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PROGRESS REPORT

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The loss of green color during chlorophyll degradation—a prerequisite to prevent cell death?

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During plant senescence, chlorophyll (Chl) is degraded to non-fluorescent Chl catabolites (NCCs; Fig. 1a). These linear tetrapyrroles accumulate in the vacuoles of senescing cells and, in many plant species, represent the final products of Chl catabolism (Matile et al. 1988; Kräutler 2003). Despite the billions of tons of Chl disappearing this way every year and the fascinating autumnal color change of deciduous trees resulting from it, most reactions underlying conversion of Chl to NCC have only recently been elucidated (for recent reviews, see Hörtensteiner 1999; Takamiya et al. 2000; Kräutler 2003). Chl breakdown is a multi-step pathway (Fig. 1a) aiming to increase pigment solubility and to abolish the photodynamic properties of Chl by complete disruption of the conjugated π-electron system. Taking this into account, Chl breakdown can be apostrophized as Chl detoxification (Hörtensteiner 1999).

As inferred from the structures of NCCs (Kräutler et al. 1991), the most remarkable structural change is the oxygenolytic opening of the porphyrin macrocycle of Chl. This reaction is catalyzed by the joint action of two enzymes, pheophorbide *a* oxygenase (PaO) and red Chl catabolite reductase (RCCR) converting pheophorbide (pheide) *a* to a primary fluorescent Chl catabolite (pFCC; Fig. 1a; Rodoni et al. 1997a). Several lines of evidence suggest that the two enzymes interact during catalysis, thereby channeling the first porphyrin cleavage product, red Chl catabolite (RCC). Thus, in vitro, RCC does not accumulate in the absence of PaO. In addition, RCCR is sensitive to oxygen, although PaO requires O_2 for incorporation into pheide *a* (Rodoni et al. 1997a; Wüthrich et al. 2000). PaO has been demonstrated to be a non-heme iron-containing monooxygenase, that specifically introduces one oxygen atom of O_2 at the α-methine bridge of pheide *a* (Hörtensteiner et al. 1995, 1998). In addition, PaO is specific for pheide *a* with pheide *b* inhibiting in a competitive manner (Hörtensteiner et al. 1995). PaO is located at the inner envelope membrane of senescing chloroplasts (Matile and Schellenberg 1996). In contrast, RCCR is a soluble chloroplast protein (Wüthrich et al. 2000), suggesting that the site of conversion of pheide *a* to pFCC is at the stromal periphery of the envelope. RCCR stereospecifically reduces the C1/C20 double bond of RCC, thereby forming two possible C1 stereoisomers of pFCC. The source of RCCR determines which one is formed; thus, for example, RCCR of *Arabidopsis thaliana* produces a different isomer than RCCR isolated from tomato (Hörtensteiner et al. 2000b).

Exploiting the biochemical characteristics of PaO, 21 candidate genes for PaO in *Arabidopsis* were identified recently by using functional genomics (Pružinská et al. 2003). One of them, *Accelerated cell death 1* (*Acd1*), was subsequently shown to exhibit PaO activity in vitro after expression in *Escherichia coli*. The properties of the heterologously expressed protein were identical to native PaO. In contrast, a homologue of ACD1, At4g25650, did not exhibit PaO activity (Pružinská et al. 2003). Thus, *Acd1* encodes *Arabidopsis* PaO (AtPaO). AtPaO is a Rieske-type iron–sulfur cluster-containing oxygenase. In *Arabidopsis*, five Rieske-type oxygenases are present which have rather diverse functions (Gray et al. 2002). Besides PaO and At4g25650, the function of which is unknown, chlorophyll *a* oxygenase (CAO; Tanaka et al. 1998) and choline monooxygenase (CMO; Rathinasabapathi et al. 1997) contain Rieske centers. In addition, Tic55, a component of the protein import machinery at the inner envelope (TIC) also belongs to this group of

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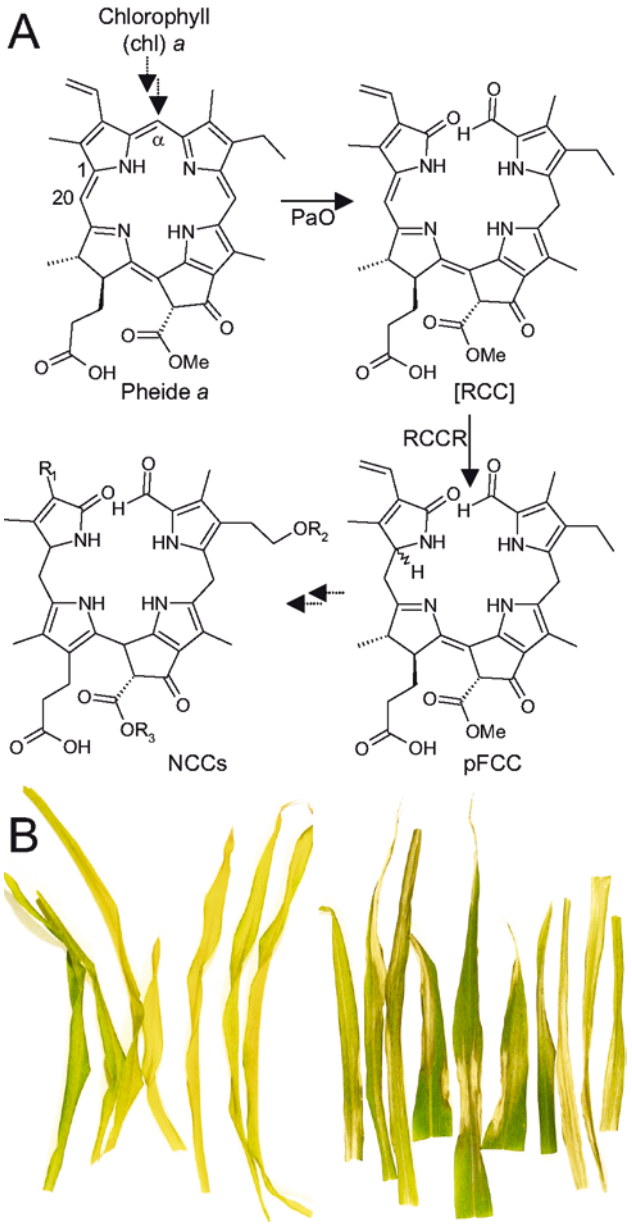

Fig. 1 **a** Pathway of chlorophyll degradation during senescence. Depicted are the structures of the Chl catabolites pheophorbide *a* (*pheide a*), red Chl catabolite (*RCC*), primary fluorescent Chl catabolite (*pFCC*), and non-fluorescent Chl catabolite (*NCC*). The key reaction is catalyzed by the joint action of pheide *a* oxygenase (*PaO*) and RCC reductase (*RCCR*) without release of the intermediate, RCC. Relevant carbon atoms and the α-methine bridge that is cleaved by PaO are labeled. R_1, R_2 and R_3 in the NCCs indicate species-specific differences (Kräutler 2003). **b** Leaves from wild-type maize at a senescent stage (*left*) and from *lls1* showing a lesion mimic phenotype (*right*)

oxygenases (Calibe et al. 1997), although an enzymatic (oxygenase) activity has not been demonstrated in this case. Rieske-type oxygenases are widely distributed in pro- and eukaryotes. In all cases, electrons necessary to drive the redox cycle of the Rieske center irons are provided from reduced ferredoxin (Fd; Schmidt and Shaw 2001). Accordingly, PaO, CAO and CMO are Fd-dependent enzymes (Schellenberg et al. 1993; Rathinasabapathi et al. 1997; Tanaka et al. 1998). Fd is kept in the reduced state by the activity of a reductase. In higher plants, the nature of this reductase is unknown so far, but possibly Fd-NADPH oxidoreductase (FNR) is involved. It has been shown that another protein of the protein import machinery at the inner envelope, Tic62, is able to bind FNR (Küchler et al. 2002).

The RCCR gene has been identified using classical protein purification and PCR-based cloning strategies (Rodoni et al. 1997b; Wüthrich et al. 2000). RCCR is a novel protein that does not have high homology to other reductases, but is distantly related to a family of Fd-dependent bilin reductases, necessary for the biosynthesis of phycobilins and the phytochrome chromophore (Frankenberg et al. 2001). In contrast to other Fd-dependent enzymes, RCCR and the bilin reductases appear to lack a metal or flavin cofactor. Thus, electron transfer is believed to occur directly from reduced Fd to the respective substrates (Frankenberg and Lagarias 2003). In this respect, RCCR would rather be active as a "chaperone", mediating the interaction of Fd and RCC (at this stage still bound to PaO), and controlling the regio- and stereoselective reduction (Kräutler 2003). When comparing the amino acid sequences of different RCCRs, no obvious domains can be identified that could be responsible for the stereospecificity. Therefore, chimeric proteins that were composed of portions of RCCR from tomato and *Arabidopsis* were produced in *E. coli* and their stereospecificity analyzed. It turned out that a Phe-to-Val exchange at position 218 was sufficient to change the stereospecificity of the *Arabidopsis* RCCR (I. Anders and S. Hörtensteiner, unpublished). Interestingly, Phe^{218} lies adjacent to a stretch of four amino acids that is absent in the bilin reductases (Frankenberg et al. 2001).

The rather complex PaO/RCCR reaction represents a key step of the entire Chl catabolic pathway. Thus, PaO activity is restricted to senescence (Schellenberg et al. 1993; Hörtensteiner et al. 1995), whereas activities of other enzymes of the pathway, such as chlorophyllase (catalyzing the initial removal of phytol from Chl) or RCCR are constitutive (Trebitsh et al. 1993; Rodoni et al. 1997a; Jakob-Wilk et al. 1999). Surprisingly, both PaO mRNA and protein are present in non-senescent leaf tissue (Gray et al. 2002; Pružinská et al. 2003). Although PaO expression is up-regulated to some extent upon senescence induction, at the same time the increase in activity is a magnitude higher (Pružinská et al. 2003). From this it is concluded that PaO is regulated on the posttranscriptional level as well. So far, the nature of this proposed regulation has not been elucidated.

The joint reaction of PaO and RCCR is responsible for the loss of green pigment color. In this respect, it is most important for Chl detoxification during senescence. The importance of Chl catabolism for plant survival can be inferred from the analysis of Chl catabolic mutants. Different mutants have been identified that are defective in either PaO or RCCR. These include *Ara-*

bidopsis accelerated cell death 1 (*acd1*; Greenberg and Ausubel 1993) and maize *lethal leaf spot 1* (*lls1*; Gray et al. 1997), in which the maize homologue of *AtPaO* is affected (Fig. 1b), and *Arabidopsis acd2*, which is defective in RCCR (Greenberg et al. 1994; Mach et al. 2001). All of these mutants develop cell death lesions on their leaves in an age-dependent fashion. The phenotype is similar to the induction of defense reactions in pathogen resistance; thus, respective mutants are termed lesion mimic mutants (Mach et al. 2001). The affected genes were believed to be involved in a cell death suppression mechanism either directly (affecting a signal cascade?) or through, for example, the removal of toxic molecules (Greenberg and Ausubel 1993; Gray et al. 1997). In favor of the latter was the finding that in *lls1* a cell death-inducing signal was derived from plastids and lesion formation was light dependent (Gray et al. 2002). Indeed, both PaO and RCCR mutants accumulate Chl catabolites (pheide *a* in *lls1* and *acd1*, and RCC in *acd2*) upon dark-induced senescence. In addition, the content of these catabolites positively correlates with cell death progression of the respective mutants (Pružinská et al. 2003; A. Pružinská and S. Hörtensteiner, unpublished results). Thus, in these mutants, the accumulation of photoreactive Chl catabolites can be suggested to cause the production of reactive oxygen species that in turn induce cell death (Mach et al. 2001). Surprisingly, other mutants have been described that do not develop an apparent cell death phenotype although they also accumulate pheide *a* due to reduced PaO activities (Vicentini et al. 1995; Thomas et al. 1996). Besides pheide *a*, these mutants also accumulate chlorophyllides, indicating that they have a genetic defect that is different from *acd1* or *lls1*. On the other hand, several lesion mimic mutants have been identified that are affected in genes of Chl biosynthesis (Hu et al. 1998; Meskauskiene et al. 2001; Ishikawa et al. 2001).

Altogether, it can be concluded that functional Chl metabolism, i.e. biosynthesis, turnover and degradation, is important to prevent the accumulation of photodynamic intermediates. Furthermore, it becomes obvious that Chl degradation via the PaO/RCCR pathway is a vitally important process during plant senescence. Quite likely it is also involved in cellular responses to a variety of stresses that are linked to Chl breakdown, such as the hypersensitive reaction. The photodynamic properties of Chl enable the conversion of light energy to chemical energy during photosynthesis, but during senescence, photodynamism may turn into a threat. Thus, parallel to the ''invention'' of Chl and the evolution of oxygenic photosynthesis, plants evolved a mechanism for the detoxification of Chl. In unicellular photosynthesizers, such as *Chlorella protothecoides*, RCCR is absent and, consequently, RCC-like compounds are excreted into the medium (Engel et al. 1991; Hörtensteiner et al. 2000a). However, the development of multicellular plants required, in addition to PaO, the appearance of RCCR to enable the safe disposal of Chl catabolites inside the vacuole.

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