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Leaf senescence in a non-yellowing mutant of *Festuca pratensis*: Proteins of photosystem II

Paul I. Hilditch[1], Howard Thomas[1,*], Barry J. Thomas[1], and Lyndon J. Rogers[2]

[1] Plant and Cell Biology Department, Institute for Grassland and Animal Production, Welsh Plant Breeding Station, Plas Gogerddan, Aberystwyth, Dyfed, SY23 3EB, and
[2] Department of Biochemistry, University College of Wales, Aberystwyth, Dyfed, SY23 3DD, UK

Abstract. The senescence of leaves is characterized by yellowing as chlorophyll pigments are degraded. Proteins of the chloroplast also decline during this phase of development. There exists a non-yellowing mutant genotype of *Festuca pratensis* Huds. which does not suffer a loss of chlorophyll during senescence. The fate of chloroplast membrane proteins was studied in mutant and wild-type plants by immune blotting and immuno-electron microscopy. Intrinsic proteins of photosystem II, exemplified by the light-harvesting chlorophyll a/b-binding protein (LHCP-2) and D1, were shown to be unusually stable in the mutant during senescence, whereas the extrinsic 33-kilodalton protein of the oxygen-evolving complex was equally lable in both genotypes. An ultrastructural study revealed that while the intrinsic proteins remained in the internal membranes of the chloroplast, they ceased to display the heterogeneous lateral distribution within the lamellae which was characteristic of nonsenescent chloroplasts. These observations are discussed in the light of possible mechanisms of protein turnover in chloroplasts.

Key words: *Festuca* (mutant, senescence) – Leaf senescence – Light-harvesting chlorophyll a/b-binding protein – Mutant (*Festuca*) – Photosystem II – Protein turnover (photosystem II) – Senescence (leaf)

Introduction

The prime function of a leaf is the interception of light energy and the use of that energy in the assimilation of atmospheric CO_2. Should a given leaf no longer be able to carry out this function efficiently, it may be to the advantage of the plant as a whole to discard that organ. If so, the leaf will enter the final phase of its development, senescence (for a review, see Stoddart and Thomas 1982). This is characterized by the degradation of cellular constituents, notably proteins, and the redistribution of mobilised material to other parts of the plant. Most of the protein of leaf cells resides in the chloroplasts and a significant proportion of this protein is complexed with light-harvesting pigments, the chlorophylls. In the normal course of senescence, these pigments are broken down and the colour of the leaf changes from green, usually to yellow. There exists a mutant genotype of the grass *Festuca pratensis* in which this breakdown of chlorophyll is impaired (Thomas and Stoddart 1975), although the loss of photosynthetic activity and other vital functions proceeds normally (Hilditch et al. 1986a). The objective of the present study was to assess the extent to which the breakdown of certain chloroplast proteins during senescence is affected by the mutation. Immunochemical techniques were used to examine the organization of photosystem II (PSII). The chlorophyll a/b-binding protein (LHCP-2) is the most abundant membrane protein of the chloroplast and, with its associated pigments, serves as the light-harvesting complex for PSII. The LHCP-2 is an intrinsic protein of the thylakoid membrane, encoded by the nuclear genome (Timko et al. 1985). The D1 protein is also an intrinsic thylakoid protein, but is encoded by the genome of the chloroplast. It is thought to function as the photochemical reaction centre of PSII (for a review, see Barber and Marder 1986). The 33-kilodalton (kDa) protein is an extrinsic membrane protein component of the PSII oxygen-evolving complex and is situated on the

* To whom correspondence should be addressed

Abbreviations: kDa = kilodalton; LHCP-2 = light-harvesting chlorophyll a/b-binding protein; M_r = relative molecular mass; PSII = photosystem II; SDS = sodium dodecyl sulphate

lumenal surface of the thylakoid membrane (Ljungberg et al. 1984). Examining the behaviour of proteins in close proximity to each other but with contrasting spatial dispositions in the thylakoid membrane allows inferences to be made concerning the mode and site of action of proteolytic processes during chloroplast disassembly.

Materials and methods

Plant material. Plants of *Festuca pratensis* cv. Rossa (normal) and Bf 993 (mutant) were grown in hydroponic culture as described by Hilditch et al. (1986a). Rossa is a commercial fescue variety; Bf 993 is a Welsh Plant Breeding Station experimental line (Thomas 1987). Senescence was induced in 1-cm segments of leaves which were incubated in darkness at 20° C on moist filter paper in Petri dishes (Thomas 1987).

Protein extraction and analysis. For each sample, typically 40 leaf segments were frozen and homogenized in liquid nitrogen. Buffer was added, and homogenization continued until the mixture was completely thawed. In all experiments the extraction buffer contained 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) pH 7.5; 20 mM dithiothreitol; 1 mM disodium ethylenediaminetetraacetate (EDTA); 50 $\mu g \cdot ml^{-1}$ phenylmethylsulphonyl fluoride and 1% (v/v) 2-mercaptoethanol. For the extraction of total proteins, sodium dodecyl sulphate (SDS) was included in the buffer at 1% (w/v), and the mixture incubated at 40° C for 1 h before centrifugation at $12000 \cdot g_{av}$ at 20° C for 10 min. In some experiments, soluble proteins were separated from lamellar proteins; here SDS was not included in the initial extraction buffer, and centrifugation at $12000 \cdot g_{av}$ at 5° C immediately followed thawing of the tissue. The supernatant was designated the soluble fraction. Particulate proteins (mostly thylakoidal in origin) were extracted from the pellet by incubation with buffer containing SDS at 40° C as described above. Soluble protein samples were denatured by one freeze-thaw cycle following the addition of 0.32 volumes of 313 mM Tris pH 6.8; 10% (w/v) SDS; 25% (w/v) glycerol; 25% (v/v) 2-mercaptoethanol. Lamellar proteins were similarly prepared for electrophoresis using 0.25 volumes of a denaturing medium without mercaptoethanol and with urea in place of glycerol. Proteins were analysed by denaturing gel electrophoresis as described by Hilditch et al. (1986b).

Antisera. All antisera used in this study were polyclonal sera raised in rabbits. Antiserum to *F. pratensis* LHCP-2 was prepared as described previously (Hilditch 1986). Anti-D1 was obtained as an antiserum to a D1-galactosidase fusion protein from *Escherichia coli* transformed with cloned D1 genes from *Pisum sativum* (Nixon et al. 1986). This antiserum and one against *P. sativum* 33-kDa extrinsic protein were the kind gifts of D. Chapman, Imperial College, London.

Electroblotting. Following separation by SDS-polyacrylamide gel electrophoresis, proteins were transferred electrophoretically to nitrocellulose membrane (SM11306 BL; Sartorius, Belmont, Surrey, UK) in a Bio-Rad (Watford, Herts., UK) Transblot apparatus, used as directed by the manufacturers. Transfer was carried out overnight at a constant voltage of 60 V, current 100–120 mA. The buffer was cooled to below 5° C throughout.

Immune staining. After transfer the nitrocellulose membrane was washed in Tris-buffered saline (TBS: 10 mM Tris pH 7.4; 140 mM NaCl) for 20 min, and then in a 2% (w/v) solution in TBS of spray-dried skimmed milk (J. Sainsbury, London, UK) for 1 h. The primary antiserum was diluted, typically 100- to 1000-fold, in a solution of bovine serum albumin (BSA; 3% w/v) in TBS. After a 1-h incubation at room temperature, the membrane was washed six times in TBS. Secondary antibody (horseradish-peroxidase-conjugated swine anti-rabbit-Ig serum; Dako, High Wycombe, Bucks., UK) diluted 500-fold in 3% BSA in TBS, was added and similarly incubated. A substrate solution containing 0.2 $mg \cdot ml^{-1}$ 3-amino-9-ethylcarbazole in 50 mM sodium acetate pH 5.0 was added after two washes in TBS. The reaction was initiated by the addition of 30% H_2O_2 to 0.03% (v/v).

Immuno-electron microscopy. Tissue was sliced in fixative solution (67 mM cacodylate buffer pH 7.4 containing 1.85% [w/v] paraformaldehyde and 4.6% glutaraldehyde) into pieces about 1 mm square. The segments were then subjected to mild vacuum to remove air, and chilled to 4° C. After 2.5 h, the tissue was transferred to 30% ethanol (v/v in fixative) at 0° C; after a further 1 h to 50% ethanol at −10° C and chilled to −35° C over 2 h (Wells 1985). Subsequently the tissue was placed in 70% ethanol at −20° C and chilled to −35° C overnight. After transfer to 95% ethanol and 2 h at −35° C, the specimens were transferred into dry 100% ethanol, still at −35° C. Some 2 h later, the ethanol was replaced with fresh 100% ethanol, and incubated at −35° C for 3 h. At this time the ethanol was replaced with a 1:1 mixture of ethanol and resin (Lowicryl K4M; Chemische Werke Lowi, Waldkreiburg, FRG), and the tissue maintained at −35° C overnight. The next day the solution was changed to a 2:1 mixture of resin and ethanol; the following day to a 3:1 mixture. Finally the tissue was placed in 100% resin, which was changed twice. Polymerisation was induced by exposure to ultraviolet light for 3 d at −35° C and 2 d at about 10° C. Gold or silver sections were cut from the embedded blocks using a 45° glass knife, and picked up on 200-mesh gold grids with a support film of collodion or parlodion coated with carbon. Glow-discharging of the grids was used to improve adhesion of the sections. Grids were floated on primary antiserum diluted 1:20 (v/v) in 50 mM Tris pH 7.4, 1% (w/v) BSA, 0.1% (w/v) gelatine, 0.015% (v/v) Tween 20. After 1 h the grids were washed with a slow stream of distilled water, and blotted dry. They were next floated on secondary antiserum (a 1:20 dilution of anti-rabbit Ig, 15-nm gold particles; either Janssen, Beerse, Belgium, or BioCell Research Laboratories, Cardiff, UK) for 1 h, and washed. Staining was achieved with 2% (w/v) uranyl acetate for 10 min followed by lead-citrate solution (Reynolds 1963) for 2 min. Sections were examined by transmission electron microscopy using a Jeol (Tokyo, Japan) 100S microscope operated at 80 kV, Control grids were treated similarly but were floated initially either on 1:20 diluted pre-immune rabbit serum or on buffer without antiserum.

Results and discussion

Western blotting. The non-yellowing mutant genotype Bf 993 of *F. pratensis* retains its photosynthetic pigments during leaf senescence (Thomas and Stoddart 1975). Consequently, it is of interest to consider what effect the lesion has upon the behaviour of the protein components of the chloroplast. The major chlorophyll-binding protein LHCP-2 has been investigated previously by enzyme-linked immunoassay (Hilditch 1986); it was demonstrated that immunologically recognisable LHCP-2 re-

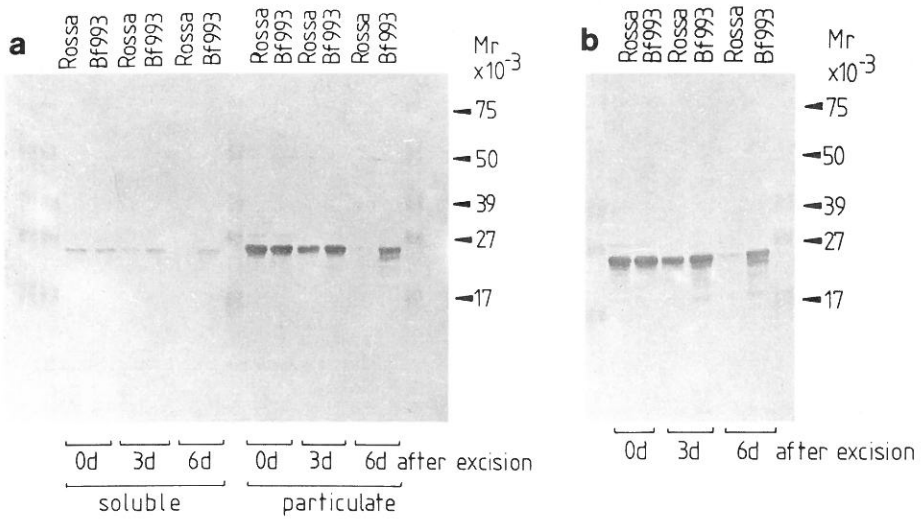

Fig. 1 a, b. Protein extracts from senescing leaves of mutant (Bf 993) and wild-type (Rossa) genotypes of *Festuca pratensis*, fractionated by SDS-polyacrylamide gel electrophoresis, blotted and stained for LHCP-2. **a** Soluble and lamellar extracts. **b** Total protein extract. Relative molecular masses (M_r) were determined with reference to pre-stained protein standards (Bio-Rad)

mained in senescing leaves of Bf 993, while the LHCP-2 content of leaves of the wild-type Rossa declined in a similar fashion to chlorophyll.

Figure 1 a presents the results of an experiment in which soluble and lamellar protein fractions from senescing leaves of both genotypes were blotted and probed with the anti-LHCP-2 serum. Considering first the lamellar proteins: in agreement with the data of Hilditch (1986) there was a marked decline in intensity of the major band with increasing senescence in wild-type Rossa, but little change in intensity in mutant Bf 993. A small number of fainter bands were visible, and the main band appeared to be split into more than one entity. It is likely that this splitting of the major LHCP-2 band is a consequence of the heterogeneity among LHCP-2 polypeptides (see Barber and Marder 1986); there is evidence for immunological similarity between the various isoforms (Slovacek and Harvey 1984). There is also a faint band some small distance above the LHCP-2 position (i.e. of higher molecular weight). The identity of this species is unclear, but it may represent one of the polypeptides of the related LHC-1 family that either crossreacted with the anti-LHCP-2 (Evans and Anderson 1986) or was originally co-purified with the LHCP-2 antigen. Bands of lower molecular weight than LHCP-2 are particularly evident in the samples from senescent leaves of Bf 993. It is likely that these are products of endopeptidase cleavage.

The separations of soluble proteins blotted with anti-LHCP-2 also presented in Fig. 1 a, revealed small concentrations of the antigen, mirroring the changes in the lamellar samples. These are presumed to represent contamination of the soluble protein fractions with lamellar proteins during extraction. No immunologically reactive fragments were detectable in the soluble fractions. In the same experiment, total protein fractions were prepared and blotted for LHCP-2 (Fig. 1 b). The results were similar to those obtained with blots of isolated lamellar proteins, except that lower molecular-weight proteolytic fragments were much more numerous and prominent.

Are the antigenic fragments observed in Fig. 1 true products of in vivo proteolysis occurring in senescing tissue? In preparing soluble and lamellar protein fractions, homogenisation of the leaf material and dispersion in buffer was immediately followed by centrifugation. This separates the thylakoid proteins from the major proteolytic enzymes which were released from the vacuole and remained in the soluble fraction. Moreover, low temperatures were maintained throughout the extraction process. On the other hand, the centrifugation step was omitted when isolating total protein and the entire homogenate was incubated at 40° C for 1 h. Despite the presence of SDS and protease inhibitors, endopeptidase (probably released from the vacuole) were clearly capable of attacking proteins in the extract. The (vacuolar) acid endopeptidase of grass leaves is known to be rather indifferent to such inhibitors (Thomas and Huffaker 1981; Miller and Huffaker 1981). This explanation is supported by observations of the behaviour of the large subunit of ribulose bisphosphate carboxylase in the soluble and total-protein fractions following electrophoresis (data not shown). A degradation product, roughly 2- to 3-kDa smaller, was visible in separations of total SDS-solubilised proteins but was almost undetectable in the lamella-free supernatant fraction which had not been incubated at 40° C with detergent.

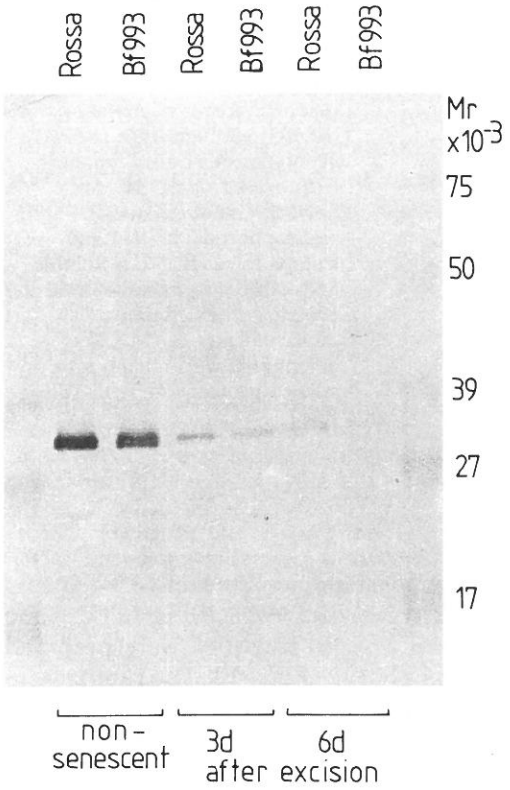

Fig. 2. The 33-kDa polypeptide of PSII visualised in lamellar protein extracts from senescing leaves of Bf 993 and Rossa by Western blotting

It seems reasonable to conclude, therefore, that the major proportion of LHCP-2 fragments detected in the total-protein extract, and by implication the similar fragments present in considerably lower amounts in the lamellar preparation, were the work of endopeptidases during extraction and processing and were not present in vivo. Their enhancement in extracts from senescent leaves of Bf 993 is readily explained, since levels of acid endopeptidase increase during senescence in both Rossa and Bf 993 (Thomas 1982b).

A Western blot from an experiment in which lamellar protein extracts were probed using anti-33-kDa serum is shown in Fig. 2. No antigenic components were detected in soluble protein extracts. This protein was degraded with similar kinetics in both Rossa and Bf 993, an observation consistent with earlier studies of PSII activity (Hilditch et al. 1986a) that failed to find significant differences between the two genotypes in the stability of the oxygen-evolving complex during senescence. The extrinsic nature of the 33-kDa protein renders it susceptible to degradation in Bf 993, whereas intrinsic membrane proteins are unusually stable in this genotype.

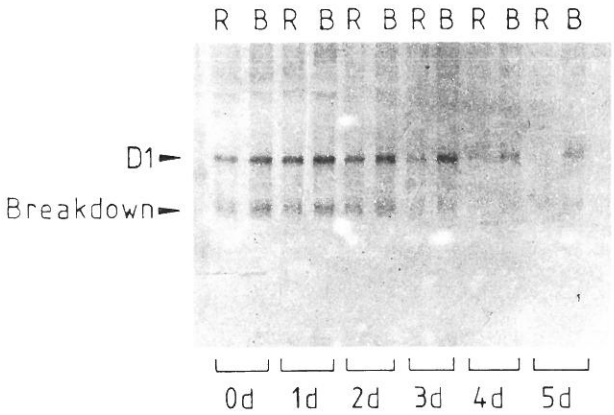

Fig. 3. Western blot of D1 protein in lamellar extracts from leaf tissue of Bf 993 (*B*) and Rossa (*R*) senescing in the dark

Antiserum to a D1-galactosidase fusion protein (Nixon et al. 1986) consistently identified not only a polypeptide of molecular weight around 32 kDa, but also a species of about 24 kDa (Fig. 3). Greenberg et al. (1987) have described a similar component and showed it to be a product of light-dependent in-vivo proteolytic breakdown from the N terminus of the protein. The fragment remained in the membrane of Bf 993 and was as stable as other thylakoid proteins in the dark, persisting in Rossa until 3 d, and up to 6 d in Bf 993. In the light, however, it is degraded at a similar rate to its parent molecule, and is thus not accumulated. This implies that not only the first, but also the second, step in the rapid proteolysis of D1 protein is light-requiring and that the initial step takes place in situ, without prior removal of the protein from the bilayer. Furthermore, the proteolytic fragment in the mutant shares with the intact D1 protein the property of abnormal stability in darkness (Fig. 3).

These immunological data are in broad agreement with the inferences made from radiolabelling studies of the turnover of D1 (Hilditch et al. 1986b). The stability of D1 protein during dark-induced senescence is considerably enhanced in the mutant Bf 993. It is apparent, however, that the stability of D1 in the mutant is not indefinite: levels of the protein in senescent leaves of Bf 993 were clearly lower than in fresh tissue.

Immuno-electron microscopy. Immuno-electron micrographs of chloroplasts from nonsenescent leaves of both genotypes, stained for LHCP-2 protein are presented in Fig. 4. No consistent or appreciable differences were observed in morphology or number of chloroplasts from mutant and wild-type before the onset of senescence. The LHCP-2 anti-

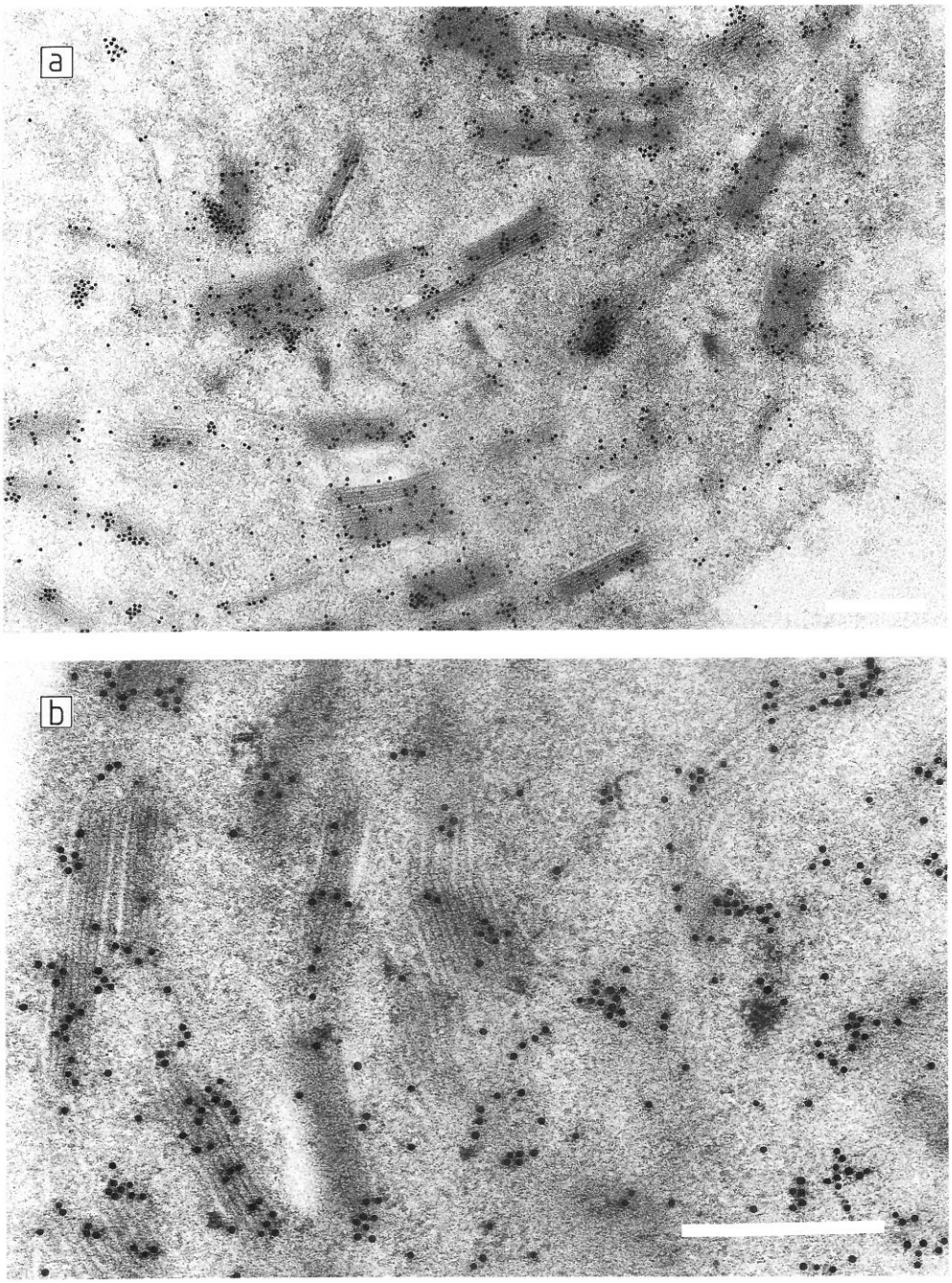

Fig. 4a, b. Micrographs of chloroplasts from nonsenescent tissue of **a** Rossa and **b** Bf 993, stained with anti-LHCP-2 and gold-labelled secondary antibody. Concentrations of antigen are visible in the appressed membrane region. Bars = 500 nm

gen was clearly concentrated in the appressed (stacked) regions of the thylakoid membranes.

Figure 5a is a micrograph of a chloroplast of Rossa tissue, which had been subjected to 4 d of dark-induced senescence. By this stage the internal structure of the organelle had become severely disrupted. Internal membranes had degenerated into chaotically arranged fragments displaying none of the organised structure seen in the nonsenescent organelles. There was a pronounced accumulation of electron-transparent holes corresponding to the locations of plastoglobuli, lipid globules characteristic of chloroplast senescence (Tevini and Steinmüller 1985). The specimen presented in Fig. 5a had been stained with anti-LHCP-2, but because the levels of antigen are considerably reduced in tissue at this stage of senescence, little gold labelling was apparent.

The photosynthetic membranes of Bf 993 chloroplasts are more stable than those of Rossa during

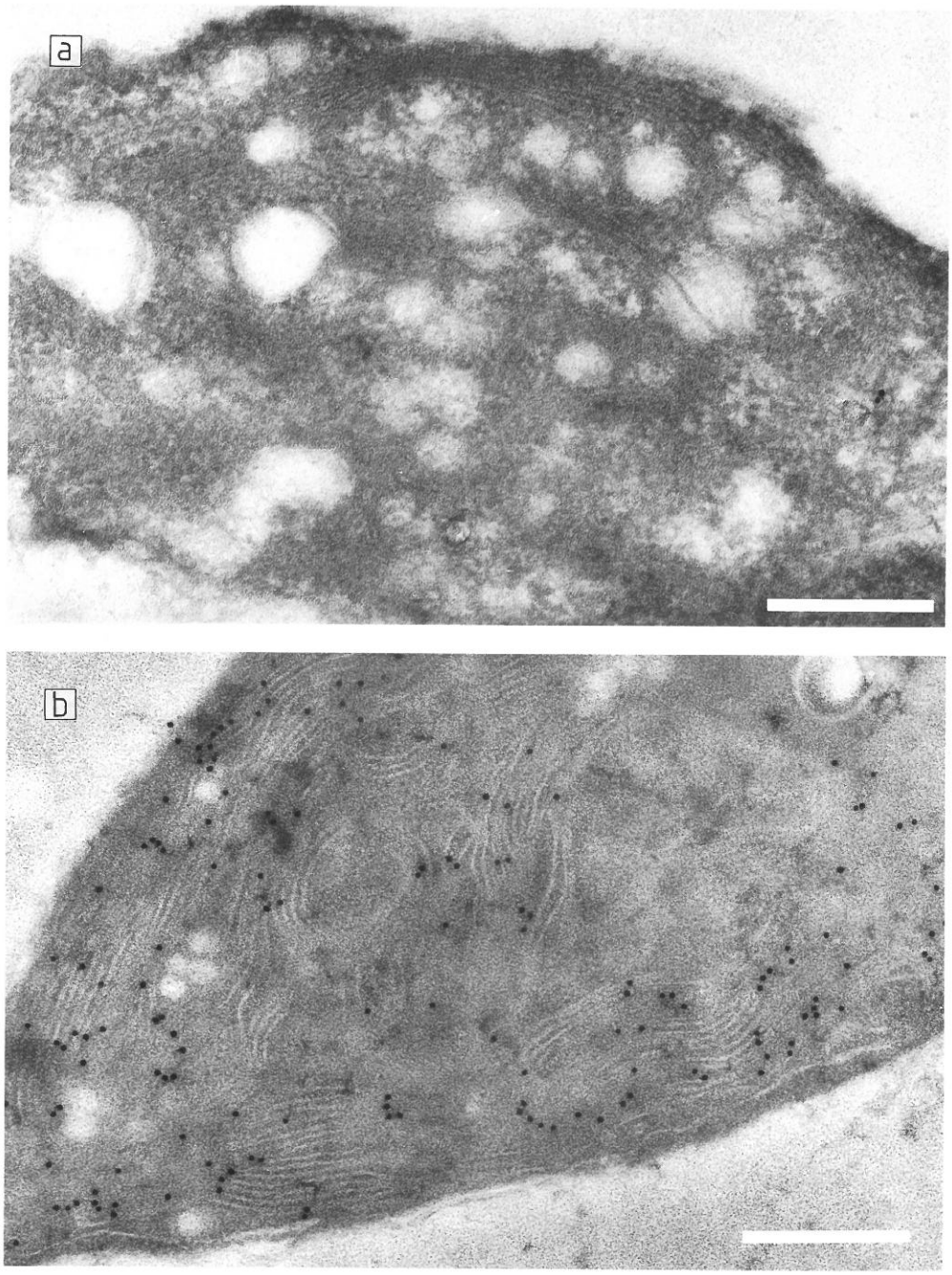

Fig. 5a, b. Micrographs of chloroplasts from tissue of **a** Rossa and **b** Bf 993 which had been senescing in the dark for 4 d, immunogold stained with anti-LHCP-2. Bars = 500 nm

senescence but do not survive dark incubation unchanged (Thomas 1977). The micrograph of Fig. 5b shows an organelle of Bf 993 in which the defined granal membrane stacks of nonsenescent plastids have been replaced by single membrane pairs, often lying in parallel with each other, but not so closely appressed as those in the typical granum. The number and size of plastoglobuli were greatly reduced in senescing chloroplasts of Bf 993 compared to those of Rossa. This specimen also was stained with anti-LHCP-2, revealing the continued presence of this protein, although the differential localization within the membrane had disappeared, lateral distribution of gold label throughout the membrane being essentially uniform.

The antisera to D1 and 33-kDa proteins were also used in ultrastructural studies (data not shown). The D1 protein displayed heterogeneous distribution within the membrane in nonsenescent

chloroplasts similar to that of LHCP-2; it too lost this organisation in senescent tissue of Bf 993 becoming randomly distributed throughout the membranes. The 33-kDa protein had largely disappeared from chloroplasts of both genotypes following their 4-d dark incubation.

Conclusions

The behaviour of a number of chloroplast membrane proteins during senescence in mutant Bf 993 and wild-type Rossa has now been studied using a variety of immunological techniques. Collating the information obtained from these studies with that gleaned from experiments of protein chemistry (Hilditch et al. 1986b; Thomas 1982a), the following generalization can be made concerning the effect of the non-yellowing lesion on chloroplast proteins: (i) the non-yellowing mutation confers exceptional stability during senescence on *intrinsic* thylakoid membrane proteins; (ii) *extrinsic* membrane proteins, whether on stromal or lumenal sides of the membrane, do not exhibit a comparable stability; (iii) the behaviour of *soluble* proteins in the chloroplast stroma is unaffected by the lesion.

It is not yet possible to say by what means the non-yellowing lesion confers differential stability on the protein components of this organelle. The defining phenotype of Bf 993 is a marked stability of photosynthetic pigment during senescence. These pigments are, however, complexed with proteins within the membrane, which are also stable in the mutant. Moreover, the pigment-protein complexes are closely associated with the galactolipids of the bilayer, which also seem to be unusually stable (Harwood et al. 1982). The problem lies in identifying which, if any, of these components is the *target* of the lesion.

The picture of thylakoid disassembly that emerges from our studies contrasts with that of Gepstein and co-workers (Roberts et al. 1987; Ben-David et al. 1983) who also used immunological approaches. They have interpreted functional and structural decline of bean and oat leaf chloroplasts during senescence almost exclusively in terms of disrupted protein synthesis and selective depletion of membrane components. In contrast, the behaviour of the *Festuca* mutant during senescence strongly indicates that declining synthesis alone cannot account for net protein loss: positive activation of (probably senescence-specific) degradation processes must also occur.

It is clear that we know rather little about the means by which intrinsic polypeptides are degraded. Are they removed from the bilayer for processing by soluble proteinases? If so, how are the thermodynamic obstacles overcome? Alternatively, do there exist specialized and as yet undiscovered proteinases which are capable of attacking membrane proteins in situ? D1 protein certainly appears to be the substrate for such a proteinase in the light – but this proteinase is probably extremely specific, as is the thylakoid-associated proteolytic activity which is apparently responsible for the breakdown of the enzyme protochlorophyllide reductase during chloroplast biogenesis (Griffiths and Walker 1986). The degradation of membrane proteins must require the attentions of factors which are different from those which catabolise soluble proteins, and it is possible that it is this type of activity which is deficient in mutant Bf 993. Work in progress is investigating the molecular genetics of the mutation.

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