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A novel blue fluorescent chlorophyll catabolite accumulates in senescent leaves of the peace lily and indicates a split path of chlorophyll breakdown

Bernhard Kräutler [a,b,*], Srinivas Banala [a,b,1], Simone Moser [a,b,2], Clemens Vergeiner [a,b], Thomas Müller [a,b], Cornelius Lütz [c], Andreas Holzinger [c]

[a] Institute of Organic Chemistry, University of Innsbruck, A-6020 Innsbruck, Austria
[b] Centre of Molecular Biosciences, University of Innsbruck, A-6020 Innsbruck, Austria
[c] Institute of Botany, Department of Physiology and Cell Physiology of Alpine Plants, University of Innsbruck, A-6020 Innsbruck, Austria

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ABSTRACT

Colorless, non-fluorescent Chl-catabolites (NCCs) are the typical, ubiquitous products of chlorophyll (Chl)-breakdown in senescent leaves. However, a fluorescent Chl-catabolite (FCC) accumulated in de-greened leaves of *Spathiphyllum wallisii* (Peace Lily), which showed a weak blue luminescence. The FCC, named *Sw*-FCC-62, was 'hypermodified' with an unprecedented 6-(2-[3,4-dihydroxy-phe-nyl]-ethyl)-β-glucopyranosidyl ester at the propionyl group. Such esters stabilize FCCs against their typical and rapid, spontaneous isomerization to NCCs. Chl-breakdown in *Sp. wallisii* thus branches off from the 'common' path in leaves, and furnishes unique and 'persistent' FCCs. Our findings on 'hypermodified' FCCs also call for attention as to possible physiological roles of Chl-catabolites in plants.

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1. Introduction

Breakdown of chlorophyll (Chl) is a visual hallmark of leaf senescence [1–3]. Non-fluorescent Chl-catabolites (NCCs) occur in leaves of various higher plants as characteristic colorless, tetra-pyrrolic degradation products of Chl [4–6]. NCCs were also detected in ripening apples and pears [7]. Chl-breakdown was thus suggested to follow a common path in senescent leaves and ripening fruit, and to yield the 'invisible' NCCs as its 'final' stage [7,8] (Fig. 1).

In striking contrast, in freshly senescent yellow leaves of the peace lily (*Spathiphyllum wallisii*) a major fluorescent Chl-catabolite (FCC) accumulated, as reported here (see Fig. 2). Minute amounts of fluorescent compounds were observed earlier during active stages of leaf de-greening as presumed Chl-breakdown products [9]. Two of these were identified as FCCs, unstable precursors of NCCs [10,11]. In banana peels (*Musa acuminata*) and in senescent banana leaves, accumulation of 'persistent' FCCs was discovered recently [12–14].

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

High performance liquid chromatography (HPLC)-grade solvents were from Acros Organics (Geel, Belgium), other chemicals from Fluka (Buchs, Switzerland). Ultrapure water, 18 M Ω cm^{-1},
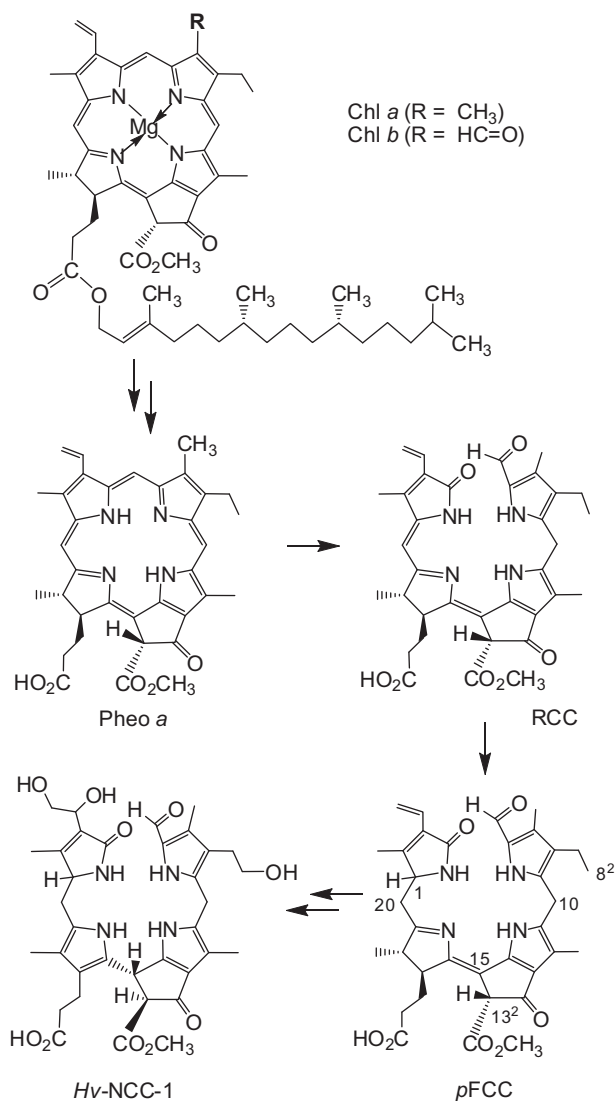
Fig. 1. Structural overview of a typical path of Chl-breakdown in senescent leaves: the Chls are degraded by enzyme catalyzed processes via pheophorbide a (Pheo *a*) and the red Chl-catabolite (RCC) to the 'primary' fluorescent Chl-catabolite (*p*FCC), or its (C-1)-epimer, *epi-p*FCC [4,22]. FCCs with a free propionic acid group are indicated to be imported into the vacuole and to isomerize rapidly to non-fluorescent Chl-catabolites (NCCs), such as *Hv*-NCC-1. *Hv*-NCC-1 is a tetrapyrrolic catabolite in barley leaves (*Hordeum vulgare*) [6] and is derived from *p*FCC [4].

was from a Millipore apparatus, Sep-Pak-C18 Cartridges from Waters Associates; pH-values were measured with a WTW Sentix 21 electrode and a WTW pH535 digital pH meter.

2.1.2. Plant material

A peace lily (*Sp. wallisii*) (see Supplementary material (Supp-Mat), Fig. S1) grew inside under normal day light, and developed naturally senescent leaves sporadically (see Fig. 2). Yellow senescent *Sp. wallisii* leaves were freshly cut and frozen with liquid nitrogen and either stored at −80 °C, or they were extracted directly (see below).

2.2. Chromatographic methods

HPLC: Hewlett Packard HPLC-system, P1100 pump, online degasser, Agilent1100 diode detector and fluorescence detector. Injection loop 1 ml (Rheodyne valve), Hypersil ODS 5 μm 250 × 4.6 mm

i.d. column (at 20 °C) connected to a Phenomenex ODS 4 mm × 3 mm i.d. precolumn, flow rate 0.5 ml min^{-1}. Data were collected and processed with HP Chemstation. *Solvent systems: analytical HPLC*: 100 mM K-phosphate (pH 7)/methanol (MeOH)/ water. *Semi-preparative HPLC*: acetonitrile/water (see SuppMat for details).

2.3. Extraction and isolation of Chl-catabolites

10.8 g (wet weight) of freshly harvested yellow *Sp. wallisii* leaves were frozen in liquid N_2 and were mixed with 5 g of sea sand. The cold mixture was ground finely under dim light. MeOH (6 ml) was added, and the cold slurry was filtered through a Buchner funnel. Extraction with MeOH was repeated 4 times. The collected extracts were concentrated to 3 ml at reduced pressure. The resulting mixture was diluted with 7 ml of water and the suspension was centrifuged for 5 min at 12 000 rpm (about 12 700×g). The precipitate was removed and the yellow-orange supernatant was centrifuged again. The clear supernatant was transferred into a flask and the volume reduced to roughly 4 ml on a rotary evaporator at <20 °C. The concentrated extract was stored overnight at 4 °C for further use.

For analysis and isolation of Chl-catabolites, the extract, obtained as described, was partitioned into 12 portions of about 0.3 ml, which were diluted with 0.7 ml of water each, before injection into the HPLC-system (using the solvent system for semi-preparative work). A fraction was collected in each run that contained an FCC, according to on-line spectra (*Sw*-FCC-62). An NCC fraction (*Sw*-NCC-58) was isolated similarly. Two less abundant other fractions, an FCC (*Sw*-FCC-66) and an NCC (*Sw*-NCC-51) were identified tentatively by their UV- and fluorescence spectra [15,16]. The fraction containing *Sw*-FCC-62 was concentrated to 3 ml under reduced pressure and re-purified by HPLC, using the solvent system described above. The sample of isolated *Sw*-FCC-62 (1.27 μmol, calculated based on log (ε_{318nm}, in MeOH) = 4.29 of *Mc*-FCC-56 [12]) was used for the subsequent spectroanalytical characterization. A sample of *Sw*-NCC-58 was isolated and characterized similarly (see below and SuppMat).

2.4. Spectroscopic analysis of Chl-catabolites

2.4.1. General

Ultraviolet/visible (UV/Vis): Hitachi U-3000. Fluorescence: Varian Cary Eclipse. *Circular dichroism (CD)*: Jasco J715. *Nuclear magnetic resonance (NMR)*: Varian Unity Inova 500 MHz spectrometer. *High resolution-electrospray ionization-mass spectra (HR-ESI-MS)*: Bruker Fourier transform ion cyclotron resonance (FT-ICR), polyethylene glycol mass standard, (+)-ion mode, MeOH as solvent. *Matrix assisted laser desorption-ionization-time of flight (MAL-DI-TOF)*: Bruker Ultraflex, (+)-ion mode, 2,5-dihydroxybenzoic acid as matrix.

2.4.2. Spectroscopy of Sw-FCC-62

UV/Vis (in MeOH) λ_{max} (ε_{rel}) = 379sh (0.50), 358 (0.68), 316 (1.00). Fluorescence (*ca.* 30 μM in MeOH, λ_{ex} at 356 nm): λ_{max} = 441 nm (see Fig. 2). ^{1}H NMR- and ^{13}C NMR-spectra, see SuppMat Figs. S3 and S4). HR-ESI-MS: 943.3976 ([M + H]$^+$), m/z_{calc} ($C_{49}H_{59}N_4O_{15}^+$) = 943.3971 (*ESI-MS-MS* in SuppMat).

2.4.3. Spectroscopy of Sw-NCC-58

UV/Vis (in MeOH) λ_{max} (ε_{rel}) = 220sh (1.36), 245 (1.04), 316 (1.00). CD (in MeOH, 2.4 10^{-5} M) $\lambda_{min/max}$ (nm)/($\Delta\varepsilon$) = 226 (12.6), 250 (−4.4), 259 (−4.1), 282 (−12.1), 324 (5.6). MALDI-MS: m/z (%) = 667.33 ([M+Na]$^+$), 645.33 ($C_{35}H_{40}N_4O_8^+$ = [M+H]$^+$), 522.16 ([M−ringA]$^+$).
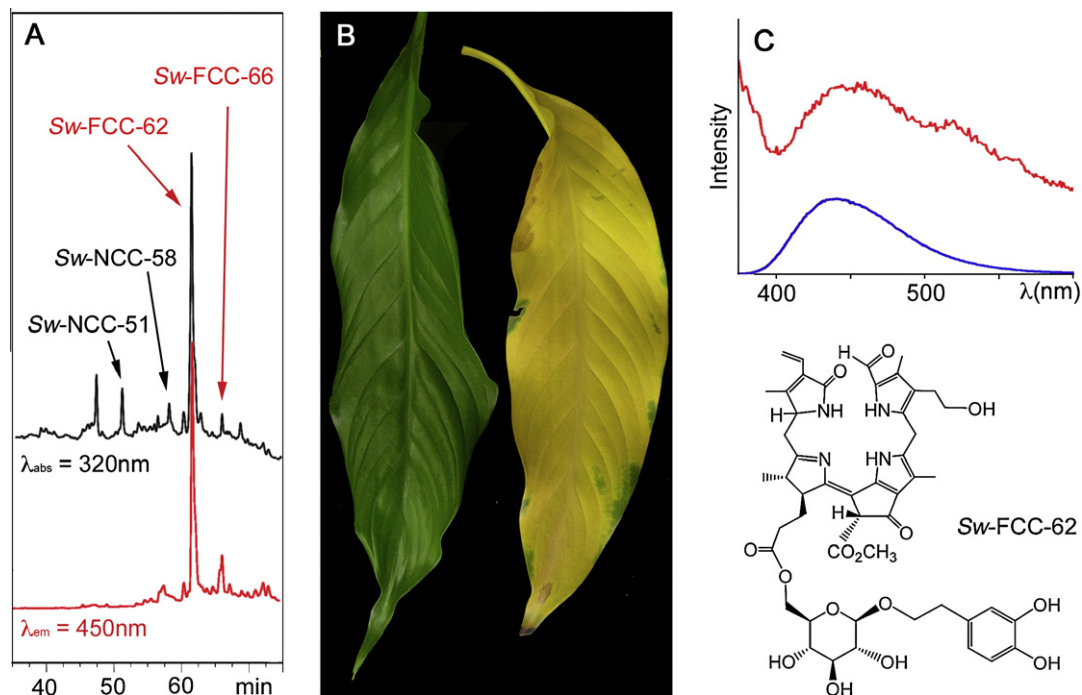
Fig. 2. (A) HPLC-analysis of senescent *Sp. wallisii* leaves. Black trace: Chl-catabolites detectable by absorbance at $\lambda_{abs} = 320$ nm; red trace: detection by luminescence at $\lambda_{em} = 450$ nm. (B) Pictures of a green and of a yellow (senescent) *Sp. wallisii* leaf. (C) In vivo fluorescence spectrum of a yellow *Sp. wallisii* leaf (red trace), fluorescence spectrum of *Sw*-FCC-62 in MeOH solution (at 293 K, blue trace) and formula of *Sw*-FCC-62.

2.5. Light and fluorescence microscopy

Cross and epidermal surface sections of green and senescent yellow *Sp. wallisii* leaves were cut with a razor blade. Sections were viewed at a Zeiss Axiovert 200 M microscope, equipped with a Zeiss Axiocam MRc5 (Carl Zeiss AG, Jena, Germany). For fluorescence images either filter set 01 (excitation BP 365/12 nm, emission LP 397 nm) or filter set 09 (excitation BP 450–490 nm, emission LP 515 nm) were used (see Fig. 4).

3. Results

3.1. Identification of major Chl-catabolites

A fresh extract of senescent leaves of the peace lily (*Sp. wallisii*) contained a major FCC at retention time (t_R) = 62 min (named *Sw*-FCC-62), a minor FCC component at t_R = 66 min (*Sw*-FCC-66), and two NCC-fractions, at t_R = 51 and 58 min, as revealed by HPLC (see Fig. 1). From 10.8 g of a yellow leaf *Sw*-FCC-62 (1.27 µmol) was isolated and classified by its UV/Vis- and fluorescence spectra (see Fig. 2). The molecular formula of *Sw*-FCC-62 ($C_{49}H_{58}N_4O_{15}$) was deduced from observation of $[M+H]^+$ at m/z = 943.398 $[(m/z)_{calc}(C_{49}H_{59}N_4O_{15}^+)$ = 943.397]. A fragment ion at m/z = 789.21 indicated loss of the aglycon moiety.

A 500 MHz ^{1}H NMR spectrum of *Sw*-FCC-62 showed the set of the characteristic signals of an FCC-moiety. The constitution of *Sw*-FCC-62 was deduced from detailed 2-dimensional NMR experiments [17,18] (see SuppMat and Fig. S4). Signals of all 35 non-exchangeable protons of the tetrapyrrole moiety were assigned, identifying it as a functionalized FCC (see e.g., [15,19]). The complex signals of 14 additional hydrogen atoms identified a glucopyranose moiety and a 2-(3,4-dihydroxyphenyl)-ethyl unit as peripheral modification of this *Sw*-FCC, as well as the β-configuration at the anomeric center of the sugar unit. The attachment site of this functionalized glucopyranoside unit at the propionate group of the FCC-core was deduced from a ^{1}H, ^{13}C-heteronuclear correla-

tion. *Sw*-FCC-62 was thus identified as a $3^1,3^2$-didehydro-8^2-hydroxy-13^2-(methoxycarbonyl)-17^3-[6′-β-glucopyranosyl-($1′ \rightarrow 1″$)-(2-[3,4-di-hydroxyphenyl]-ethyl)]-1,4,5,10,17,18,20-(22*H*)-octahydro-4,5-secophytoporphyrin (Figs. 2 and 5).

A similar spectroscopic analysis of *Sw*-NCC-58 showed its molecular formula as $C_{35}H_{40}N_4O_8$, and its constitution as a $3^1,3^2$-didehydro-8^2-hydroxy-13^2-(methoxy-carbonyl)-1,4,5,10,15,20-(22*H*,24*H*)-octahydro-4,5-seco-phytoporphyrin. This indicated *Sw*-NCC-58 to carry a free propionic acid function and to have the same molecular constitution as *Cj*-NCC-1 [20,21] (see Fig. 3). The CD-spectrum of *Sw*-NCC-58 was similar to that of *Cj*-NCC-1 [20], indicating a common configuration at C(15) and C(13^2) [19]. However, HPLC-analysis indicated *Sw*-NCC-58 and *Cj*-NCC-1 to be different. The structural mismatch between *Sw*-NCC-58 and *Cj*-NCC-1 was thus assigned to the stereochemistry at C(1) [10,11], classifying these two NCCs as C(1)-epimers.

3.2. Optical analysis of green and yellow leaves of Sp. wallisii

De-greened, yellow leaves of *Sp. wallisii* showed weak blue luminescence with a broad emission maximum near 450 nm (see Fig. 2). Green and yellow, senescent *Sw*-leaves were also studied by bright field and *epi*-fluorescence microscopy (see Fig. 4). Senescent leaves displayed a marked blue fluorescence in mesophyll cells (in palisade parenchyma as well as in spongy parenchyma).

4. Discussion

In this study, naturally de-greened (yellow) leaves of the tropical evergreen *Sp. wallisii* were examined for their Chl-catabolites and FCC was found to accumulate prominently. In most earlier investigations of senescent leaves, FCCs were only observed as minor components and as transient precursors of NCCs [4,15,22,23]. The main FCC from *Sw*-leaves, named *Sw*-FCC-62, was remarkably abundant (>13% of the Chl in a green leaf). The structure of *Sw*-FCC-62 was deduced and it was revealed to carry
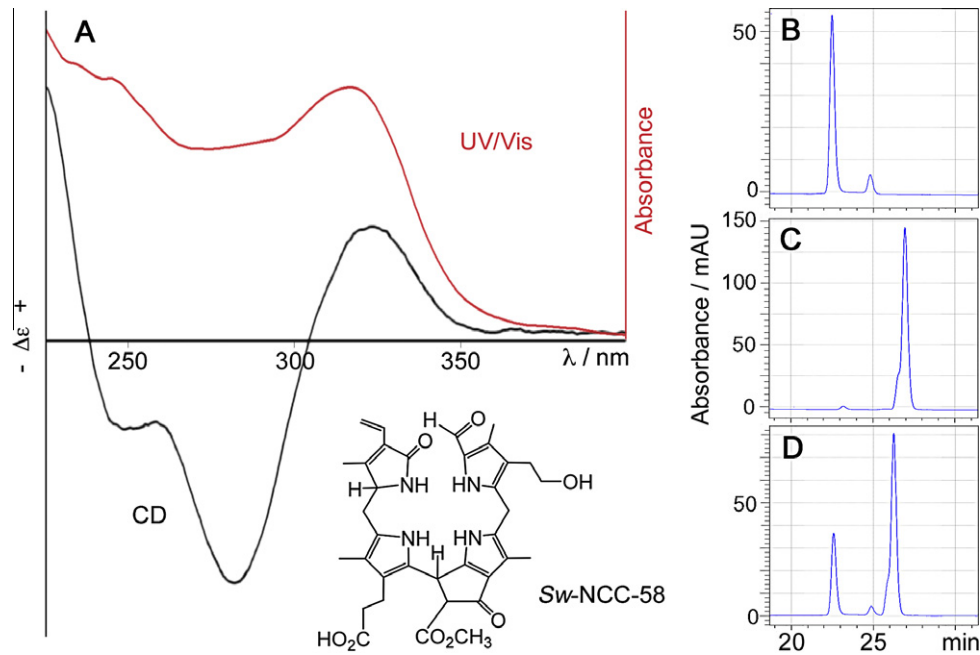
Fig. 3. (A) UV-spectrum (red) and CD-spectrum (black trace) and formula of *Sw*-NCC-58, (B) HPLC-analysis of *Sw*-NCC-58, (C) of *Cj*-NCC-1 and (D) of a mixture of these two epimeric NCCs (bottom).
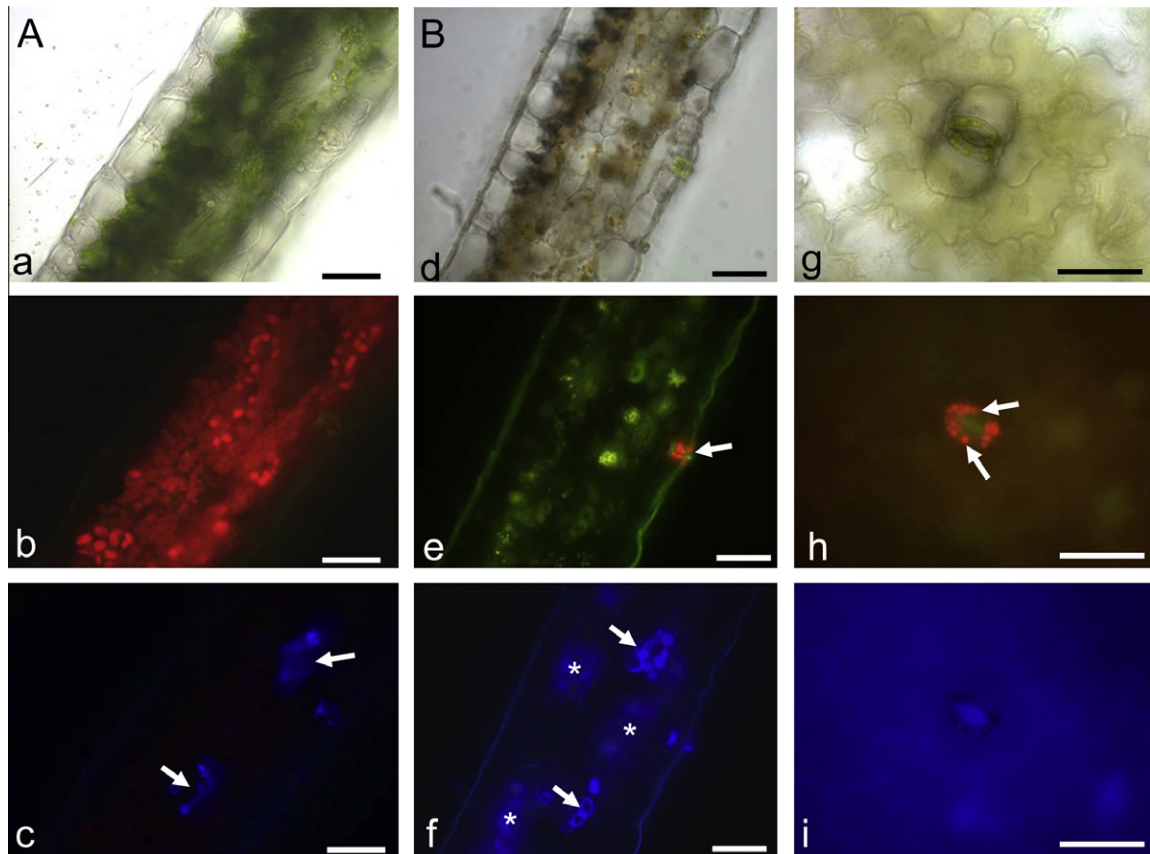

Fig. 4. (A) Green leaf of *Spathiphyllum wallisii*. Light microscopic, bright field (a) and fluorescence microscopic (b and c) images of a cross section of a green leaf, (b) excitation with blue-green light, notice chloroplast fluorescence throughout the mesophyll, (c) excitation with UV-light (see text for details), notice blue fluorescence in cell walls of xylem and fiber tissue (arrows). (B) Senescent yellow leaf of *Sp. wallisii.* Light microscopic, bright field and fluorescence microscopic images of cross section (d–f) and epidermal surface section (g–i). The first image in a row shows bright field images (d and g), the second image shows fluorescence microscopy, blue-green light excitation (e and h), and the third image UV-excitation (f and i); whereas red chloroplast fluorescence is virtually missing in the mesophyll, it remains in stomatal guard cells (arrows in e and h); blue fluorescence is seen in the palisade parenchyma cells (marked by asterisks), which is to distinguish from the blue cell wall fluorescence of xylem and fiber tissue (arrows). Bars 50 μm.

a complex β-glucopyranosyl-(1→1)-2-(3,4-dihydroxyphenyl)-ethyl ester function at the propionyl side chain. Precedence for a natural esterification of a tetrapyrrole with such a group is lacking, and Sw-FCC-62 is a new type of 'hypermodified' FCC. β-(2-[3,4-Dihydroxyphenyl]-ethyl)-glucopyranose (also called 'dopaol-β-glucopyranoside') is a natural product, that occurs e.g., in (extracts of) several species of the genus Chelone (Scrophulariaceae) [24], the stem bark of Syringa velutina [25], and the thalloid liverwort Marchantia polymorpha [26].

Sw-FCC-62 is also a new member of the seemingly unique 'persistent' FCCs, which are resistant against their (chemical) conversion to the corresponding NCCs [19,21]. Related FCCs were found to accumulate and induce blue luminescence in yellow M. acuminata bananas [12], in senescent sections of peels of overripe bananas [14], and in senescent banana leaves [13] (see SuppMat, Fig. S2).

In typical senescent Sw-leaves (as depicted in Fig. 2) NCCs were detected as minor components. One of these NCCs, named Sw-NCC-58, was deduced to be the C(1)-epimer of Cj-NCC-1, an NCC from the deciduous tree Cercidiphyllum japonicum [20,21]. Chl-breakdown in Sp. wallisii was thus indicated to (involve red Chl-catabolite reductase RCCR-1 [27] and to) pass through the stage

of pFCC [10]. In contrast, in peels of ripening bananas, the complementary stereo-chemical lineage was revealed [28], which passes the stage of epi-pFCC [11], the C(1)-epimer of pFCC.

Accumulation of the blue luminescent Sw-FCC-62 in senescent Sw-leaves indicates Chl-breakdown to end up in an FCC, to a large extent. The presence of ester functions at the propionyl side chain in this 'persistent' FCC and the detection of lesser amounts of NCCs in yellow Sw-leaves, are both in line with the hypothesis on the natural transformation of FCCs to NCCs [21,22]: In senescent leaves, NCCs are indicated to result in the acidic medium of the vacuoles from a rapid stereo-selective isomerization of FCCs, which is accelerated by their free propionic acid group [4,19]. Indeed, NCCs, the non-fluorescent tetrapyrrolic 'end' products of Chl-breakdown carry a propionic acid group and are found in the vacuoles [3,29].

Most peripheral modifications observed in NCCs are likely to occur already at the stage of the corresponding FCCs and to result from transformations catalyzed by cytosolic enzymes (see e.g. [22]). Polar FCCs were thus suggested to arise from pFCC (or epi-pFCC) before their transport into the vacuole [4,22]. Hydroxylation at C(8^2) of the ethyl group of ring B (to give 8^2-OH-pFCC or 8^2-OH-epi-pFCC) appears to be the first (typical) modification of the pri-
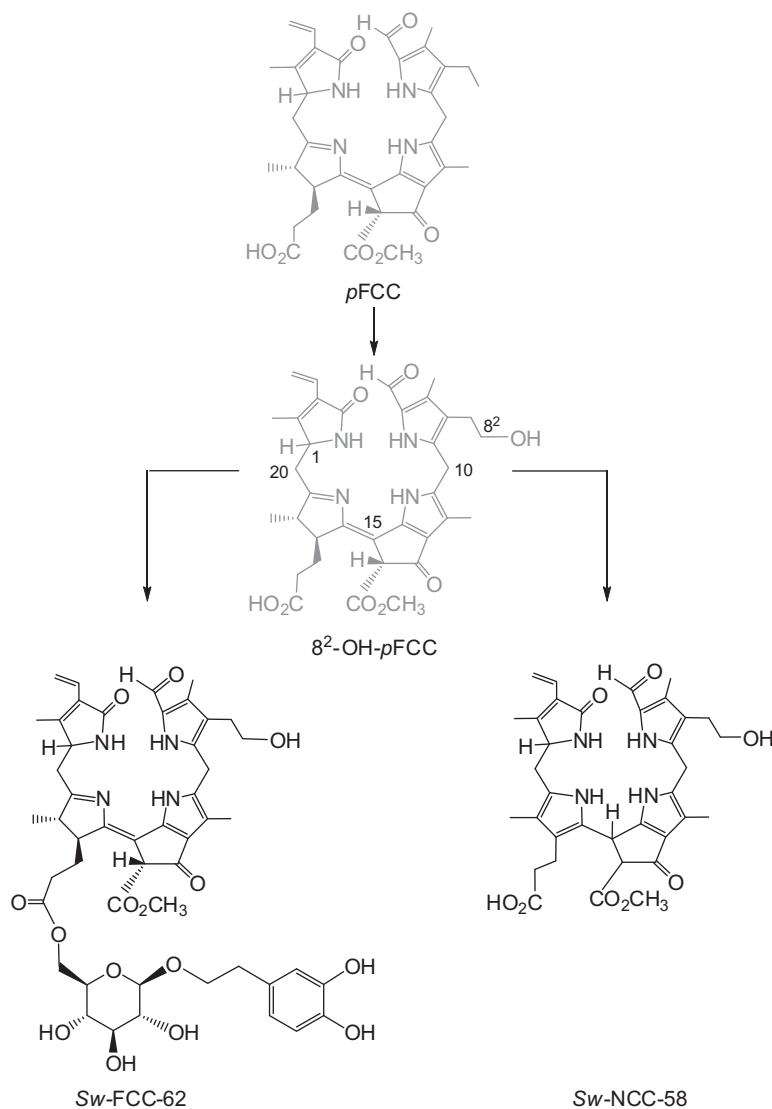

Fig. 5. In senescent leaves of Sp. wallisii the 'primary' FCC (pFCC) and its hydroxylation product (82-OH-pFCC) are likely (but unobserved) intermediate FCCs. 8^2-OH-pFCC is suggested to be imported into the vacuole and isomerized there to Sw-NCC-58, or to be converted to Sw-FCC-62, the accumulating, 'persistent' FCC with a propionate ester function.

mary FCCs (*p*FCC or *epi-p*FCC). This chemically demanding reaction is indicated by the structures of (the correspondingly functionalized) NCCs to occur in senescent leaves very effectively, and only minute amounts of *p*FCCs (or '*p*NCCs', their isomerisation products) are detectable in the extracts [4,22]. The hydroxylating enzyme has not been identified, but it has been suggested to be active in the cytosol, or even in the gerontoplast (see Fig. 6) [4,30].

In senescent *Sw*-leaves the direct formation of *Sw*-FCC-62 is presumed to occur next and, possibly, is achieved by a single enzymatic esterification of the hypothetical 8^2-OH-*p*FCC. The inferred enzyme, which remains to be identified, is likely to reside in the cytosol, where modifications of FCCs are generally believed to occur [22]. By this key step *Sw*-FCC-62 would thus be steered off from the 'common path' of Chl-breakdown. The unobserved 8^2-OH-*p*FCC would thus be a hypothetical branching point of Chl-breakdown and it would represent the last 'common progenitor' of the later Chl-catabolites: the hypothetical 8^2-OH-*p*FCC is thus suggested (i) to either isomerise to *Sw*-NCC-58, subsequent to transport into the vacuole via an active ABC-transporter [22], or, (ii) to be transformed into the 'persistent' *Sw*-FCC-62 in the cytosol (see Figs. 5 and 6). Indeed, the remarkable ester function of the latter and the non-acidic cytosolic medium help preserve it by slowing down its 'chemical' isomerization to an NCC [19]. No evidence is available for a functionalized NCC isomeric to *Sw*-FCC-62 that could result from an eventual dislocation of *Sw*-FCC-62 into the vacuole. Similar to other FCCs, which are localized outside of the vacuole [23], the 'persistent' *Sw*-FCC-62 may thus accumulate in the cytosol.

De-greened *Sp. wallisii* leaves showed the blue luminescence typical of FCCs (see Fig. 2). Green (Fig. 4A) and yellow, senescent *Sw*-leaves (Fig. 4B) were thus studied by microscopic analyses. In senescent *Sw*-leaves a marked blue fluorescence was observed in mesophyll cells (palisade parenchyma as well as spongy parenchyma). Thicker cell walls of tracheids and fibers (Fig. 4B-f) also showed blue fluorescence (ascribed to cell wall phenolics, lignin and coumaric acid derivatives [31–33]), as was also seen in green leaves (Fig. 4A-c). Senescent leaves lacked the red Chl- fluorescence; only in the guard cells of their stomata were intact chloroplasts marked by their red fluorescence (Fig. 4B-e and h). Indeed, stomata remain functional during (early phases of) senescence [34]. Green *Sw*-leaves showed bright red Chl-fluorescence in the whole mesophyll, which lacked the marked blue fluorescence of the senescent cells (Fig. 4A-b).

In senescent *Sw*-leaves also yellow fluorescent 'spots' were observed (Fig. 4B–e). They might be putative lipid droplets, as also seen in senescent banana leaves [13]. Indeed, in senescent leaves chloroplasts develop into a distinct plastid type, the gerontoplasts, which are formed by loss of thylakoid membranes and accumulation of lipid globules [29,35]. At a late stage, rupture of the gerontoplast envelope was observed to lead to release of globules to the cytoplasm [35].

Esterified FCCs were now detected in the monocotyls *Sp. wallisii* (here) and in *Musa acuminata* [12]. The ester stabilizes FCCs against their (chemical) transformation to NCCs and, possibly, preserving FCCs for an unknown biological role [12–14]: The ester function of *Sw*-FCC-62 is a β-gluco-pyranosyl-(1→1)-2-(3,4-dihydroxy-phenyl)-ethyl unit, and belongs to the class of the phenylethyl-glycosides [24–26]. Interestingly, phenylethyl-glycosides were known in dicotyls, for which they were suggested to be useful as taxonomic markers [36].

Chl-breakdown has been considered, first of all, a detoxification process [3,4,22,30] and a physiological function of the linear tetrapyrroles from Chl-breakdown is unknown. This is clearly intriguing in view of the importance of the structurally related natural tetrapyrroles from heme breakdown (biliverdin, phycobilins) [37,38]. Indeed, the ubiquitous NCCs have been shown to be effective antioxidants [7], of possible physiological benefit. The related ('persis-
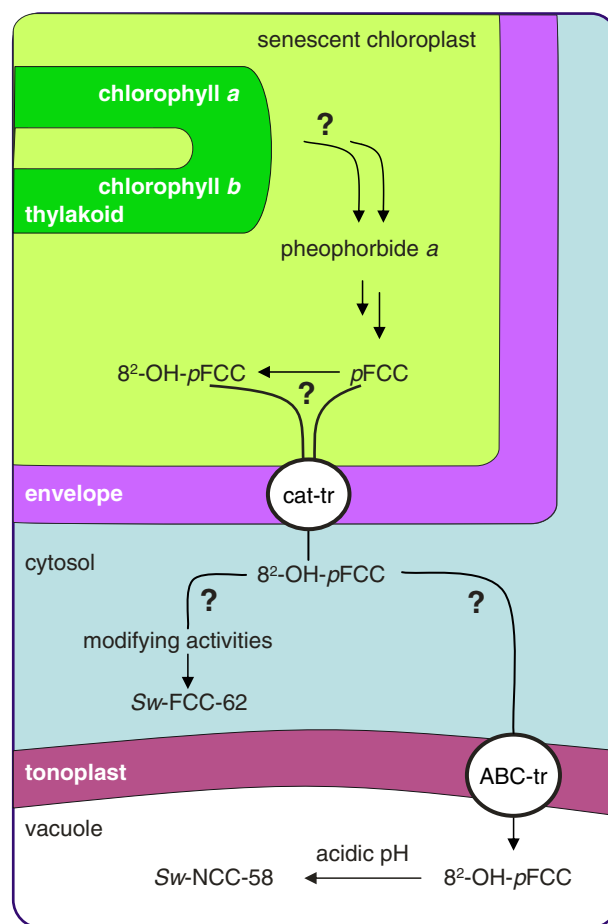

Fig. 6. Topographical model of a hypothetical path of Chl-breakdown [4,22], as suggested to be relevant in senescent leaves of *Sp. wallisii*. Chls are degraded by chloroplast enzymes to *p*FCC (or - possibly - to the hydroxylated 8^2-OH-*p*FCC). FCCs (*p*FCC or 8^2-OH-*p*FCC) are exported into the cytosol with assistance of the 'catabolite' transporter (cat-tr) in the envelope. 8^2-OH-*p*FCC may then be transported by a tonoplast-bound ABC-transporter (ABC-tr) into the vacuole, where it isomerizes spontaneously to *Sw*-NCC-58. Alternatively, 8^2-OH-*p*FCC would be converted in the cytosol into *Sw*-FCC-62 by a hypothetical enzymatic esterification.

tent') FCCs were also suggested to possibly contribute to the viability of higher plants, and they invite considerations of a further biological function in plants [12–14].

Few plants are known to have 'visibly' luminescent senescent leaves, e.g., *Ginkgo biloba* [39] or bananas [13], but FCCs were only detected in the latter. The optical perception of de-greened leaves, fruit (and flowers) helps guide the crucial interactions of plants with insects, birds and other animals (see e.g. [40,41]). 'Persistent' FCCs, the newly discovered blue luminescent plant pigments, are sources of optical signals [12–14], and may be useful as natural molecular *in-vivo* markers and signals of ripening and of programmed cell death.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.09.011.

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