The Structure and Function of Grana-Free Thylakoid Membranes in Gerontoplasts of Senescent Leaves of *Vicia faba* L.

H. Oskar Schmidt

Zentralinstitut für Genetik und Kulturpflanzenforschung der AdW der DDR, 4325 Gatersleben

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In the course of yellowing (senescence) the leaves of *Vicia faba* L. lose 95% of their chlorophyll. Gerontoplasts develop from chloroplasts and aggregate with the pycnotic mitochondria and the cell nucleus in the senescent cells (organelle aggregation). The gerontoplasts contain only a few, unstacked thylakoid membranes but a large number of carotinoid-containing plastoglobuli, which after the degration of chlorophyll presumably assume the light protection of the cells. The thylakoid membranes of the gerontoplasts were isolated by means of a flotation method. Their polypeptide composition is characterized by a high proportion of light-harvesting complex. Evidence of relatively high photochemical activity shows that functional thylakoid membranes are present in the premortal senescence state of leaves and this suggests that there is functional compartmentation of the hydrolytic processes in this stage of the leaves' development.

Introduction

During the senescence (yellowing) of leaves, genetically programmed hydrolytic processes regulated by hormones take place. These lead to the degradation of the cell organelles and chloroplasts and finally to lysis of the cells [1, 2]. The leaf alters its most important function within the plant during this stage of development: the photosynthesizing apparatus is degraded; nitrogen compounds and carbohydrates are mobilized in the leaf and moved to the plant's storage organs or, in the case of annuals, to the soil substrate. From being a CO_2 assimilation organ the leaf becomes a supplier of nutrients to the plant.

In spite of the progression of the degradation processes during leaf yellowing, the leaf cells, including the cell organelles and membranes in the cells at each particular stage of degradation, remain functional for a prolonged period (up to several weeks).

Extensive biochemical investigations and examinations by electron microscopy have been carried out on the degradation of chloroplasts, at least in the initial stages of senescence [3–6]. As a rule, the chloroplasts pass through a series of typical degradation stages. The content of ribulose diphosphate carboxylase-oxygenase in the "mature" chloroplasts of green leaves drops with the onset of senescence. Subsequently degradation of the thylakoid membranes and of chlorophyll commences. Lipids and carotinoids released in the process collect, at least to some extent, in globulare osmiophilic droplets designated as plastoglobuli according to Lichtenthaler and Sprey [7]. The "senescent chloroplast" containing irregularly arranged grana stacks and numerous plastoglobuli, is formed; it usually also contains starch grains and is typical of senescent leaves with its yellow-green colour.

The further degradation of the thylakoid membranes leads to a type of plastid that has only a few unstacked thylakoids, does not normally contain any starch, and is filled mainly with plastoglobuli. This premortal stage of chloroplast development was described by Sitte [8, 31] as "gerontoplast". These organelles contain only traces of chlorophyll and accordingly their function also differs from that of the chloroplasts (see Discussion).

Numerous investigations have been carried out on grana-containing thylakoid membranes from mature and senescent chloroplasts [9–12] but none are available on thylakoids of gerontoplasts. In the present work these thylakoids were isolated by means of a flotation method in order to examine the structure and function of these final degradation products of the thylakoid system of chloroplasts.

Present address: Boschstraße, Postfach 220, D-6703 Limburgerhof.

Reprint requests to Dr. O. Schmidt.

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Materials and Methods

Test object

Vicia faba L. cv. Dornburger Ackerbohne was grown for about 5 weeks in a hydroculture in a con-

trolled environment chamber up to the 12-leaf stage. The shoots of these plants were cut above and below the 3rd leaf node and placed in bottles containing distilled water. In a controlled environment chamber at 2000 lux and 18 °C the leaves on these shoot sections turned yellow reproducibly within 7–9 days.

Electron microscopy

Leaf sections and isolated thylakoid membranes were fixed with 3% glutaraldehyde and 1% OsO_4, embedded in Epon and examined on a JEM 2 (Jeol, Japan).

Number of chloroplasts

The determination of the chloroplast count per cell was carried out according to Possingham and Sauer [30].

Isolation of thylakoid membranes

The chloroplasts were isolated using a flotation method [13]. Leaves that had turned completely yellow (2 mg chlorophyll/cm^2) were homogenized in isolation buffer (75 mM tris-HCl, pH 7.8, 0.5 M sucrose, 0.2% human serum albumin, 0.2% polyvinylpyrrolidone, 0.1 mM diisopropylfluorophosphate and 0.1 mM iodoacetamide), pressed through nylon gauze and centrifuged for 2 min at $600 \times g$. The plastids were separated from the supernatant by centrifugation (30 min at $20{,}000 \times g$), suspended in 50 mM tris-HCl, pH 7.5, 31% sucrose (w/w), 0.1 mM diisopropylfluorophosphate and 0.1 mM iodoacetamide, and introduced into the middle layer of a multi-stage density gradient (17%, 21%, 25%, 31%, 33%, 35% and 42% sucrose (w/w), in 50 mM tris-HCl, pH 7.5). As the result of centrifugation (25 min at $40{,}000 \times g$), gerontoplasts floated to the surface of the gradient; senescent chloroplasts were found in the 25% and 31% layers. Whole and fragmented mature chloroplasts sedimented into the 42% layer. The gerontoplasts were collected and shocked osmotically with distilled water. The thylakoids were then pelleted by centrifugation (30 min at $40{,}000 \times g$).

Assay of photosynthetic activity

The electron transport was measured from water, benzidine and DCPIP/DCMU to anthraquinon sulfone acid, respectively, as described by Metzger and Ohmann [14].

Electrophoretic separation

The membranes were dissolved in 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 50 mM tris-HCl, pH 8.9, and the proteins were separated in slab gels (10% polyacylamide and 5 M urea) as described by Schmidt [15]. Staining was carried out with Coomassie Brilliant blue G 250. Densitograms were prepared with the aid of a Zeiss Schnell-photometer III (VEB Carl Zeiss, Jena, GDR).

Results

Yellowing of the leaves of Vicia faba

The senescence of the field bean *Vicia faba* L. proceeds progressively, *i.e.* the lower, oldest leaves begin to turn yellow on the flowering plant, while leaves and blossoms are being formed at the tip of the shoot. In the case of plants growing outdoors, the weather causes most of the yellowing leaves to drop off before they have turned completely yellow and reached the final premortal senescent stage.

In order to obtain sufficient quantities of yellowed leaves for the isolation of gerontoplasts, green leaves were removed from the plant together with a piece of shoot and brought reproducibly to complete yellowing in a controlled environment chamber. The leaves used for isolating gerontoplasts were completely yellow but were still turgescent (premortal state of senescence). Under the chosen conditions they exhibited the same features of senescence as leaves on outdoor plants, both externally and under a light microscope. During yellowing the chlorophyll content of the leaves dropped from 35 ± 2 µg (S.E.) chl/cm^2 (= 100%) to 1.5 ± 0.4 µg chl/cm^2 (4.3%). In addition, the ratio of chl a to chl b decreased from 2.8 ± 0.1 to 2.2 ± 0.1, the protein content decreased from 382 ± 14 µg/cm^2 to 88 ± 14 µg/cm^2 (23.0%) and the nitrogen content from 165 ± 5 µg/cm^2 to 45 ± 2 µg/cm^2 (27.3%). The number of chloroplasts per palisade cell remained constant: 98 ± 5 were counted in green leaves and 107 ± 7 in yellow leaves. Since no chloroplast division takes place during senescence, it may be concluded that there is no degradation of complete chloroplasts by phagocytosis or lysis during this period and that thylakoid membranes are always degraded inside the chloroplasts.

Microscopic examination of chloroplasts and gerontoplasts

Examination of green leaves under a light microscope showed that the chloroplasts in the cell were dispersed uniformly over the surface (Fig. 1a). Under an electron microscope the chloroplast of *Vicia faba* proved to be typical of higher plants (Fig. 2a). In the course of senescence the diameter of the plastids decreased from 6 μm to about 2 μm: They also moved into the part of the palisade cells

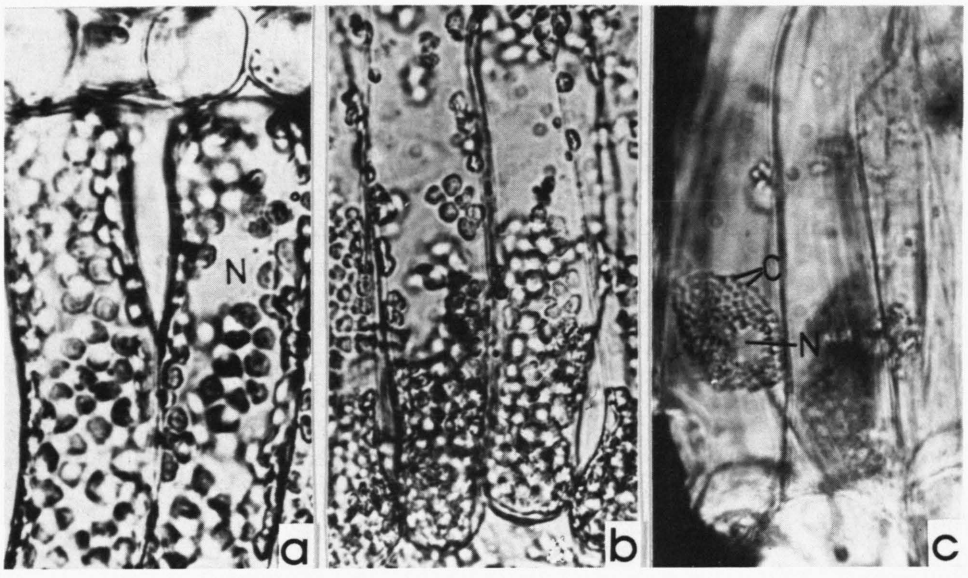

Fig. 1. Electron micrographs of plastids in palisade cells from leaves of various stages of senescence. a) Green leaf (35 μg chl/cm^2); b) yellow-green leaf (7 μg chl/cm^2); c) yellow leaf (1.5 μg chl/cm^2). Magnification: ×570. C = chloroplast, N = nucleus.

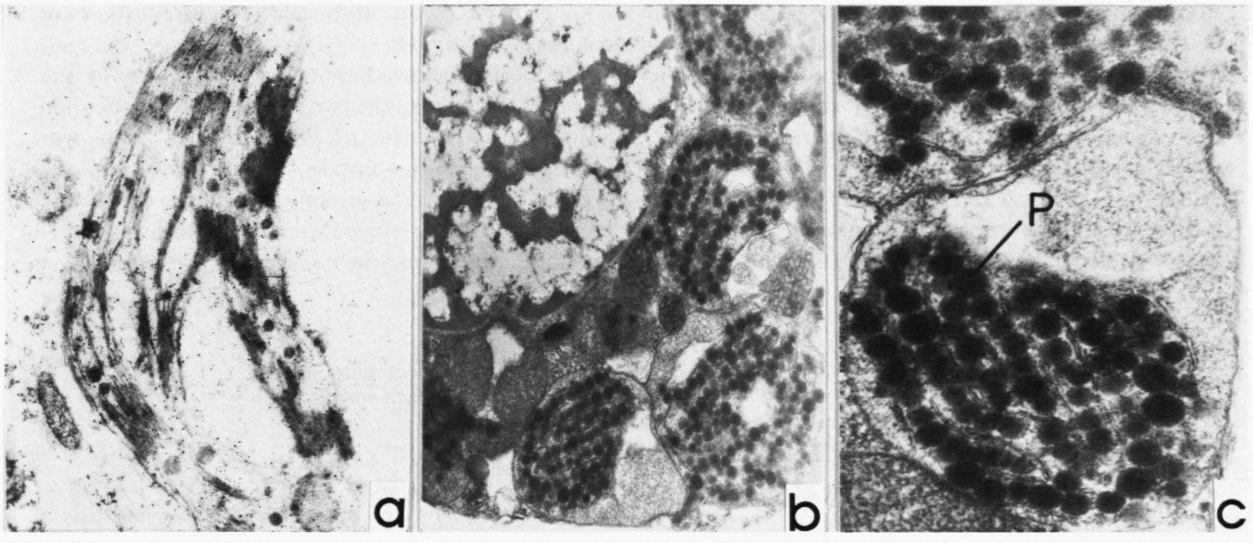

Fig. 2. Electron micrographs of chloroplasts and gerontoplasts from leaves of *Vicia faba*. a) Chloroplast from a green leaf (35 μg chl/cm^2) × 13,300; b) cell organelle aggregation: cell nucleus with gerontoplasts and pycnotic mitochondria from a yellow leaf (1.5 μg chl/cm^2) × 7500; c) gerontoplast as b) × 20,000.

away from the epidermis (Fig. 1b), in which the cell nucleus was also located. In yellow leaves the plastids enclose the cell nucleus as a continuous casing (Fig. 1c). This aggregation of gerontoplasts may also be found in living senescent cells of outdoor plants.

Electron micrographs showed that there are also mitochondria between the gerontoplasts and that these were smaller than those of green leaves and had a reduced membrane system (pycnotic mitochondria) (Fig. 2b). The gerontoplasts contained no starch grains and only a few unstacked thylakoid membranes which were organized in parallel within the organelle, and between which were rows of plastoglobuli (Fig. 2c).

The thylakoid membranes of the chloroplasts and gerontoplasts

In order to investigate the functionality of thylakoid membranes of gerontoplasts, gerontoplasts were isolated by means of a flotation method. This method enables gerontoplasts to be separated from mature and senescent chloroplasts as well as from broken chloroplasts formed during homogenization [13]. This separation is necessary since there are always also mature chloroplasts as well as gerontoplasts in leaves that have turned yellow, *e.g.* in the stomata and cells along the vascular bundles [16, 17]. The isolated thylakoids of the gerontoplasts were disintegrated with SDS and the proteins separated electrophoretically. Fig. 3b shows the corresponding densitogram. A comparison with the protein spectrum of thylakoids from green leaves showed that the spectra were similar, and that most of the proteins were present in both green and senescent leaves. However, there were quantitative differences. For example, gerontoplasts had a relatively higher proportion of the chlorophyll *b*-containing LHC. This led probably to the lowered chl *a*/chl *b* ratio of 2.2 in

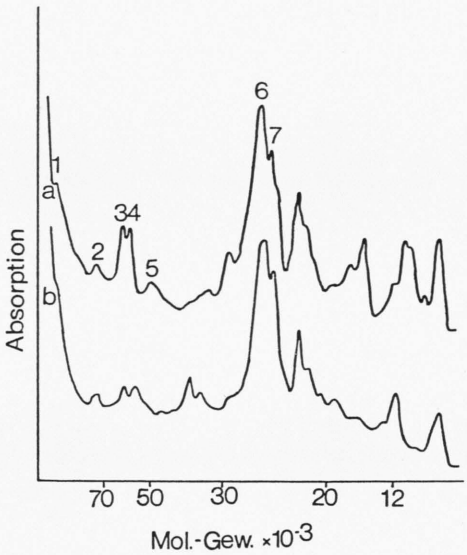

Fig. 3. Densitograms of electrophoretically separated polypeptides of thylakoid membranes from a) chloroplasts of green leaves (35 μg chl/cm²) and b) gerontoplasts of yellow leaves (1.5 μg chl/cm²). Identified polypeptide bands (see [15]). (1) Chl_a–AP1 (PS I); (2) Chl_a–P1 (PS I); (3) α-subunit of ATP synthase; (4) β-subunit of ATP synthase; (5) Chl_2–P2 (PS II); (6) $Chl_{a/b}$–AP2a (LHC); (7) $Chl_{a/b}$–AP2b (LHC).

yellow leaves. Since the presence of these functionally important proteins does not permit any conclusions to be drawn about the functional integrity of the membranes, the photochemical activities of thylakoids from chloroplasts and gerontoplasts were compared (Table I).

The results showed that, with reference to the chlorophyll content, the two types of membranes had a similarly high activity for PS I and PS II. The lower value for the water-splitting system of gerontoplasts is probably due to the prolonged isolation procedure and also to plant substances that interfere (polyphenols and hydrolytic enzymes). The conclusion

Table I. Photochemical activity of thylakoid membranes from chloroplasts and gerontoplasts (μM O_2 consumption/h × 200 μg chl; means from two independent experiments, each with three parallels).

	PS I DCPIP/DCMU – AQ		PS II + PS I Benzidine –– AQ		Water-splitting system + PS II + PS I HO ––– AQ	
Chloroplasts	601	100%	118	100%	90	100%
Gerontoplasts	623	104%	135	114%	58	64%

can therefore be drawn that the unstacked thylakoid membranes in the gerontoplasts are photochemically active and presumably also capable of photosynthesis. The cytological and biochemical findings are evidence that orderly metabolism takes place in the cell even in this premortal stage of senescence, and that the hydrolytic processes do not impair the functionality of the membranes.

Discussion

The constant number of plastids per cell found in *Vicia faba* during senescence, and the structure of the gerontoplasts, substantially agree with findings obtained for other herbaceous plants [13, 19, 20]. What is noticeable is the aggregation of gerontoplasts and pycnotic mitochondria around the cell nucleus in the yellow leaves, which was also observed in outdoor plants and in leaves turning yellow due to a deficiency of nitrogen [13]. This aggregation of organelles may be interpreted as a manifestation of an intensive exchange of metabolites between the organelles.

A comparable aggregation of mitochondria and chlorophyll-free plastids was described by Wellburn and Wellburn [21] in a chlorophyll-free mutant of *Hordeum vulgare*. The aggregation may also have the function of protecting the organelles from light by providing mutual shade [22].

Gerontoplasts containing unstacked thylakoids had not previously been isolated preparatively. The results now obtained with *Vicia faba* show that these membranes still possess photochemical activity. Apparently, as long as thylakoid membranes are present, they are also functional. Their polypeptide composition is distinguished by an increased content of light-harvesting complex, and a decreased portion of other membrane components. This finding agrees with studies on senescent leaves of *Festuca pratensis* and *Glycine max*. in which the plastids were isolated by sedimentation methods so that there were both senescent chloroplasts and gerontoplasts in the sediment. A relative enrichment of the LHC during senescence was also observed in these studies [10–12].

Thomas [10] and Thomas and Stoddard [5] surmised that, during senescence, degradation of the peripheral proteins of the thylakoid membrane predominated and there was an enrichment of integral membrane proteins. The findings obtained with *Vicia faba* contradict this theory, since the degradation of peripheral proteins (*e.g.* plastocyanine, ferredoxin-NADP reductase and water-splitting system) is bound to lead to the complete loss of the membrane's photochemical activity. However, the loss of peripheral proteins can occur during the isolation of the thylakoid membranes, if the high protease activity in the homogenate of senescent leaves [13] is not inhibited. When membranes are incubated with proteases *in vitro*, the peripheral membrane proteins are particularly sensitive to proteolysis [15]. The functional thylakoids detected in the gerontoplasts of *Vicia faba* show that the compartmentation of metabolism is maintained up to the leaves' premortal senescence stage, so that proteolytic degradation of the thylakoid membrane proteins [23] and functional membranes can exist side by side as it occurs in green leaves (protein turn over) [1].

Since about 95% of the chlorophyll is degraded in the course of the leaves turning yellow, the extent to which the cell components are protected from photooxidation is considerably diminished. The other pigments remaining in the cells assume greater importance in protecting the cells from such damage. Therefore, the accumulation of carotinoids in the plastoglobuli of the gerontoplasts is probably not a deposit of substances of no value to the cell but a protective measure for ordered metabolism in senescent leaves. This supposition is supported by the synthesis of additional pigments (xanthophylls and anthocyanins) which is observed in senescent leaves, particularly in those exposed to considerable amounts of light [8, 24].

Additionally, of the functions that chlorophyll fulfills in a green leaf (photosynthesis pigment, protection from UV, supply of heat for transpiration, removal of light from competitive vegetation), the carotinoids in the senescent leaf also take over the supply of heat for maintaining transpiration and metabolism – especially at low air temperatures – by absorbing sunlight. Seen from this angle, the colouring of the foliage in autumn is probably not merely a "whim of nature" but a protective reaction that ensures the ordered degradation of thylakoid membranes and other cell components. Gerontoplasts should not therefore be regarded as functionless degradation products of chloroplasts, but as the specific organelle which, with the aid of the plastoglobuli, takes over the major part of light absorption of leaves in the senescent phase.

The electron microphotographs of *Vicia faba* (Fig. 2b) confirm that both the thylakoid membranes of the chloroplasts and the inner membranes of the mitochondria are degraded in the course of senescence. Respiratorily active, pycnotic mitochondria are formed after inner membrane breakdown [3]. Detailed reports on the degradation of mitochondria are also available from studies on animals. In the case of the resorption of muscle tissue (*e.g.* as the result of starvation or disuse) degradation of mitochondria and other cell components takes place, and the organelles present at any particular stage remain functional [25]. This is also found to be the case in senescent leaves [3].

From a molecular and physiological point of view, muscle degradation and the subsequent resorption of tissue components is surprisingly parallel in many respects to the senescence of leaves and the corresponding mobilization and translocation of cell components. At present, however, the aging and death of whole animal organisms is often considered to be the animal physiological parallel to leaf senescence though there seems to be fundamental differences with respect to the molecular mechanisms [26–29].

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