of NCCs have been identified from over 10 plant species. They exhibit an open tetrapyrrole backbone which is identical in all NCCs, but depending on the plant species, NCC patterns can be complex, such as in *Arabidopsis thaliana* (Arabidopsis) with to date five identified NCCs, or simple with one major NCC, as in the deciduous tree *Cercidiphyllum japonicum*. The reason for this complexity arises from the fact that NCCs vary with regard to the chemical constitution of one or more of three side positions depicted as $R^1$–$R^3$ in Fig. 1. Further variability in NCCs is provided by the chiral center of C1, which renders pyrrole ring A in either *R* or *S* configuration (Fig. 1). This depends on the plant species and is the result of the species-specific stereospecificity of red Chl catabolite reductase (RCCR) (Pružinská et al. 2007; Hörtensteiner et al. 2000), which introduced the C1-stereocenter during reduction of red Chl catabolite (RCC) to *p*FCC (see below). Thus, the occurrence of two possible stereoisomers, *p*FCC and *epi*-*p*FCC, ultimately results in C1-isomeric NCCs. With the exception of *At*-NCC-3 from Arabidopsis (Müller et al. 2006), all NCCs identified so far are derived from Chl *a* as indicated by a methyl group at C7 (Kräutler 2003; Kräutler and Hörtensteiner 2006). This specificity is explained by the fact that conversion of Chl *b* to Chl *a* is a prerequisite for Chl breakdown through the PAO pathway (see below).

Having originally been identified from senescing leaves, NCC structure analysis has recently been extended to ripening fruits. The group of B. Kräutler resolved the constitutions of different NCCs from the peels of ripening apples and pears and, in addition, further, so far structurally uncharacterized, NCCs were identified in the peels of

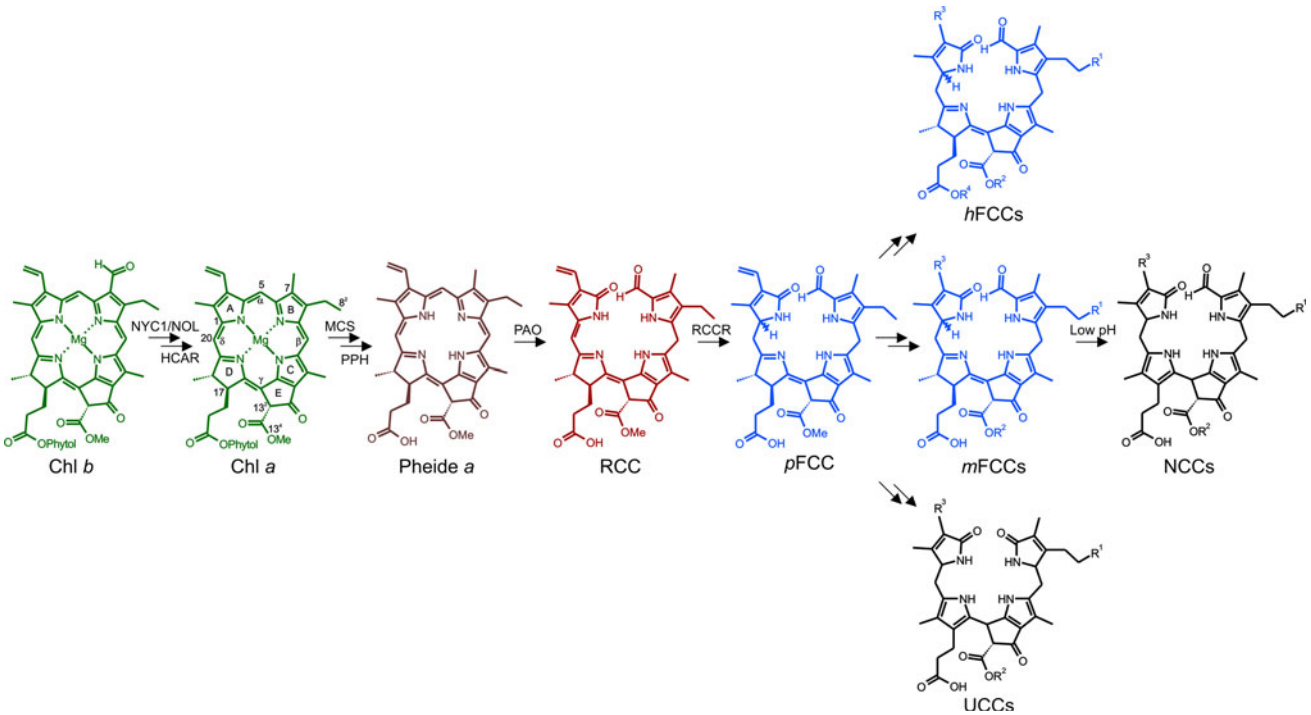


**Fig. 1** The PAO pathway of chlorophyll breakdown. Depicted are the chemical structures of chlorophyll and of chlorophyll catabolites as well as chlorophyll catabolic enzymes involved in the pathway. Note that besides the linear PAO pathway ending in the formation of NCCs, divergent paths of the pathway have been proposed recently after the identification of UCCs and *h*FCCs that occur as persistant catabolites in different plant species. Possibly these catabolites are derived from *p*FCC. Pyrrole rings (A–D), the isocyclic ring (E), methine bridges ($\alpha$-$\delta$) and relevant carbon atoms are labeled in Chl *a*. $R^1$-$R^4$ in FCCs, NCCs and UCCs indicate modifications as outlined in Table 1. *Chl* chlorophyll, *HCAR* hydroxy-Chl *a* reductase, *hFCC* hypermodified fluorescent Chl catabolite, *MCS* metal chelating substance, *mFCC* modified fluorescent Chl catabolite, *NCC* nonfluorescent Chl catabolite, *NOL* NYC1-like, *NYC1* non-yellow coloring1, *PAO* pheide *a* oxygenase, *pFCC* primary fluorescent Chl catabolite, *Pheide* pheophorbide, *PPH* pheophytinase, *RCC* red Chl catabolite, *RCCR* RCC reductase, *UCC* urobilinogenoidic Chl catabolite

yellow bananas (*Musa cavendish*) (Moser et al. 2008a; Müller et al. 2007; Kräutler 2008). These analyses demonstrate that the PAO pathway of Chl breakdown not only occurs during leaf senescence but also during fruit ripening.

NCCs accumulate inside the vacuoles of senescing cells (Matile et al. 1988; Hinder et al. 1996). They are derived from FCCs, which, after vacuolar import (Hinder et al. 1996), rapidly convert to NCCs by an acid-catalyzed isomerization (Oberhuber et al. 2003) (Fig. 2). Thus, in contrast to NCCs, FCCs are generally of low abundance in senescing leaves. Despite this, FCCs could be identified in different species (Ginsburg and Matile 1993; Pružinská et al. 2005; Bachmann et al. 1994) and the constitutions of *p*FCC and of some *m*FCCs have been resolved (Table 1). Interestingly, within a given plant species, the C1-stereochemistry and side modifications found in FCCs and NCCs are identical (Pružinská et al. 2005). This supports the view that modifications of these side positions occur at the level of FCCs. The fluorescence properties of FCCs are due to a Schiff's base (C=N–C=C) configuration of pyrrole ring D and the unsaturated $\gamma$-methine bridge (Fig. 1). Upon isomerization of a FCC to its respective NCC this configuration is lost. Interestingly, it has recently been shown that the speed of FCC-to-NCC isomerization is, at least in part, determined by the structural configuration around the isocyclic ring E (Fig. 1); Arabidopsis *mes16* mutants that are devoid of MES16, a methyl esterase that specifically demethylates the $C13^2$ carboxymethyl group of FCCs (see below), accumulate rather high concentrations of $O13^4$-methylated FCCs. As a consequence, senescent *mes16* leaves strongly fluoresce under UV-light (Christ et al. 2012). Senescent leaves of wild type Arabidopsis mostly accumulate NCCs that are $O13^4$-desmethylated (Pružinská et al. 2005). In vitro FCC-to-NCC isomerization experiments on *p*FCC and $O13^4$-desmethyl *p*FCC under slightly acidic conditions demonstrated that the latter was isomerized three-times faster, indicating that a free carboxyl group at $C13^2$ promotes the isomerization to a NCC (Christ et al. 2012).

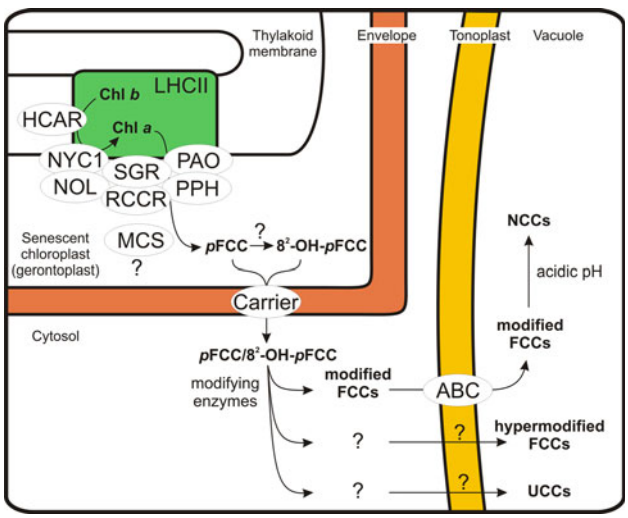


**Fig. 2** Cellular model of the PAO pathway of chlorophyll breakdown. The model shows the main route of breakdown leading to the formation of NCCs. Within gerontoplasts, breakdown of Chl to *p*FCC putatively occurs in a dynamic protein complex involving SGR and the Chl catabolic enzymes. Note, that interaction of HCAR with the other components has not yet been investigated and that the molecular nature of MCS is unknown. The model also indicates divergent paths identified in different plant species that lead to the formation of *h*FCCs or UCCs. These catabolites possibly derive from *p*FCC and possibly accumulate in the vacuole. *ABC* ATP-binding cassette transporter, *Carrier* putative catabolite transporter at the envelope, *SGR* stay-green protein. For further abbreviations see the legend of Fig. 1. Putative steps are labeled with a *question mark*

## Chlorophyll catabolic enzymes

### Reduction of chlorophyll *b* to chlorophyll *a*

Except *At*-NCC-3 (Müller et al. 2006), all FCCs and NCCs are derived from Chl *a*. This is explained, at least in part, by the substrate specificity of PAO for pheophorbide (Pheide) *a* (Hörtensteiner et al. 1995) and suggests that conversion of Chl *b* to Chl *a* is a prerequisite for further degradation via PAO. However, mutants that are devoid of PAO specifically accumulate Pheide *a* (Tanaka et al. 2003; Pružinská et al. 2003), indicating that conversion of Chl *b* to Chl *a* occurs upstream of Pheide formation.

Chl *b*-to-Chl *a* conversion is carried out in two reductive steps with C7-hydroxymethyl Chl *a* as an intermediate. The two enzymes catalyzing these consecutive reactions, Chl *b* reductase and hydroxymethyl Chl *a* reductase (HCAR), have different biochemical properties; while the latter is a ferredoxin-dependent, stroma-localized enzyme, Chl *b* reductase localizes to the thylakoid membrane and requires NADPH as electron source (Ito et al. 1996; Scheumann et al. 1998, 1999). Genes encoding both enzymes have recently been identified (Kusaba et al. 2007; Meguro et al. 2011). In Arabidopsis and rice, Chl *b* reductase is encoded by two genes, *NON-YELLOW COLORING1* (*NYC1*) and *NYC1-LIKE* (*NOL*) (Horie et al. 2009; Kusaba et al. 2007; Sato et al. 2009). NYC1 and NOL are members of the family of short-chain dehydrogenases/reductases and share around 50 % sequence identity. *NYC1* expression correlates with leaf senescence (Kusaba et al. 2007). Rice or Arabidopsis *nyc1* mutants exhibit a stay-green phenotype with high retention of light harvesting complex II (LHCII) subunits and Chl *b* during senescence. This supported the assumption that NYC1 encodes Chl

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

| Name | $R^{1c}$ | $R^{2c}$ | $R^{3c}$ | $R^{4c}$ | C1-epimer[d] | Source[e] | Reference |
|---|---|---|---|---|---|---|---|
| *p*FCCs | | | | | | | |
| *p*FCC | H | $CH_3$ | Vinyl | H | 1 | E | Mühlecker et al. (1997) |
| *epi*-*p*FCC | H | $CH_3$ | Vinyl | H | *epi* | E | Mühlecker et al. (2000) |
| *m*FCCs | | | | | | | |
| *At*-FCC-1[a] | OH | H | Vinyl | H | 1 | L | Pružinská et al. (2005) |
| *At*-FCC-2[a] | H | H | Vinyl | H | 1 | L | Pružinská et al. (2005) |
| *h*FCCs | | | | | | | |
| *Mc*-FCC-49[b] | *O*-glucosyl | $CH_3$ | Vinyl | Daucic acid | *epi* | F | Moser et al. (2009) |
| *Mc*-FCC-56[b] | OH | $CH_3$ | Vinyl | Daucic acid | *epi* | F | Moser et al. (2008a) |
| *Ma*-FCC-61[b] | OH | $CH_3$ | Vinyl | Digalactosylglyceryl | *epi* | L | Banala et al. (2010) |
| *Sw*-FCC-62[b] | OH | $CH_3$ | Vinyl | Dihydroxyphenylethylglucosyl | 1 | L | Kräutler et al. (2010) |
| NCCs | | | | | | | |
| *At*-NCC-1[a] | *O*-glucosyl | H | Vinyl | H | 1 | L | Pružinská et al. (2005) |
| *At*-NCC-2[a] | OH | H | Vinyl | H | 1 | L | Pružinská et al. (2005) |
| *At*-NCC-3[a] | OH[f] | H | Vinyl | H | 1 | L | Pružinská et al. (2005) |
| *At*-NCC-4[a] | *O*-glucosyl | $CH_3$ | Vinyl | H | 1 | L | Pružinská et al. (2005) |
| *At*-NCC-5[a] | H | H | Vinyl | H | 1 | L | Pružinská et al. (2005) |
| *Bn*-NCC-1[a] | *O*-malonyl | H | Vinyl | H | 1 | L | Mühlecker and Kräutler (1996) |
| *Bn*-NCC-2[a] | *O*-glucosyl | H | Vinyl | H | 1 | L | Mühlecker and Kräutler (1996) |
| *Bn*-NCC-3[a] | OH | H | Vinyl | H | 1 | L | Mühlecker and Kräutler (1996) |
| *Bn*-NCC-4[a] | H | H | Vinyl | H | 1 | L | Pružinská et al. (2005) |
| *Cj*-NCC-1[a] | OH | $CH_3$ | Vinyl | H | *epi* | L | Curty and Engel (1996) |
| *Cj*-NCC-2[a] | H | $CH_3$ | Vinyl | H | *epi* | L | Oberhuber et al. (2003) |
| *Hv*-NCC-1[a] | OH | $CH_3$ | Dihydroxyethyl | H | 1 | L | Kräutler et al. (1991) |
| *Lo*-NCC-1[a] | OH | $CH_3$ | Vinyl | H | nd | L | Iturraspe et al. (1995) |
| *Ls*-NCC-1[a] | OH | $CH_3$ | Vinyl | H | nd | L | Iturraspe et al. (1995) |
| *Ms*-NCC-2[a] | OH | $CH_3$ | Vinyl | H | *epi* | F | Müller et al. (2007) |
| *Nr*-NCC-1[a] | *O*-glucosylmalonyl | $CH_3$ | Vinyl | H | *epi* | L | Berghold et al. (2004) |
| *Nr*-NCC-2[a] | *O*-glucosyl | $CH_3$ | Vinyl | H | *epi* | L | Berghold et al. (2004) |
| *Pc*-NCC-1[a] | *O*-glucosyl | $CH_3$ | Vinyl | H | *epi* | F | Müller et al. (2007) |
| *Pc*-NCC-2[a] | OH | $CH_3$ | Vinyl | H | *epi* | F | Müller et al. (2007) |
| *So*-NCC-1[a] | OH | H | Dihydroxyethyl | H | *epi* | L | Berghold et al. (2002) |
| *So*-NCC-2[a] | OH | $CH_3$ | Dihydroxyethyl | H | *epi* | L | Oberhuber et al. (2001) |
| *So*-NCC-3[a] | OH | H | Vinyl | H | *epi* | L | Berghold et al. (2002) |
| *So*-NCC-4[a] | OH | $CH_3$ | Vinyl | H | *epi* | L | Berghold et al. (2002) |
| *So*-NCC-5[a] | H | $CH_3$ | Vinyl | H | *epi* | L | Berghold et al. (2002) |
| *Sw*-NCC-58[b] | OH | $CH_3$ | Vinyl | H | 1 | L | Kräutler et al. (2010) |
| *Zm*-NCC-1[a] | *O*-glucosyl | $CH_3$ | Dihydroxyethyl | H | *epi* | L | Berghold et al. (2006) |
| *Zm*-NCC-2[a] | *O*-glucosyl | $CH_3$ | Vinyl | H | *epi* | L | Berghold et al. (2006) |
| UCCs | | | | | | | |
| *Hv*-UCC-1[a,g] | OH | $CH_3$ | Dihydroxyethyl | H | 1 | L | Losey and Engel (2001) |
| *Ap*-UCC-1[a,g] | OH | $CH_3$ | Dihydroxyethyl | H | *epi* | L | Müller et al. (2011) |

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

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

[c] $R^1$–$R^4$ indicate residues at C3, $C8^2$, $C13^2$ and $C17^3$ side positions, respectively, of FCCs, NCCs and UCCs as shown in Fig. 1

[d] C1 stereochemistry refers to the type of *p*FCC, i.e. *p*FCC (1) or *epi*-*p*FCC (*epi*), formed in the respective species or genus; nd, not determined

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

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

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

*b* reductase, although Chl *b* reductase activity could so far not be demonstrated with recombinant NYC1 (Horie et al. 2009; Kusaba et al. 2007). A possible explanation is the hydrophobic nature of NYC1 proteins; they contain three predicted transmembrane spanning domains, fitting the proposed biochemical features of Chl *b* reductase. In contrast, rice and Arabidopsis NOL were both shown to exhibit Chl *b* reductase activity when expressed in *E. coli* (Sato et al. 2009; Horie et al. 2009). NOL, although predicted to be a soluble protein, co-purifies with the thylakoid membrane, which is explained by physical interaction between NYC1 and NOL (Sato et al. 2009; Sakuraba et al. 2012). Interestingly, rice *NOL* mutants have a stay-green phenotype similar to *nyc1* mutants, but in Arabidopsis, loss-of-function of *NOL* does not result in retention of Chl. This indicates that in these two species, NOL could have different roles; in rice NYC1 and NOL are equally important probably by forming a heteromeric complex which is a prerequisite for activity, while in Arabidopsis, NYC1 function seems to predominate NOL.

*HCAR* was recently cloned from Arabidopsis (Meguro et al. 2011). It encodes a chloroplast-localized protein that contains an iron-sulfur cluster and a FAD-binding pocket. Recombinant HCAR catalyzed reduction of C7-hydroxymethyl Chl *a* to Chl *a* in the presence of reduced ferredoxin, in agreement with its proposed biochemical properties (Scheumann et al. 1998). Interestingly, HCAR has high sequence homology to cyanobacterial divinyl reductases, which catalyze reduction of the C8 vinyl moiety of divinyl-protochlorophyllide to an ethyl group during Chl biosynthesis. This indicates that during evolution HCAR was recruited from divinyl reductases to enable Chl *b* to Chl *a* conversion in algae and land plants (Meguro et al. 2011).

## Mg-dechelation and dephytylation

Pheide *a* has been identified as a genuine intermediate of Chl breakdown (Langmeier et al. 1993; Vicentini et al. 1995a) and the establishment of an in vitro assay that converts Pheide *a* to *p*FCC in an PAO-dependent manner (Hörtensteiner et al. 1995) demonstrated that removal of phytol and the central magnesium atom of Chl precedes the chlorine ring opening reaction of PAO. Until recently, uncertainty existed about the order of these two reactions, with phytol removal preceding Mg-chelation being the favored hypothesis (Tanaka and Tanaka 2006; Hörtensteiner 2006). In agreement with this, chlorophyllase, which hydrolyzes phytol from Chl, was believed to be active during senescence-related Chl breakdown (Takamiya et al. 2000). Chlorophyllase genes (*CLH*) were cloned from different species including Arabidopsis and in all cases chlorophyllase activity could indeed be demonstrated with the recombinant enzymes (Tsuchiya et al. 1999; Jakob-Wilk et al. 1999; Arkus et al. 2005; Tang et al. 2004; Chen et al. 2008). Some of the cloned CLHs were predicted to localize outside the chloroplast, i.e. in the cytosol or the vacuole, and subsequent localization experiments gave ambiguous results. For example, the two Arabidopsis CLHs, AtCLH1 and AtCLH2, localized to the cytosol when tagged with green fluorescent protein (Schenk et al. 2007), while lemon CLH was shown by in situ immunofluorescence to reside inside the chloroplast in lemon flavedo tissue and to co-purify with chloroplast membranes after heterologous expression in tobacco mesophyll protoplasts (Harpaz-Saad et al. 2007; Azoulay Shemer et al. 2008). Contradictory results were also obtained in experiments with de-regulated *CLH* expression; absence of AtCLH1 and/or AtCLH2 in respective mutants (Schenk et al. 2007) as well as silencing of *AtCLH1* (Kariola et al. 2005) or *AtCLH2* (Liao et al. 2007) had little effect on senescence-related Chl breakdown, while antisense-suppression of broccoli *CLH* delayed rates of postharvest broccoli head yellowing (Chen et al. 2008). Furthermore, expression patterns of neither *AtCLHs* nor *CLHs* of broccoli or Chinese flowering cabbage correlate with Chl breakdown (Liao et al. 2007; Zimmermann et al. 2004; Büchert et al. 2011; Zhang et al. 2011). By contrast, CLH was convincingly shown to participate in Chl breakdown during fruit ripening of *Citrus* species (Azoulay Shemer et al. 2008; Harpaz-Saad et al. 2007). Further analysis is demanded to elucidate the role of CLHs in leaves and fruits, ideally in a plant species that allows simultaneous analysis of leaf senescence and fruit ripening, such as tomato.

Recently three independent groups (Schelbert et al. 2009; Ren et al. 2010; Morita et al. 2009) succeeded in identifying a candidate esterase for phytol cleavage in Arabidopsis and rice, and in one case was the recombinant Arabidopsis enzyme biochemically analyzed (Schelbert et al. 2009). It exhibited esterase activity towards Pheophytin (Phein) *a* or Phein *b,* yielding the respective Pheide pigment, but did not accept Chls as substrate. This pointed to an intriguing specificity towards metal-free pigments and the enzyme was therefore termed pheophytin pheophorbide hydrolase (pheophytinase; PPH). Consistent with this, Arabidopsis *pph* mutants accumulated Phein during leaf senescence (Schelbert et al. 2009). Arabidopsis PPH, alternatively named CRN1 by Ren et al. (2010), and its rice ortholog, termed NCY3 (Morita et al. 2009), were shown to localize to the chloroplast and deficiency in respective mutants caused a stay-green phenotype. In summary, there is striking evidence that during leaf senescence, at least in Arabidopsis, dephytylation occurs after Mg removal, by the Phein-specific action of PPH.

The mechanism of Mg-dechelation has not been resolved in detail until now. Different types of activities

have been described in the past, one attributed to a heat-stable low-molecular weight compound, termed metal-chelating substance (MCS) (Shioi et al. 1996a; Suzuki and Shioi 2002), the other attributed to heat-labile proteins, termed Mg-releasing proteins (MRP) (Suzuki and Shioi 2002; Vicentini et al. 1995b). However, MRP activity could so far only been shown when using chlorophyllin, i.e. an artificial form of Chl, as substrate, but not with Chlide. This was interpreted as MCS being active in vivo (Kunieda et al. 2005). Considering the fact that Mg-dechelation likely occurs before dephytylation (Schelbert et al. 2009), it is possible that Chlide is not the natural substrate for Mg-dechelation. Therefore, re-examination of MCS and MRP-like activities with Chl as substrate is demanded. Recently, an Arabidopsis gene, At5g17450, was proposed as a candidate for MCS. At5g17450 was identified to be co-expressed, together with PPH and PAO, with proteins that localize to plastoglobules (Lundquist et al. 2012). Plastoglobules are thylakoid-associated lipoprotein particles that are suggested to have an important role in lipid metabolism during different developmental phases, but particularly during senescence (Bréhélin et al. 2007).

## Pheophorbide *a* oxygenase

PAO is a Rieske-type monooxygenase (Pružinská et al. 2003; Hörtensteiner et al. 1998), which contains an additional mononuclear iron center that is responsible for the activation of molecular oxygen (Schmidt and Shaw 2001). Electrons required to supply the iron-redox cycle of PAO are provided by reduced ferredoxin (Ginsburg et al. 1994; Pružinská et al. 2003). PAO proteins contain two C-terminally located transmembrane domains and based on the distribution of its activity, PAO was considered to localize to the chloroplast envelope (Matile and Schellenberg 1996). However, recent re-examination using PAO-green fluorescent protein analysis and immunoblot analysis of chloroplast membrane fractions separated by sucrose density gradients demonstrated PAO to reside in the thylakoid membrane (Sakuraba et al. 2012). As mentioned above, PAO exhibits an intriguing specificity for Pheide *a*, with Pheide *b* being a competitive inhibitor. In 2003, two groups succeeded in identifying Arabidopsis PAO at the molecular level (Pružinská et al. 2003; Tanaka et al. 2003). PAO is identical to ACCELERATED CELL DEATH (ACD) 1 (Greenberg and Ausubel 1993) and the ortholog of LETHAL LEAF SPOT 1 in maize (Gray et al. 1997). The absence of PAO in mutants or antisense lines from different plant species results in premature cell death (Gray et al. 2002; Pružinská et al. 2003, 2005; Greenberg and Ausubel 1993; Tanaka et al. 2003; Spassieva and Hille 2002). Phototoxicity of Pheide *a*, which was shown in many cases to accumulate to high concentrations, is considered to trigger the observed cell death phenotype in a light-dependent manner (Pružinská et al. 2005). However, Arabidopsis PAO-antisense lines were recently shown to exhibit light-independent cell death and a cell death signaling mechanism involving Pheide *a* was proposed (Hirashima et al. 2009). Components of such a pathway have not yet been identified.

## Red chlorophyll catabolite reductase

As mentioned above, RCCR catalyzes the C1/C20 reduction of RCC to *p*FCC. RCCR is a soluble protein of about 30 kDa and, like for PAO, electrons are supplied from reduced ferredoxin. RCCR is distantly related to a family of ferredoxin-dependent bilin reductases (FDBR) (Frankenberg et al. 2001) and the recent elucidation of the crystal structures of Arabidopsis RCCR (AtRCCR) in the absence and presence of RCC confirmed a high degree of structural similarity to these FDBRs (Sugishima et al. 2009, 2010). For the FDBRs, a reaction mechanism has been proposed that involves direct transfer of an electron from ferredoxin to the substrate giving rise to a tetrapyrrole radical intermediate, followed by substrate protonation through a highly conserved glutamate residue present in the FDBRs (Tu et al. 2004, 2008). A similar radical mechanism and involvement of a glutamate residue (glutamate$_{154}$ of AtRCCR) have also been proposed for RCCR (Sugishima et al. 2009), and site-directed mutagenesis confirmed the requirement of glutamate$_{154}$ of AtRCCR for in vitro and in vivo activity (Pattanayak et al. 2012).

The above-mentioned, highly stereospecific formation of *p*FCC, yielding one of two possible C1-stereoisomers, *p*FCC or *epi*-*p*FCC, is defined by RCCR. The analysis of RCCRs from more than 50 plant species (Hörtensteiner et al. 2000; Pružinská et al. 2007), demonstrated presence of either type-1 (forming *p*FCC) or type-2 (forming *epi*-*p*FCC) RCCR in a given species. This specificity was analyzed in vitro with chimeric proteins produced between Arabidopsis (type-1) and tomato RCCR (type-2). This analysis identified phenylalanine$_{218}$ of AtRCCR as crucial for stereospecificity; when changed to valine (as present in tomato RCCR), AtRCCR was switched from *p*FCC to *epi*-*p*FCC production (Pružinská et al. 2007). Interestingly, phenylalanine$_{218}$ is located within the RCC binding pocket in the crystal structure of AtRCCR (Sugishima et al. 2009), but structure analysis of the variant with valine at position 218 did not convincingly explain the altered stereospecificity (Sugishima et al. 2010).

RCCR was molecularly identified more than ten years ago (Wüthrich et al. 2000) and was shown to be identical to *ACD2* (Greenberg et al. 1994; Mach et al. 2001). *acd2* mutants develop a cell death phenotype which highly correlates with the accumulation of RCC and RCC-like

pigments (Pružinská et al. 2007). Cell death is light-dependent and coincides with the production of singlet oxygen (Pattanayak et al. 2012). However, the fact that cell death in *acd2* also occurred in Chl-free root protoplasts, called into question the role of RCCR/ACD2 as a Chl catabolic enzyme (Yao and Greenberg 2006). Complementation of *acd2* with AtRCCR enzymes that exhibited different C1-stereospecificities as outlined above, resulted in corresponding stereospecifically uniform patterns of catabolites (Pružinská et al. 2007). This clearly demonstrated in vivo participation of RCCR in Chl breakdown during senescence.

RCCR was experimentally shown to localize to the chloroplast (Wüthrich et al. 2000), but it also partially localizes to mitochondria, particularly upon stresses, such as pathogen infection or protoporphyrin IX treatment (Yao and Greenberg 2006). In addition, cell death in *acd2* involves an early mitochondrial oxidative burst (Yao et al. 2004). Mitochondrial localization of ACD2 was believed to play a role in preventing this oxidative burst (Yao and Greenberg 2006) and indeed, specific and exclusive targeting of ACD2 to mitochondria rescues the cell death phenotype of *acd2* (Pattanayak et al. 2012). The mechanism of this protection has not been resolved yet, but it is speculated that pro-cell death signals, i.e. possibly RCC, could be mobile in the cell to trigger cell death via mitochondria and mitochondrial-localized ACD2/RCCR could detoxify this signal (Pattanayak et al. 2012).

### $C13^2$-methylester hydrolysis

The diversity of FCCs and NCCs indicate that many different enzymatic reactions occur downstream of *p*FCC (or *epi-p*FCC). For example, in Arabidopsis at least three steps are required to account for the five NCCs found in this species. However, to date only the enzyme hydrolyzing the $C13^2$-methylester has been identified at the molecular level. $C13^2$-demethylated catabolites have so far only been identified in the Brassicaceae, and in the past an enzyme termed pheophorbidase has been described, which is capable of hydrolyzing the $C13^2$-methylester of Pheide (Suzuki et al. 2006). The product of the reaction, $C13^2$-carboxyl pyropheophorbide, was shown to spontaneously decarboxylate to pyropheophorbide (Shioi et al. 1996b), a proposed product of Chl breakdown mainly found in algae and during post harvest senescence (Ziegler et al. 1988; Aiamla-or et al. 2010). Pheophorbidase was cloned from *Raphanus sativus* (Suzuki et al. 2006); it is a serine-type esterase and is highly similar to the members of the Arabidopsis methyl esterase (MES) protein family. In a recent study (Christ et al. 2012), recombinant MES16 was shown to hydrolyze the $C13^2$ methylester of both Pheide *a* and *p*FCC (or *epi-p*FCC), but not of a NCC. However, MES16 localized to the cytosol and was specifically active on FCCs in vivo. Consequently, *mes16* mutants exclusively formed $O13^4$-methylated FCCs and NCCs. Specific action on the level of FCCs was confirmed by chloroplast mislocalization of MES16 in *pao1*, an Arabidopsis mutant deficient in PAO; while *pao1* specifically accumulated Pheide (Pružinská et al. 2005; Tanaka et al. 2003), chloroplast-localization of MES16 caused the accumulation of mainly pyropheophorbide during senescence. This indicated that, although MES16 could theoretically act on Pheide, different localization of Pheide (in the chloroplast) and MES16 (in the cytosol) disables contact between them in vivo (Christ et al. 2012).

## The stay-green protein

In many plant species, stay-green mutants have been identified that show retention of Chl during senescence. As mentioned above, mutations in PPH or NYC1 show such stay-green phenotypes. However, many stay-green mutants from different species are defective in another gene, termed *STAY-GREEN* (*SGR*). Among them are mutants of bell pepper, tomato, rice, meadow fescue, Arabidopsis (termed *nye1*) as well as Gregor Mendel's famous green cotyledon mutant of pea [for a recent review, see Hörtensteiner (2009)]. It is likely that molecular defects in *SGR* are present in further stay-green mutants, such as soybean $d_1d_2$ (Guiamét et al. 1991) and Arabidopsis *ore10* (Oh et al. 2003).

SGR proteins from different species are highly similar and localize to the chloroplast (Hörtensteiner 2009; Park et al. 2007; Sato et al. 2007; Ren et al. 2007). However, a function for SGR remains uncertain. SGR has been shown to physically interact with known Chl catabolic enzymes (CCEs), i.e. NYC1, NOL, PPH, PAO and RCCR, and to also specifically interact with LHCII subunits of the photosynthetic apparatus. At the same time, CCEs interacted among each other and also with LHCII (Sakuraba et al. 2012). Although a stable high molecular weight complex containing all protein components could not be demonstrated, these findings pointed to the possibility of SGR being required to recruit CCEs to the thylakoid membrane as a prerequisite for Chl breakdown. CCE protein–protein interaction at the thylakoid membrane might allow metabolic channeling of Chl and its degradation intermediates to prevent possible pigment phototoxicity. When physical interaction between PAO and RCCR, as an example, was analyzed by bimolecular fluorescence complementation in Arabidopsis protoplasts, interaction could be demonstrated in wild type protoplasts, but not in *nye1* protoplasts, i.e. in the absence of SGR. This points to a crucial structural rather than direct biochemical role for SGR in Chl breakdown.

Besides exhibiting a leaf stay-green phenotype, a *Medicago truncatula SGR* mutant was also affected in nodule senescence (Zhou et al. 2011). This surprising finding indicates that the role of SGR may go beyond its requirement for Chl breakdown; it possibly could have a more general role in nitrogen remobilization, maybe by recruiting proteases for the degradation of protein (complexes) during senescence. In line with this is the fact that all SGR mutants analyzed so far, retain high levels of LHCII subunits (Aubry et al. 2008; Jiang et al. 2007) and a role of SGR in destabilizing Chl-binding protein complexes as a prerequisite for the subsequent degradation of apoproteins and Chl has been suggested (Park et al. 2007; Hörtensteiner 2009). In Arabidopsis, levels of SGR were shown to positively correlate with the extent of development of disease or hypersensitive response symptoms during *Pseudomonas syringae* infections (Mur et al. 2010; Mecey et al. 2011) and phototoxic Pheide *a* was considered to contribute to cell death execution (Mur et al. 2010). These results are in agreement with the light dependency shown for several plant-pathogen interactions and indicate a link between plant responses to pathogen infections and Chl breakdown.

## Subcellular localization of the pathway

The early reactions of the PAO pathway of Chl breakdown, i.e. at least up to the formation of *p*FCC or *epi*-*p*FCC, take place in senescing chloroplasts (termed gerontoplasts) (Fig. 2). In line with this is the observation that intact isolated gerontoplasts are capable of synthesizing *p*FCC (Matile et al. 1992). In addition to *p*FCC, gerontoplasts produce a second more polar FCC (Ginsburg et al. 1994; Schellenberg et al. 1990), which most likely represents the C8-hydroxylated form of *p*FCC (B. Christ and S. Hörtensteiner, unpublished). C8-hydroxylation is found in most FCCs and NCCs structurally characterized so far (Table 1), indicating this modification to be a common reaction of the PAO pathway present in all plant species. By contrast, all further side modifications are species-specific and, as far as they have been characterized (Hörtensteiner 1998; Christ et al. 2012), are indicated to take place in the cytosol. This implies that *p*FCC or C8-hydroxyl-*p*FCC is exported from gerontoplasts. Release of FCCs has been studied in isolated organelles and was shown to require the (extra-plastidial) presence of ATP or UTP (Matile et al. 1992). The molecular nature of Chl catabolite transporter(s) at the chloroplast envelope is unknown, however it is interesting to note that its specificity likely is restricted to linear tetrapyrrole-type catabolites, i.e. RCCs and FCCs. This is deduced from the observation that *acd2* mutants accumulate RCCs in the vacuole (Pružinská et al. 2007). On the other hand, Pheide *a* accumulating in *pao1* is not demethylated by cytosol-localized MES16, but when MES16 is mis-localized to the plastid $O13^4$-demethylation occurs, indicating Pheide to be retained within the gerontoplast (Christ et al. 2012).

Based on their primary amino acid sequence and from biochemical studies, plastid-located CCEs were considered to localize to different sub-chloroplast fractions, i.e. thylakoid (NYC1), stroma (NOL, PPH, RCCR) and envelope (PAO) (Scheumann et al. 1999; Sato et al. 2009; Rodoni et al. 1997; Matile and Schellenberg 1996; Schelbert et al. 2009). As a consequence, Chl pigment shuttling mechanisms between the thylakoid and the envelope have been postulated in the past (Matile et al. 1999; Hörtensteiner 2006). As mentioned above, a detailed study on protein–protein interaction among SGR and CCEs demonstrated that SGR and all CCEs (including PAO) attach to the thylakoid membrane during Chl breakdown (Sakuraba et al. 2012). They possibly form a dynamic multi-protein complex together with LHCII, which likely allows metabolic channeling of phototoxic Chl breakdown intermediates upstream of nontoxic *p*FCC (Fig. 2).

Final Chl catabolites are deposited in the vacuoles of senescing cells (Matile et al. 1988; Hinder et al. 1996). This is certainly true for NCCs, which have been shown to arise from respective FCCs through isomerization catalyzed by the acidic vacuolar sap (Oberhuber et al. 2003). Transport across the tonoplast has been shown to be a primary active process (Hinder et al. 1996), implying the involvement of ATP binding cassette (ABC) type transporters. After expression in yeast, two Arabidopsis transporters, ABCC2 and ABCC3, were shown to be capable of transporting a NCC from canola (Lu et al. 1998; Tommasini et al. 1998). However, an Arabidopsis *ABCC2* mutant was only marginally affected in senescence (Frelet-Barrand et al. 2008), pointing to a possible functional redundancy of different transporters.

## Gene regulation

Chl breakdown is an integral process of leaf senescence, however it is only part of a plethora of structural changes and metabolic processes that are turned on during leaf senescence. Collectively, these processes are termed the 'senescence syndrome' (Lim et al. 2007). Initiation and progression of the syndrome is executed by a complex regulatory network, but to date only a few transcriptional regulators have been identified (Uauy et al. 2006; Wu et al. 2012; Guo and Gan 2006). Transcriptional regulation of Chl breakdown is an integral process within this network. Thus, genes for SGR and most of the CCEs are up-regulated during leaf senescence, independent of the mode of

senescence induction (Buchanan-Wollaston et al. 2005; Van der Graaff et al. 2006; Breeze et al. 2011). Furthermore, the transcriptionally regulated genes of the PAO pathway, i.e. *SGR*, *NYC1*, *PPH*, *PAO* and *MES16* (Ren et al. 2007, 2010; Schelbert et al. 2009; Pružinská et al. 2005; Horie et al. 2009; Christ et al. 2012) are highly co-regulated in Arabidopsis and consequently cluster closely together when performing gene network analyses, for example using the ATTEDII platform (Obayashi et al. 2009). Possibly, among the hundreds of transcription factors that have been shown to be up-regulated during leaf senescence (Balazadeh et al. 2008) are some that specifically target the PAO pathway, but these have so far not been identified.

## Variations of the PAO pathway: new types of 'final' chlorophyll catabolites

### *Hypermodified* FCCs

New types of FCCs, termed *hypermodified* FCCs (*h*FCCs) were recently identified in ripening banana fruits and in senescent banana and peace lily leaves (Banala et al. 2010; Moser et al. 2008a, 2009; Kräutler et al. 2010). In contrast to the transient occurrence of *m*FCCs as intermediary catabolites in senescing leaves that are converted to NCCs (see above), *h*FCCs persist in ripening or senescing tissues. This is explained by the structure of these FCCs with the C17 propionic acid group being conjugated with different moieties (Table 1). As a likely consequence of these C17-modifications, the acid-induced isomerization to NCCs does not occur (Moser et al. 2009), likely because, as proposed by Oberhuber et al. (2003), a free propionic acid side chain at C17 is required for this isomerization. However, it remains to be shown whether *h*FCCs, like NCCs, indeed accumulate inside the vacuole (Fig. 2). The identification of *h*FCCs indicates the existence of a second variant fate of Chl catabolites within the PAO pathway, i.e. C17-modified catabolites persist as FCCs, whereas unmodified ones are converted to NCCs. Interestingly, in banana and peace lily, both NCCs and *h*FCCs occur simultaneously pointing to the possibility that *h*FCCs might have some, so far unidentified, physiological role (Moser et al. 2009).

### UCC, a new type of final chlorophyll catabolite?

In many plant species abundance of NCCs was shown to increase with senescence progression. This supports the view that NCCs (and in some species *h*FCCs) are the final products of Chl breakdown. However, in a recent study on senescent Norway Maple leaves, a new type of Chl-derived linear tetrapyrrole has been identified as the sole Chl catabolite accumulating to amounts that represent more than 50 % of the original Chl present in green maple leaves (Müller et al. 2011). Structure analysis demonstrated this catabolite to exhibit an urobilinogenoidic constitution, i.e. the $\alpha$-methine carbon that bridges pyrroles A and B in Chl and that is retained in FCCs and NCCs as a formyl group attached to ring B is absent in this new catabolite, which is therefore tentatively termed urobilinogenoidic Chl catabolite (UCC) (Fig. 1). Interestingly, an UCC that is structurally related to *Hv*-NCC-1 has been identified in small amounts in senescent barley leaves and it was speculated to be a degradation product of the latter possibly occurring by unspecific oxidation (Losey and Engel 2001). However, the maple UCC differs from all so far analyzed NCCs by an opposite stereochemistry at the $\gamma$-methine carbon, indicating it to derive from an upstream intermediate of Chl breakdown rather than being a degradation product of a (so far unknown) maple NCC (Müller et al. 2011). This indicates that in Norway Maple, a divergent path of the PAO pathway might exist that ends in the formation of UCCs and not NCCs as final degradation products of Chl. It remains to be demonstrated to which extend UCCs represent final Chl catabolites also in other plant species and whether they also accumulate inside the vacuole (Fig. 2).

## Conclusions and outlook

With the resolution of the first structure of a final Chl catabolite twenty years ago (Kräutler et al. 1991) light begun to be shed on the 'biological enigma' of Chl breakdown. Today, the core steps of the PAO pathway have been characterized biochemically and at the molecular level, and respective catabolic intermediates and end products have been identified and structurally characterized from different plant species. Genes for SGR and CCEs are commonly present in land plants, indicating that the PAO pathway is highly conserved from higher plants down to moss. Comparison of CCEs to available genomic sequences of lower plants indicate that RCCR-, PPH- and/or PAO-like proteins might also be present in algae and cyanobacteria (Schelbert et al. 2009; Gray et al. 2004; Pružinská et al. 2007; Thomas et al. 2009), however it remains to be demonstrated whether any of these homologs indeed endoces a catalytically active CCE.

Despite the major progress made recently, several aspects of Chl breakdown remain to be solved. The surprising recent identification of an UCC as the sole degradation product of Chl in Norway Maple and of persistent *h*FCCs e.g. in banana implies that the PAO pathway is more complex than the proposed linear conversion of Chl to NCCs would have implied. An intriguing possibility is

that Chl catabolites could not merely be by-products of Chl detoxification, but might also have some other biological role (Hörtensteiner and Kräutler 2011). NCCs have high antioxidative potential (Müller et al. 2007), and *h*FCCs contribute to the optical appearance of leaves and fruits (Kräutler et al. 2010; Moser et al. 2009). In addition, a naturally occurring oxidation product of the major NCC of *C. japonicum* is yellow colored and contributes to the autumnal leaf color in this deciduous tree (Moser et al. 2008b). However, biological roles for Chl catabolites remain to be experimentally proven. A further future challenge from a biochemical perspective is the molecular identification of the enzymes that convert *p*FCC to *m*FCCs, *h*FCCs and/or UCCs. Except MES16 (Christ et al. 2012), none of the respective genes have been cloned. Regarding the core part of the PAO pathway, i.e. Chl-to-*p*FCC conversion, the mechanism of Mg-dechelation is unclear and remains to be elucidated at the molecular level. Likewise, although transport of Chl catabolites at both the chloroplast envelope and the tonoplast have biochemically been demonstrated, the molecular nature of the transporters is unknown. In summary, future research is demanded to uncover all aspects of the multi-faceted PAO pathway and to fully understand its possible physiological roles.

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