Catabolites of Chlorophyll in Senescent Leaves

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Summary

Pink pigments were discovered thin-layer-chromatographically in senescent, but not in mature pre-senescent, leaves of Rossa, a yellowing genotype of *Festuca pratensis*. These pigments appeared to be absent from senescent leaves of Bf 993, a non-yellowing genotype. Similar pigments were found to be present in senescent segments of primary barley leaves and the abundance was positively correlated with the rate of chlorophyll breakdown. It was concluded that these pigments represent intermediary products of chlorophyll breakdown. A procedure for the enrichment of chlorophyll catabolites from chloroform extracts is described. The compounds were quantified either by measuring the absorption at 526 nm or colorimetrically following reaction with diazotized 4-nitro-o-anisidine. Analysis of the pink pigments by HPLC resolved 3 major and several minor compounds. The putative chlorophyll catabolites are acidic in nature and the pink appearance is due to a conspicuous absorption maximum at 526 nm.

Key words: Festuca pratensis, Hordeum vulgare, leaves, senescence, chlorophyll degradation, chlorophyll catabolites.

Introduction

The yellowing of senescent leaves is one of the most conspicuous phenomena in the development of plants. There is hardly a study on leaf senescence in which the loss of chlorophyll is not chosen as a convenient parameter. Despite the importance of chlorophyll in photosynthesis and its prominent rôle as an index of senescence, surprisingly little is known about its breakdown. Whereas the synthesis of chlorophyll has been elucidated in great detail, the biochemistry of chlorophyll catabolism is practically unknown. This gap of knowledge is largely due to the fact that intermediary non-green products of chlorophyll breakdown have so far not been detected in leaves. Only derivatives having an intact porphyrin ring structure such as chlorophyll a–l (Maunders et al., 1983), pheophytin a, pheophorbide a (Schoch et al., 1981; Schoch and Vielwerth, 1983) have been identified as possible natural products of chlorophyll a.

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Abbreviations: CC, chlorophyll catabolite; BA, N^6-benzyladenine; ABA, abscisic acid; ACC, amino cyclopropane carboxylic acid; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.

The discovery of a non-yellowing genotype of *Festuca pratensis* (Thomas and Stoddart, 1975) has opened new possibilities to approach the problem of chlorophyll catabolism. As the mutation concerns a single recessive nuclear gene (Thomas, 1987 a) it is feasible, at least in theory, to eventually identify the gene product which is likely to play a decisive rôle in the yellowing process. Several features of senescence in the non-yellowing genotype of meadow fescue have been described (Thomas, 1987 b). During an investigation of the pigments of senescing *Festuca* leaf tissue, pink compounds were observed on thin layer chromatograms of extracts from the normally yellowing cultivar Rossa. They occurred in trace amounts but appeared to be absent from non-senescent Rossa leaves as well as from leaves of the mutant genotype Bf 993, whether these were senescent or not. The suspicion that these pink pigments represent catabolites of chlorophyll was confirmed when similar pigments were discovered in senescent barley leaves and in the yellowing autumnal leaves of a large number of species. In this first account we describe the preparation and quantification of these putative non-green catabolites of chlorophyll (CCs) along with some preliminary data on their properties.

Materials and Methods

Festuca pratensis cv. Rossa (yellowing) and genotype BF 993 (non-yellowing) and *Hordeum vulgare* cv. Gerbel were cultivated at a 12 : 12 h photoperiod and a thermoperiod of 21 : 16 °C. The relative humidity was c. 70 % and the light intensity was varied between 22 Wm^{-2} (6.00–7.30 and 16.30–18.00) 60 Wm^{-2} (7.30–9.00 and 15.00–16.30) and 120 Wm^{-2} (9.00–15.00). Primary leaves of barley were harvested at day 10 after sowing, leaves of meadow fescue when the formation of the ligule had just been completed.

Induction of senescence

Leaf segments were placed in Petri dishes on filter paper moistened either with distilled water or with 10^{-5} M aequeous solutions of abscisic acid (ABA) or 6-benzylaminopurine (N^6-benzyladenine, BA). The dishes were incubated either in permanent darkness at 23 °C or under conditions of natural daylight-dark cycle of the laboratory. Prior to the incubation the segments were surface sterilized by immersion for 5 min in 0.5 % sodium hypochlorite containing 0.1 % Tween detergent and thoroughly washed with sterilized distilled water.

Extraction and preparation of CCs

Leaf segments were placed in a mortar and ground in the presence of methanol containing 5 % formic acid (100 µl per 100 mg f.w.) and subsequently extracted with chloroform (2–3 ml per 100 mg f.w.). The extraction was repeated twice and the extract (total volume c. 8–9 ml) filtered through cotton wool and subsequently through a small column (bed volume c. 0.5 ml) of silica gel beads (Merck 60, 70–230 mesh). The pink pigments CCs were adsorbed at the top of the column whilst the chlorophyll and carotenoids were eluted. The column was rinsed with chloroform and finally the CCs were eluted with methanol. For reading the absorption at 526 nm the eluate was brought to dryness and the residue dissolved in a defined volume of methanol.

Chromatography of CC

TLC. Silica gel layers (F-1200, Schleicher and Schüll) were employed. Solvent: ethylacetate/2-butanone/formic acid/water = 50/40/5/5 by volume.

HPLC. Reversed phase C-18 columns (Hypersil 5 ODS, 25 cm) were operated with the solvent system, methanol 10 %, acetonitrile 8.2 %, tetrahydrofurane 5.8 %, H_3PO_4 1 %, and water 75 %. The flow rate was 1.0 ml min^{-1} and a Kratos spectroflow 757 monitor was employed for recording A_{520} nm.

Colorimetric determination of CC

The CCs eluted with methanol from the silica gel columns were brought to dryness and dissolved in 50 µl methanol. This solution was mixed with 2.0 ml 0.1 M Tris containing 1 % (w/v) Triton-X-100. The reagent tubes were placed on ice and 500 µl of an 0.1 % (w/v) aequeous solution of diazotized 4-nitro-o-anisidine (Echtrotsalz B, Fluka) was added. After 15 min of incubation at 0 °C followed by 20 min at room temperature the absorption was read at 520 nm. Bilirubin (stock solution in 20 mM NaOH) was used as reference and the contents of leaf segments were calculated as µg bilirubin-equivalents per 100 mg of fresh weight.

Determination of chlorophyll was carried out according to Arnon (1949).

Results and Discussion

The extraction of chlorophyll requires that neutral or slightly alkaline conditions be maintained in order to avoid the formation of pheophytins. This conventional precaution is probably a reason for the fact that the proposed catabolites of chlorophyll (CC) described subsequently have so far escaped discovery. In connection with work on perloline, a prominent alkaloid in leaves of *Festuca pratensis,* extracts with acidified organic solvents were prepared. Upon the partitioning into chloroform and subsequent TLC, pink pigments with polarities between the two galactolipid classes were observed. They appeared to be present only in extracts of senescent leaves from the yellowing genotype Rossa. Non-senescent leaves of Rossa plants appeared to contain no such pigments. More significant, however, was the observation that leaves of the non-yellowing genotype Bf 993 appeared to be devoid of pink pigments whether they were senescent or not (Fig. 1).

It was concluded that the pigments represent catabolites of chlorophyll because the established genetical lesion of Bf 993 concerns a marked reduction in competence to degrade chlorophyll. Hence, we tentatively adopted the operational term «chlorophyll catabolites» (CCs).

The prepurification procedure described in Material and Methods is based on the observation that CCs have a high affinity for silica gel when chloroform is used as the solvent. It turned out to be convenient to extract leaf tissue in such as fashion that the CCs are extracted directly with chloroform and subsequently absorbed selectively onto silica gel beads. It also became apparent, particularly upon the development of an HPLC system, that the CCs behave like acids. Therefore the initial grinding of leaf tissues in the presence of formic acid is essential for ready dissolution of the pigments in chloroform.

Substantial evidence favouring the identity of pink pigments with CCs was provided by the observation of pigments similar to those shown in Fig. 1 in senescent primary leaves of barley. In fact, a whole group of pigments having colors ranging from pink to yellow can be discerned on the TL chromatograms from barley (Fig. 2)

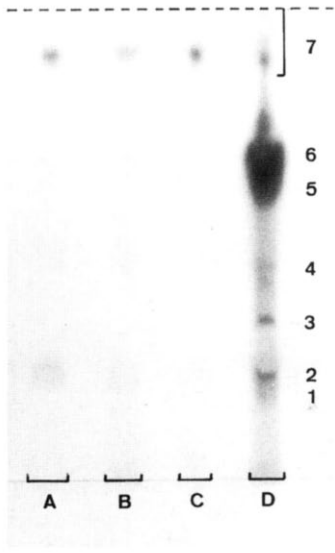

Fig. 1: Thin layer chromatogram of pigments present in chloroform extracts from *Festuca pratensis* leaves. Details of the preparation are given in «Materials and Methods». Comparison of pigments present in mature (A) and senescent (B) leaves, of the non-yellowing genotype Bf 993. Pigments from mature (C) and senescent (D) leaves of Rossa, a normal yellowing genotype. Conditions of induced senescence: incubation of segments for 3 days in permanent darkness. Colors: 1, 2, 3, 4 pink; 5, ochre; 6, yellow. 7, contaminating pheophytins and carotenoids.

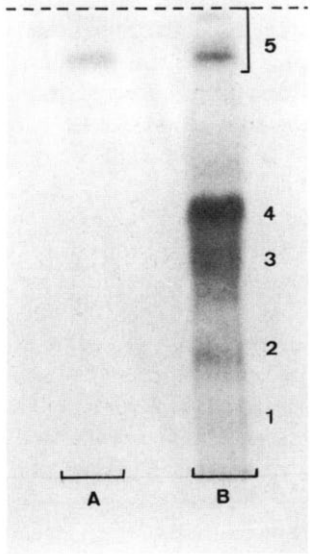

Fig. 2: Thin layer chromatogram of pigments present in chloroform extracts from mature non-senescent (A) and senescent (B) primary leaves of barley. Enrichment of pigments as described in «Material and Methods». Conditions of induced senescence: 4 days permanent darkness. Colors: 1, 2, 3, pink; 4, ochre/yellow; 5, contaminating pheophytins and carotenoids.

as well as *Festuca*. If kept in air, the yellow and ochre colored bands gradually turn to red and brownish-red indicating that these CCs may be oxidized. It appears from the R_f values of CCs prepared from *Festuca* and *Hordeum*, respectively, that the pigments,

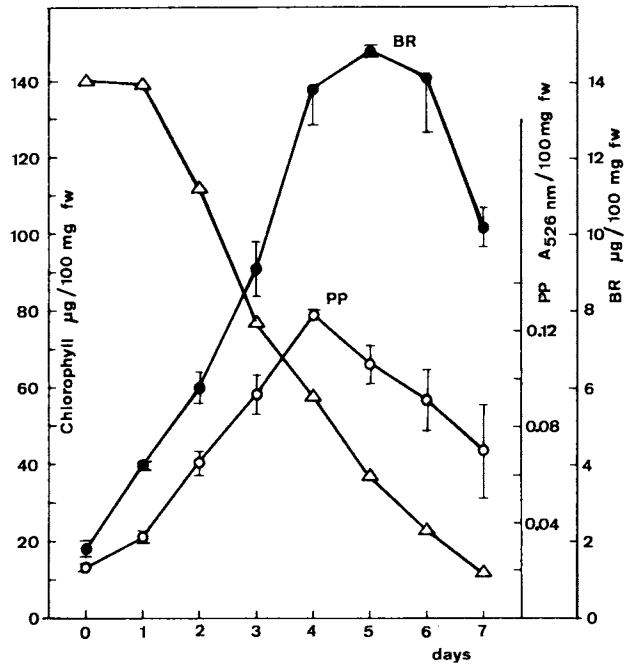

Fig. 3: Contents of chlorophyll and chlorophyll catabolites in leaf segments of barley during senescence in permanent darkness. ●——●, BR, bilirubin-equivalents; ○——○, PP, pink pigments; △——△, chlorophyll. Standard errors are given for at least 3 parallel determinations.

although very similar as far as the colors and other properties are concerned, are not identical in the two species.

In order to investigate the possible correlation between the occurrence of CCs and chlorophyll breakdown in senescent leaves, it was necessary to find a means for quantification. By analogy with the best-characterized tetrapyrrole catabolic pathway, namely that of haem, we considered the CCs were likely to represent some sort of bile pigments, and so a diazotized reagent as used in the clinical analysis of bilirubin was tried.

Diazotized 4-nitro-o-anisidine indeed turned out to be useful for a colorimetric assay. Using pigments scraped off from thin layers after chromatography it was established that all of them contribute to the color reaction with Echtrot B, yet for obvious reasons it is not yet possible to specify whether the final yields of dye are different or equal for the individual CCs. The colorimetric assay has, however, the advantage of allowing an overall estimate of the CCs present in a leaf extract. It should be mentioned that the contaminating compounds in the preparations of CCs, thylakoidal lipids, traces of carotenoids and perloline in the case of *Festuca*, are not reactive with Echtrot B.

As shown in Fig. 3 segments of primary barley leaves kept in permanent darkness contained increasing amounts of pink pigments up to the 4th day. Thereafter the

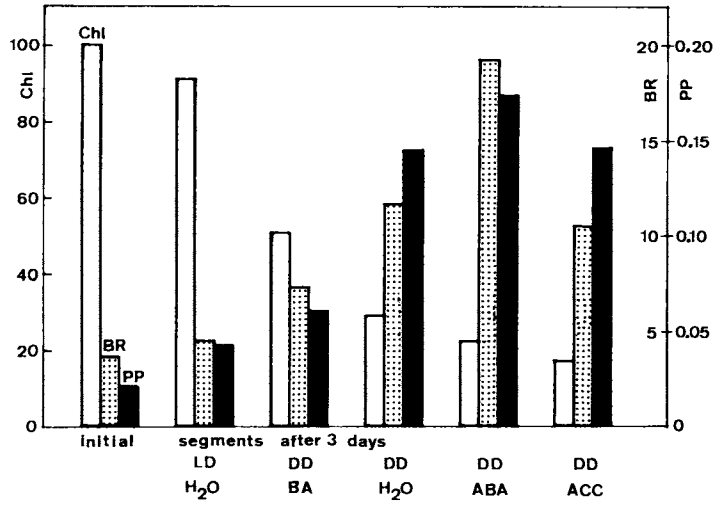

Fig. 4: Changes of chlorophyll and chlorophyll catabolites upon the incubation of barley leaf segments under various conditions. LD, natural daylight; DD, permanent darkness; BA, benzyladenine 50 μM; ABA, abscisic acid 10 μM; ACC, aminocylopropane carboxylic acid 1 mM. Chl., chlorophyll relative units; BR, bilirubin-equivalents, μg per 100 mg fresh weight; PP, pink pigments, $A_{526\,nm}$ per 100 mg fresh weight.

pink CCs gradually disappear as the rate of chlorophyll degradation slowed down. The contents of CCs estimated as bilirubin-equivalents increased in a similar fashion as the pink pigments; they remained at a high level from day 3 to day 6 of the senescence period. When segments were kept in natural daylight conditions the marked retention of chlorophyll was associated with low levels of CCs, estimated both as pink pigments and as bilirubin-equivalents (Fig. 4).

Convincing correlations between the content of CCs and chlorophyll degradation were also observed in segments treated with BA on the one hand or with ABA and ACC, respectively, on the other hand (Fig. 4). The treatments causing an accelerated catabolism of chlorophyll were associated with high levels of CCs in the leaf segments and vice versa. It should be mentioned that CCs were always detectable in the mature, non-senescent leaves. This may be interpreted as an indication, that some breakdown of chlorophyll takes place even in the mature leaves. This view is supported by demonstrations of chlorophyll turnover in mature leaves of various species (Perkins and Roberts, 1983).

The mutation responsible for the non-yellowing character of *Festuca pratensis* Bf993 is not perfectly stringent. Chlorophyll is degraded in the senescent leaves, yet at a comparatively slow rate. The data presented in Table 1 demonstrate that CCs are indeed accumulated in senescent segments of Bf993 leaves. However, the amounts of CCs extracted from the senescent Rossa segments were much higher corresponding to a higher rate of chlorophyll breakdown.

Table 1: Loss of chlorophyll and appearance of chlorophyll catabolites upon dark-induced senescence of *Festuca pratensis* leaves. Segments of the yellowing genotype Rossa and of the non-yellowing Bf 993 were incubated in permanent darkness for 4 days.

	Rossa			Bf 993		
	Chlorophyll	Pink pigments	Bilirubin equivalents	Chlorophyll	Pink pigments	Bilirubin-equivalents
day 0	281	0.033	3.8	274	0.027	3.5
day 4	78	0.241	13.5	187	0.046	6.8

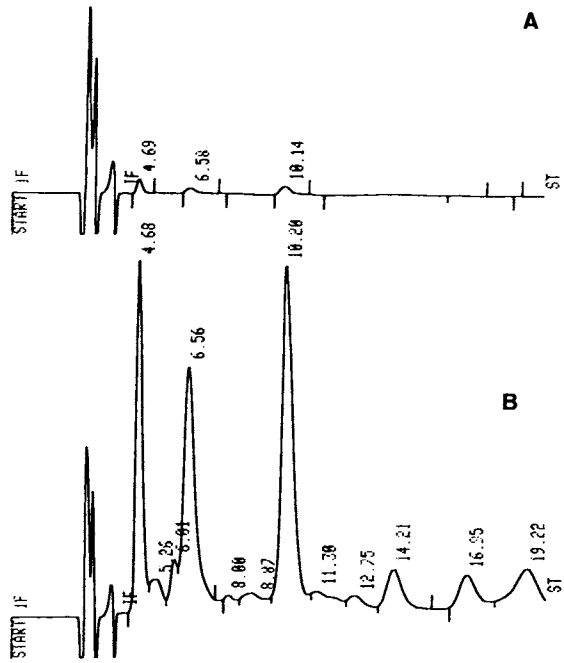

Fig. 5: HPLC chromatograms of preparations of CCs from barley primary leaf segments. A, mature, non-senescent. B, segments senesced for 4 d in permanent darkness. The absorption at 520 nm was recorded. Samples containing CCs from equal fresh weights of segments were injected.

The occurrence of CCs is not restricted to senescent leaves of the two grass species mentioned so far. Similar pink and yellow pigments were obtained from senescent leaves of annual plants such as tobacco or bean as well as from a large number of perennials and deciduous trees during normal autumnal yellowing. Hence, there is little doubt that the CCs first observed in *Festuca* are typical for all leaves that catabolize chlorophyll.

A more detailed analysis of properties of CCs, and the elucidation of their chemical structure in particular, depends on an effective separation of the individual pigments.

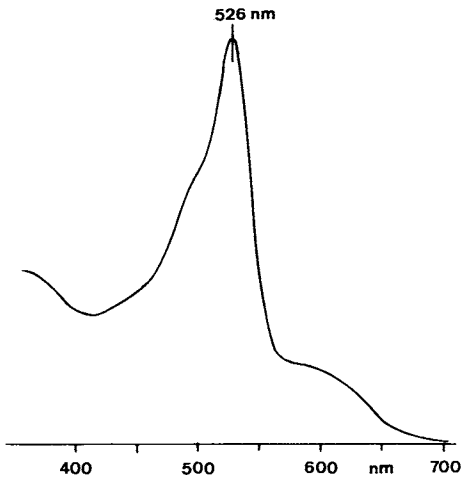

Fig. 6: Spectrum of pink pigments isolated from senescent barley leaves.

Because the resolution power of TLC is certainly inadequate for this purpose, an HPLC system was developed. Fig. 5 shows chromatograms of CCs prepared from senescent and from mature, non-senescent barley leaf segments. By monitoring at 520 nm, three major and some minor compounds were resolved corresponding to the pink pigments seen in TL chromatograms. Compounds with similar Rfs to those of senescing tissue were detected in preparations from non-yellowing leaves but they indicate that only very small quantities of CC are present unless the leaves are induced to senesce and to catabolize chlorophylls rapidly.

The inclusion of a strong acid such as orthophosphoric acid in the HPLC solvent is an absolute requirement for the chromatography of CCs on reversed phase C-18 columns. In the presence of neutral solvent systems, the pigments have no affinity for the column and are invariably eluted with the front. It is obvious that this behaviour of CCs is due to acidic properties; whether or not the reversed phase column is retentive appears to depend on the deprotonation or protonation, respectively, of the acidic groups. In acid media CCs appear to be stable. Under neutral or alkaline conditions the CCs are rather labile, to judge by the loss of color intensity and color changes. The spectrum of partially purified pink pigment from senescent barley leaves shown in Fig. 6 is characterized by a conspicuous peak at 526 nm. The spectra of the different pink pigments are very similar indicating that the compounds may be chemically closely related with each other. A prominent yellow compound which appears to be a member of the group of CCs has so far not been considered particularly. It has an absorption maximum at 420 nm and appears to be less polar than the pink pigments (Figs. 1 and 2). It is unstable and tends to change its colour from yellow to brownish-red within a few hours after TLC, possibly as a consequence of oxidation.

The mobilization of chlorophyll in senescent leaves is important with regard to the withdrawal and re-utilization of nitrogen in the developing organs and reserve tissues of the plant. On the face of it, salvage of the four nitrogen atoms per molecule of

chlorophyll might be expected to make only a minor contribution (less than 3 %) to the overall nitrogen present in the proteins of mesophyll cells. Yet, investigations of the non-yellowing genotype of *Festuca pratensis* have yielded ample evidence that not only the catabolism of chlorophyll is slow but also that of the proteins to which chlorophyll is complexed (Thomas and Hilditch, 1987). Whether the chlorophyll protects the protein from degradation or chlorophyll is not catabolized because the mutation concerns a deficiency in the specific degradation of pigment-associated proteolipids is difficult to decide. It is, however, a fact that – at least in *Festuca* – the degradation of chlorophyll and corresponding thylakoidal proteins are coupled. Hence, not only the nitrogen contained in the chlorophyll but also that contained in such major chloroplastic proteins as the light harvesting protein LHC 2 must be considered. From data given in Thomas (1983), it can be estimated that the mobilisation of chlorophyll then contributes c. 52–58 % to the total nitrogen exported from senescent leaves of the normal genotype of *Festuca* and less than 40 % in the mutant tissue.

Apart from such considerations, the elucidation of chlorophyll catabolism is a challenge because the current knowledge about the yellowing of leaves and the color changes of various fruits suggests that it is an orderly process which is in certain cases even reversible. Chlorophyll breakdown has remained enigmatic, because neither biochemical mechanisms involved nor metabolites produced have been identified so far. Such investigations are also confronted with a difficult technical problem concerning the specific radiolabelling of chlorophyll, a prerequisite for successfully establishing the catabolic pathway. Hence, the discovery of the CCs by virtue of the unique research tool represented by the non-yellowing genotype of *Festuca pratensis* may eventually allow the biochemical reactions involved in chlorophyll breakdown to be predicted. The next step is to isolate CCs in quantities sufficient for the analysis of their chemical structure. As all the chlorophyll and chlorophyll-derivatives having an intact porphyrin ring structure are green or brownish-green it is likely that the pink appearence of some major CCs is due to the opening of the porphyrin, analogous to the bile pigments of catabolism of haem. Whether or not a term like phytobilirubin is justified for the CCs remains to be established. It is hoped, however, that elucidation of the chemical structures will eventually allow a pathway of chlorophyll breakdown in senescent leaves to be established and enzymes responsible for individual reactions *in vivo* to be identified.

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