Acta Bot. Neerl. 42(4), December 1993, p. 481–490

Chlorophyll degradation by free radicals derived from linolenic acid in incubated barley thylakoids

J. CUELLO and A. LAHORA

Departamento de Biologia Vegetal, Facultad de Biologia, Universidad de Murcia, 30100 Espinardo, Murcia, Spain

SUMMARY

Using chloroplasts isolated from the senescent primary leaves of barley seedlings, the linolenic acid-dependent degradation of chlorophyll was studied in incubated thylakoids. This reaction, measured by the decrease of absorbance at 672 nm, was produced by free radicals from linolenic acid oxidation and, thus, was inhibited by *n*-propyl-gallate and α-tocopherol, general scavengers of free radicals. Although the beginning of the reaction was delayed by heating the thylakoids, this process must be of a non-enzymatic nature since it is not impeded by thermal treatment and it proved impossible to isolate a crude extract from thylakoids with a degradative enzymatic activity using a mixture of solubilized chlorophylls as substrate. The enzyme chlorophyll oxidase, described by others for thylakoids, was not found. In addition, the reaction of chlorophyll with the peroxidation products of linolenic acid seems to occur with the cooxidation of one or more carotenoids. Due to the high content of esterified linolenic acid in the thylakoid galactolipids, the degradation of chlorophyll studied here may have a physiological significance.

Key-words: chlorophyll degradation, free radicals, *Hordeum vulgare*, linolenic acid, senescence, thylakoids.

INTRODUCTION

Although chlorophyll degradation is one of the most characteristic symptoms of leaf senescence, the reactions through which this process occurs are still not clear (Thimann 1980; Hendry *et al.* 1987).

Among the various enzymes described as responsible for chlorophyll degradation (Sabater & Rodriguez 1978; Hendry *et al.* 1987; Matile *et al.* 1989) at least two, chlorophyll peroxidase and oxidase, are constituent proteins of thylakoids (Martinoia *et al.* 1982; Lüthy *et al.* 1984; Thomas *et al.* 1985). The characteristics of chlorophyll oxidase, described as a linolenic acid (LNA)-dependent enzyme, are relatively difficult to study because the enzyme is found together with its substrate in the same thylakoidal membranes. It is surprising that chlorophyll oxidase is present in the two photosystems, both with a chlorophyll *a*/chlorophyll *b* ratio higher than 8, while the light-harvesting complex with a ratio of 1·2 does not show this enzymatic activity (Lüthy *et al.* 1986b).

Abbreviations: LNA, linolenic acid; MDA, malondialdehyde.

Besides enzyme and substrate, the supposed activator (LNA) is also found in the thylakoidal membranes: the principal lipids of the photosynthetic tissues (galactosyl-diglycerides) that are found in chloroplast membranes contain very high quantities of LNA; in leaves of 18:3 plants this represents more than 90% of the total of esterified fatty acids (Douce & Joyard 1980). It has also been found that LNA is released from thylakoids in *in vitro* experiments (O'Sullivan *et al.* 1987; O'Sullivan & Dalling 1989). In addition, LNA stimulates the senescence of oat leaf sections both in light and darkness (Ueda & Kato 1982) and the senescence of the pulvinus in bean petiole explants in light (Ueda *et al.* 1991).

Using thylakoids from barley leaves, we have studied the nature of linolenic acid-dependent degradation of chlorophyll. The results show that the chlorophyll reacts with free radicals resulting from the oxidation of LNA in a non-enzymatic way, and with the probable involvement of one or more carotenoids.

MATERIALS AND METHODS

Plant material and thylakoid isolation

Barley seedlings (*Hordeum vulgare* L. var Hassan) were grown for 14 days as described by Cuello *et al.* (1984).

Chloroplasts were isolated, as described by Garcia *et al.* (1983), from 4 g of primary leaf sections and 25 ml of extraction buffer containing 0·35 M saccharose, 25 mM HEPES, 2 mM Na_2-EDTA and 2 mM sodium isoascorbate, pH 7·6. The chloroplast precipitate obtained by centrifugation of the extract at 2500 × *g* was washed twice with 5 ml extraction buffer. According to Calle *et al.* (1986), this preparation of chloroplasts is relatively free of contamination by cytoplasm (<1%) and mitochondrial (<5%) proteins.

The membranous fraction of chloroplasts was also prepared according to Garcia *et al.* (1983), the final precipitate of membranes being resuspended in 1 ml of 0·1 M sodium phosphate buffer, pH 7·4.

Measurement of chlorophyll degradation

The chlorophyll oxidase activity associated with thylakoids was measured, according to the method of Thomas *et al.* (1985), by the decrease in absorbance at 672 nm during the initial linear phase of chlorophyll degradation at 25°C. This measurement, and that of spectral changes occurring during the degradation process, were carried out in a Hitachi 150-20 spectrophotometer. The reaction was carried out in a final volume of 3 ml in 0·1 M sodium phosphate buffer, 0·05% sodium dodecyl sulphate (SDS) (w/v) and 1·4 mM LNA, pH 7·4, with an initial concentration of thylakoidal chlorophyll of approximately 0·02 g l^{-1}. Sometimes the reaction medium contained a different detergent or other possible effectors in the concentrations indicated. Apart from 4-methyl-catechol (which was assayed by adding a solution of 0·125 M in water) and LNA and α-tocopherol (which were added to the medium using ethanolic stock solutions of 20 g l^{-1} and 0·2 M, respectively), the effectors were incorporated into the reaction medium by dissolving them in the 0·1 M sodium phosphate buffer. The volumes of LNA and α-tocopherol ethanolic solutions added to the medium were insignificant (<2%) when compared with the total volume. The quantities of ethanol added to the reaction media had no discernible effect, at least during the reaction times used. Chlorophyll transformation in

a slightly acid medium was studied with thylakoids in 0·1 M sodium phosphate buffer, pH 6·2.

Isolation of crude colourless extract from thylakoids

The colourless enzymatic extract from thylakoids was obtained using a slightly modified method of Shioi *et al.* (1991) for the crude extract of *Chenopodium album* with chlorophyll-degradative activity. The thylakoid precipitate from 8 g of leaves was homogenized with 3 ml of 80% acetone (v/v) at −20°C and then centrifuged at 10 000 × *g* for 10 min. The supernatant obtained (solution of chlorophylls and other pigments) was kept for use as substrate for the reaction, while the precipitate, after being washed twice with 10 ml absolute acetone at −20°C, was resuspended in 2 ml of 0·1 M sodium phosphate buffer, pH 7·4. To dissolve this precipitate, SDS was added to the resuspension up to 0·5% (w/v), which was shaken in the cold for 60 min before centrifugation at 2200 × *g* for 5 min, the supernatant constituting the supposed colourless enzymatic extract. To assay the activity of the extract, the previously described reaction medium (3 ml) contained a mixture of chlorophylls (from the solution of 80% acetone) instead of thylakoids and, in addition, 100 μl of colourless extract.

Pigment separation and other determinations

The photosynthetic pigments were separated by thin-layer chromatography from the ether extract of the mixture of barley leaf pigments, using a thin layer of cellulose (Carlo-Erba) and the petroleum ether 60–80/acetone/*n*-propanol (90/10/0·45) solvent (Harborne 1973), the separated pigments being eluted with ethanol. These solutions and the ethanolic solutions of 1·33 g l^{-1} pure chlorophylls *a* and *b* (Sigma) were used to study the possible reaction of the individual pigments in the standard medium with LNA.

The levels of LNA peroxidation in the ethanolic solutions were measured by means of thiobarbituric acid assay for malondialdehyde (MDA) (Heath & Packer 1968). The absolute concentration of chlorophyll was measured by the method of Arnon (1949), while protein was determined by the method of Lowry *et al.* (1951) after precipitation with 10% trichloroacetic acid (w/v).

RESULTS AND DISCUSSION

SDS, the detergent usually used in the experimental media for chlorophyll oxidase, does not dissolve well in potassium phosphate buffer so, because the non-enzymatic formation of phaeophytins from chlorophyll occurs at slightly acid pH and, furthermore, LNA is less soluble in the acidic range (Lüthy *et al.* 1984), 0·1 M sodium phosphate buffer pH 7·4 (Thomas *et al.* 1985) was chosen for this work.

Firstly, the effects of the detergents, SDS and Triton X-100, on the rate of chlorophyll degradation were studied, when used in the concentrations recommended by other authors (Thomas *et al.* 1985; Lüthy *et al.* 1986a) (Fig. 1). In the selected conditions, the initial rate of chlorophyll degradation was higher with SDS than with Triton X-100, whether the latter was applied at the beginning of the reaction or when preincubating the thylakoids in the cold with the detergent. This may be because LNA dissolves better with SDS than with Triton X-100. With the simultaneous presence of SDS and 10% acetone (a solvent which, according to Shioi *et al.* (1991), stimulates the enzymatic degradation of chlorophyll up to 30% or more), the rate of degradation is identical to

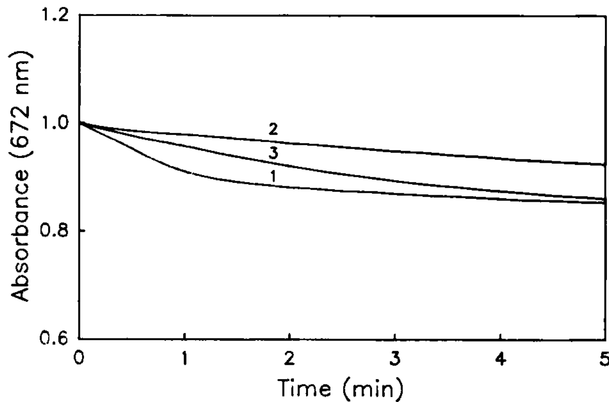

Fig. 1. Degradation of chlorophyll in thylakoids incubated in 0·1 M sodium phosphate buffer and 1·4 mM LNA, pH 7·4. Reaction in the presence of (1) 0·05% SDS, (2) 0·1% Triton X-100, and (3) 0·035% Triton X-100 (after preincubating the thylakoids for 30 min in the cold with 1·5% Triton X-100) or 0·05% SDS+10% acetone. The same ethanolic solution of LNA was used in all the experiments. The results refer to one of three assays (with no significant differences between them). The small initial variations in absorbance were not considered.

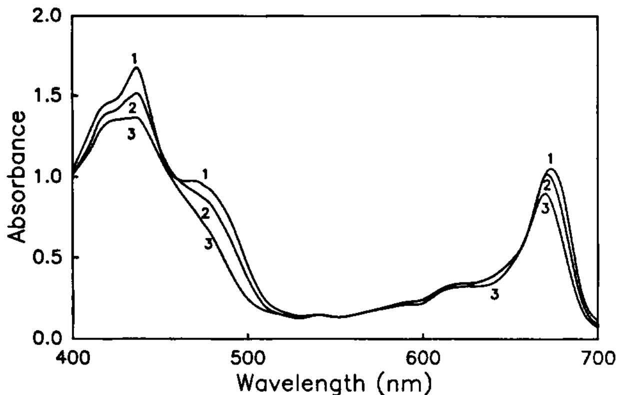

Fig. 2. Spectral changes with time of thylakoids incubated in 0·1 M sodium phosphate buffer, 0·05% SDS and 1·4 mM LNA, pH 7·4. The spectra shown represent the medium (1) without LNA, (2) immediately after, and (3) 5 min after the addition of 59 µl of a solution of LNA containing 0·161 µM MDA. The curves refer to one of three assays (with no significant differences between them). For more experimental details, see text.

that of thylakoids preincubated with Triton (Fig. 1). Furthermore, the formation of chlorophyllide in chloroplast membranes suspended in buffer increases with Triton X-100 but not with SDS (Amir-Shapira *et al.* 1986). So 0·05% SDS was included in the reaction medium in all the following experiments.

The spectral changes produced during the LNA-dependent degradation of chlorophyll are shown in Fig. 2, where the recordings from 700 to 400 nm in the standard reaction medium are depicted before and after the addition of LNA (at 0 and 5 min). The greatest decreases in absorbance are at approximately 437, 470 and 672 nm. The fall at 672 nm is due not only to chlorophyll bleaching but also, to a lesser extent, to the displacement of the absorption maximum of thylakoids to shorter wavelengths (2 nm at 5 min, compare curves 2 and 3). While the decreased absorbance at 437 and 672 nm are

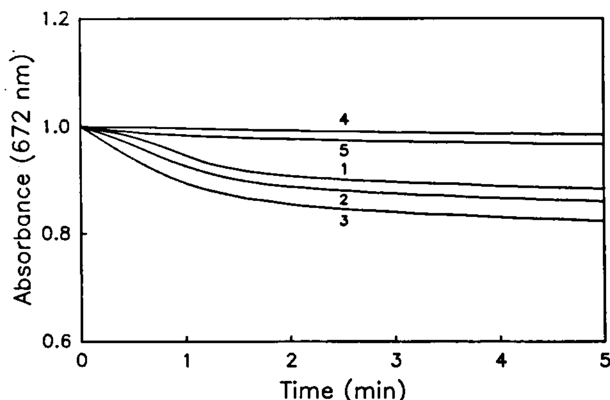

Fig. 3. Effect of the degree of LNA oxidation and of general scavengers of free radicals on chlorophyll degradation in incubated thylakoids. MDA concentrations in the ethanolic solutions of LNA were: (1) 0·022 μM (preparation from a recently opened vial), (2) 0·146 μM, and (3) 0·218 μM. The effects of (4) 2 mM α-tocopherol and (5) 10 μM *n*-propyl-gallate were assayed with the LNA solution containing 0·218 μM MDA. The results shown refer to one of three experiments (with no significant differences between them).

due to the loss of chlorophyll, the disappearance of the shoulder at 470 nm is caused, in part at least, by the reaction of carotenoids because, in addition to the fact that carotenoids are greatly absorbing at this wavelength, no more xanthophylls were detected after the chromatographic separation of a mixture of photosynthetic pigments and LNA. Furthermore, the LNA-dependent reaction(s) (Fig. 2) take(s) place through free radicals since the spectral changes are inhibited almost totally by 10 μM *n*-propyl-gallate (Fig. 3). In addition, the changes produced in the standard medium with LNA are qualitatively different from those occurring in thylakoids suspended in 0·1 M sodium phosphate buffer pH 6·2 without LNA. In the latter case, according to results not shown, absorbance increases at 400–420 nm, a peak is formed at 540 nm and the initial maximum at 672 nm does not change its wavelength. These results are typical of the non-enzymatic formation of phaeophytins and phaeophorbides in a dilute acidic medium (Hendry *et al.* 1987).

Since LNA-dependent chlorophyll degradation is inhibited by the general free radical scavenger *n*-propyl-gallate, the effect of LNA preparations of different degrees of oxidation, characterized by their MDA contents, was assayed. The results with three different solutions of LNA including the effects of the general scavengers of free radicals, α-tocopherol and *n*-propyl-gallate, are shown in Fig. 3. Both the initial rate and the degradation profile depend, at least partially, on the MDA content of the LNA preparation used. A direct relation was observed between the rate of degradation and MDA content, which increases with the time elapsed since the preparation of the LNA solution. Furthermore, the scavengers, α-tocopherol and *n*-propyl-gallate, almost totally inhibit chlorophyll degradation with any of the LNA preparations (shown in Fig. 3 for the highest MDA content), an inhibition already described for chlorophyll oxidase with these scavengers (Lüthy *et al.* 1986a). These results indicate the need to characterize the LNA preparation used with regard to its MDA content, or any other product indicative of the level of free radicals, in order to study the other factors which influence the reaction.

To investigate the possible enzymatic character of the reaction, the effect of thermal inactivation of the thylakoids on the chlorophyll degradation rate was studied (Fig. 4).

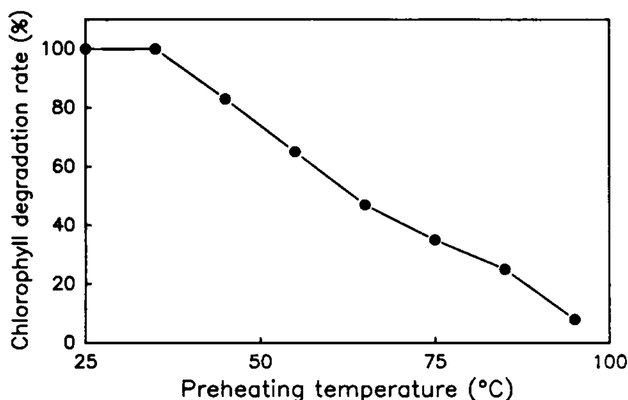

Fig. 4. Rate of chlorophyll degradation in thylakoids preheated to different temperatures. The thylakoids were exposed before the assays, to the indicated temperatures for 10 min in the presence of 0·5% SDS. The decrease in absorbance at 672 nm was measured 0·2–0·4 min after the addition of a LNA solution containing 0·218 μM MDA. The decrease observed for thylakoids preheated at 25°C (0·115) was taken as 100%. Values are the means of two independent experiments. No individual value deviates more than 12% from the mean.

It was necessary to heat the thylakoids with 0·5% SDS to prevent insolubilization. Thermal inactivation of chlorophyll degradation occurred, although with an atypical profile. Compared with pretreatment at 25°C (100% activity), the degradation rate fell to approximately 50% at 65°C while at 85°C it was still above 20%. Apart from the high temperatures necessary for a high degree of inactivation, the almost proportional inverse relation between degradation rate and temperature is not typical of the thermal inactivation of enzymes. These results contrast with those of Martinoia *et al.* (1982) who found that chlorophyll oxidase was significantly activated by preheating the thylakoids up to 50°C and was only inactivated at temperatures above 55°C. A possible explanation for this behaviour in our studies is that the chlorophyll degradation, dependent on free radicals derived from LNA, at least in part may be non-enzymatic; preheating the thylakoids would then make the chlorophyll less accessible to these radicals.

To test this hypothesis, colourless extracts of thylakoids were isolated in accordance with the procedure described by Shioi *et al.* (1991) for obtaining crude enzymatic extracts from *Chenopodium album* with chlorophyll-degradative activity (see Materials and Methods). The results obtained using this colourless extract and a mixture of solubilized chlorophylls as substrate are incompatible with an enzymatic reaction (Fig. 5). The chlorophyll is degraded with boiled extract (although with a slight delay) and even without extract (with a greater delay). Moreover, diethyldithiocarbamate (DIECA) inhibits degradation with or without extract (Fig. 5), which excludes the possibility of it acting through the inactivation of chlorophyll oxidase, as indicated by Lüthy *et al.* (1984). In addition, the spectral changes which take place with time in the medium containing solubilized chlorophylls, with or without the colourless extract, are similar to those of the medium with thylakoids (Fig. 2) although, in the case of free chlorophylls, the decreases in absorbance occur approximately at 440, 470 and 669 nm (results not shown).

To test the reaction of the individual photosynthetic pigments with the LNA peroxidation products, the pigments were isolated by thin-layer chromatography. Based on the spectral changes observed in the usual reaction medium, there was no bleaching

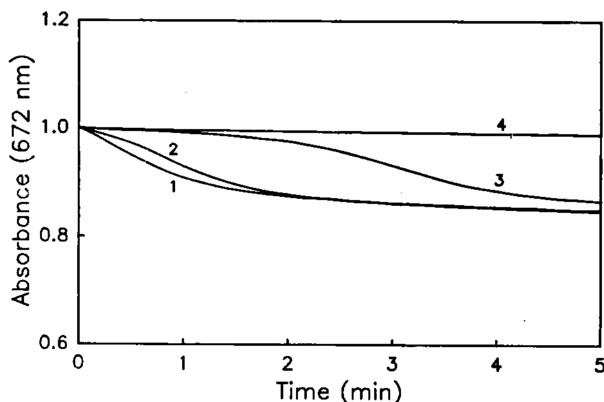

Fig. 5. Chlorophyll degradation in standard medium supplemented with 4% acetone, containing free chlorophylls and colourless extract from thylakoids. The reaction took place with (1) crude extract (100 µl in a total volume of 3 ml), (2) extract boiled for 5 min, (3) without extract, and (4) in any of the above conditions plus 0·5 mM DIECA. Initial mixtures of chlorophylls in 80% acetone and the colourless extract were obtained as described in Materials and Methods. Solutions of LNA containing 0·180 µM MDA were used. The colourless extract contained 2·83 mg protein ml^{-1}. The small initial variations in absorbance were not considered. The data refer to one of three or four experiments carried out with almost identical results.

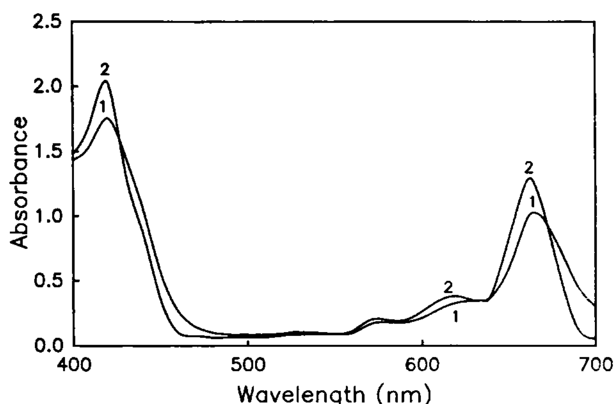

Fig. 6. Spectral changes of pure chlorophyll *a* produced by addition of LNA. The spectra are recorded in the standard medium (1) before and (2) immediately after the addition of 59 µl solution of LNA containing 0·161 µM MDA. Chlorophyll *a* concentration in the medium is approximately 0·02 g l^{-1}. The spectra refer to one of three assays carried out with almost identical results.

of chlorophyll *a*, *b* or lutein when these pigments were assayed individually with LNA or when binary combinations were made with this polyunsaturated fatty acid. In the case of individual chlorophylls, there were instantaneous increases in absorbance at their two characteristic maxima (Fig. 6 for chlorophyll *a*); these changes were not affected by 10 µM *n*-propyl-gallate. However, as indicated above, the chromatographic separation of an ether solution containing a mixture of pigments and LNA showed the disappearance of xanthophylls and part, at least, of the chlorophyll *a*. One explanation for this is that the non-enzymatic degradation of chlorophyll by LNA-free radicals might occur with the cooxidation of one or more specific carotenoids. This participation of several pigments in the oxidation process with LNA would explain the absence of

chlorophyll degradation in some pigment complexes isolated from thylakoids (Lüthy *et al.* 1986b).

To summarize, the results indicate that the concentration of free radicals in the LNA sample plays a decisive role in chlorophyll degradation in incubated thylakoids (Fig. 3). This can be demonstrated because the general scavengers of free radicals, α-tocopherol and *n*-propyl-gallate inhibit this degradation almost completely (Fig. 3). This reaction is mainly non-enzymatic and, under the assay conditions, no colourless thylakoid extract was found which catalyses the reaction of chlorophyll with the LNA peroxidation products. For reasons as yet unknown, the reaction is delayed by heating of the thylakoids (Fig. 4) or of their extract (Fig. 5). DIECA, in principle considered as an inhibitor of chlorophyll oxidase (Lüthy *et al.* 1984), acts as a scavenger of free radicals (Cornwell & Morisaki 1984; Sinaceaur *et al.* 1984). According to our experimental results, 5 mM 4-methyl catechol and 10 mM pyrogallol (known substrates of polyphenol oxidase) totally inhibit LNA-dependent chlorophyll degradation, in agreement with the role of these phenolic compounds as scavengers of free radicals. The breakdown of chlorophyll associated with lipid peroxidation has also been described as caused by bipyridylium herbicides such as diquat (Van Rensen 1975) and paraquat (Harris & Dodge 1972). Recently, the participation of lipid peroxides in chlorophyll breakdown has been demonstrated (Somashekaraiah *et al.* 1992), as has the fact that senescence or abscission is stimulated by LNA through the formation of LNA peroxides (Ueda *et al.* 1991).

The possible participation in the thylakoid medium of lipoxygenase, a generator of LNA peroxides found in chloroplasts (Galliard & Chan 1980), cannot be ignored. Moreover, there is the possible detergent effect of LNA (Thomas 1986) because this liberates intrinsic proteins from thylakoids (Garstka & Kaniuga 1991).

The results obtained might have a physiological significance in leaf senescence. High levels of LNA are esterified in thylakoid galactolipids (Douce & Joyard 1980) and it has been shown that the galactolipid LNA and/or linoleic acid content of thylakoids of chloroplasts in oat leaves increases during senescence in the dark (Dalgarn & Newman 1979). In addition, phospholipid-degrading enzymes in senescing membranes show a preference to degrade molecular species containing polyunsaturated fatty acids (Brown *et al.* 1991) which, if extended to include the galactolipids, would lead to the liberation of LNA. The *in vitro* release of LNA from thylakoids through thylakoid-associated galactolipase activity has been demonstrated (O'Sullivan *et al.* 1987; O'Sullivan & Dalling 1989), although in our case the quantity of LNA released must be very low since the SDS of the reaction medium inhibits galactolipase activity (O'Sullivan *et al.* 1987). Studies involving a non-yellowing mutant of *Festuca pratensis* support the idea that the hydrolysis of thylakoid galactolipids plays an important role in the regulation of leaf senescence (Thomas 1987; Gepstein 1988). Since lipolytic enzymes are found in membranes of non-senescent tissues (Brown *et al.* 1991), the protein synthesis in cytoplasm, which is necessary for chloroplast degradation (Stoddart & Thomas 1982; Cuello *et al.* 1984) might lead to an increase in the activity of these enzymes.

ACKNOWLEDGEMENTS

This work was financed by CICYT (Project PB 85-0318). We are grateful to Professor D. Francisco Garcia Carmona for checking the manuscript.

REFERENCES

Amir-Shapira, D., Goldschmidt, E.E. & Altman, A. (1986): Autolysis of chlorophyll in aqueous and detergent suspensions of chloroplast fragments. *Plant Science* **43:** 201–206.

Arnon, D.J. (1949): Copper enzymes in isolated chloroplasts polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* **24:** 1–15.

Brown, J.H., Palyath, G. & Thompson, J.E. (1991): Physiological mechanisms of plant senescence. In: Bidwell, R.G.S. (ed.): *Growth and Development* (Steward, F.C. (ed.): *Plant Physiology: A Treatise*, Vol. X). Academic Press, New York.

Calle, F., Martin, M. & Sabater, B. (1986): Cytoplasmic and mitochondrial localization of the glutamate dehydrogenase induced by senescence in barley (*Hordeum vulgare*). *Physiol. Plant.* **66:** 451–456.

Cornwell, D.G. & Morisaki, N. (1984): Fatty acid paradoxes in the control of cell proliferation: prostaglandins, lipid peroxides, and cooxidation reactions. In: Pryor, W.A. (ed.): *Free Radicals in Biology*, Vol. VI. Academic Press, New York.

Cuello, J., Quiles, M.J. & Sabater, B. (1984): Role of protein synthesis and light in the regulation of senescence in detached barley leaves. *Physiol. Plant.* **60:** 133–138.

Dalgarn, D.S. & Newman, D.W. (1979): Lipids of dark-treated oat (*Avena sativa*) leaf chloroplast thylakoids. *J. Exp. Bot.* **30:** 551–556.

Douce, R. & Joyard, J. (1980): Plant galactolipids. In: Stumpf, P.K. and Conn, E.E. (eds): *The Biochemistry of Plants*, Vol. 4. Academic Press, San Diego.

Galliard, T. & Chan, H.W.-S. (1980): Lipoxygenases. In: Stumpf, P.K. and Conn, E.E. (eds): *The Biochemistry of Plants*, Vol. 4. Academic Press, San Diego.

Garcia, S., Martin, M. & Sabater, B. (1983): Protein synthesis by chloroplasts during the senescence of barley leaves. *Physiol. Plant.* **57:** 260–266.

Garstka, M. & Kaniuga, Z. (1991): Effect of linolenic acid on release of the 43 KDa polypeptide and manganese from photosystem II membranes depleted of the 33 KDa extrinsic polypeptide. *Physiol. Plant.* **82:** 271–277.

Gepstein, S. (1988): Photosynthesis. In: Nooden, L.D. and Leopold, A.C. (eds): *Senescence and Aging in Plants*. Academic Press. San Diego.

Harborne, J.B. (1973): *Phytochemical Methods*. Chapman & Hall, London.

Harris, N. & Dodge, A.D. (1972): The effect of paraquat on flax on cotyledon leaves: physiological and biochemical changes. *Planta* **104:** 210–219.

Heath, R.L. & Packer, L. (1968): Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **125:** 189–198.

Hendry, G.A.F., Houghton, J.D. & Brown, S.B. (1987): The degradation of chlorophyll-*a* biological enigma. *New Phytol.* **107:** 255–302.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951): Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

Lüthy, B., Martinoia, E., Matile, P. & Thomas, H. (1984): Thylakoid-associated 'chlorophyll oxidase': distinction from lipoxygenase. *Z. Pflanzenphysiol.* **113:** 423–434.

Lüthy, B., Matile, P. & Thomas, H. (1986a): Properties of linolenic acid-dependent chlorophyll oxidation activity in thylakoid membranes. *J. Plant Physiol.* **123:** 169–180.

Lüthy, B., Thomas, H. & Matile, P. (1986b): Linolenic acid-dependent 'chlorophyll oxidase'-activity: a property of photosystems I and II. *J. Plant Physiol.* **123:** 203–209.

Martinoia, E., Dalling, M.J. & Matile, P. (1982): Catabolism of chlorophyll: demonstration of chloroplast-localized peroxidative and oxidative activities. *Z. Pflanzenphysiol.* **107:** 269–279.

Matile, P., Düggelin, T., Schellenberg, M., Rentsch, D., Bortlik, K., Peisker, C. & Thomas, H. (1989): How and why is chlorophyll broken down in senescent leaves. *Plant Physiol. Biochem.* **27:** 595–604.

O'Sullivan, J.N., Warwick, N.W.M. & Dalling, M.J. (1987): A galactolipase activity associated with the thylakoids of wheat leaves (*Triticum aestivum* L.). *J. Plant Physiol.* **131:** 393–404.

O'Sullivan, J.N. & Dalling, M.J. (1989): The effect of a thylakoid-associated galactolipase on the morphology and photochemical activity of isolated wheat leaf chloroplasts. *J. Plant Physiol.* **134:** 504–509.

Sabater, B. & Rodriguez, M.T. (1978): Control of chlorophyll degradation in detached leaves of barley and oat through effect of kinetin on chlorophyllase levels. *Physiol. Plant.* **43:** 274–276.

Shioi, Y., Tatsumi, Y. & Shimokawa, K. (1991): Enzymatic degradation of chlorophyll in *Chenopodium album*. *Plant Cell Physiol.* **32:** 87–93.

Sinaceaur, J., Ribiere, C., Nordmann, J. & Nordmann, R. (1984): Superoxide degrading activity of desferrioxamine. In: Bors, W., Saran, M. and Tait, D. (eds): *Proc. 3rd Int. Conf. on 'Oxygen Radicals in Chemistry and Biology' Neuherberg 1983*. Walter de Gruyter, Berlin.

Somashekaraiah, B.V., Padmaja, K. & Prasad, A.R.K. (1992): Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus*

vulgaris). Involvement of lipid peroxides in chlorophyll degradation. *Physiol. Plant.* **85:** 85–89.

Stoddart, J.L. & Thomas, H. (1982): Leaf senescence. In: Boulter, D. and Parthier, B. (eds): *Nucleic Acids and Proteins in Plants*. Enc. of Plant Physiol. New Series vol. 14A. Springer-Verlag, Berlin.

Thimann, K.V. (1980): The senescence of leaves. In: Thimann, K.V. (ed): *Senescence in Plants*. CRC Press, Florida.

Thomas, H. (1986): The role of polyunsaturated fatty acids in senescence. *J. Plant Physiol.* **123:** 97–105.

Thomas, H. (1987): Sid: a Mendelian locus controlling thylakoid membrane disassembly in senescing leaves of *Festuca pratensis*. *Theor. Appl. Genet.* **73:** 551–555.

Thomas, H., Lüthy, B. & Matile, P. (1985): Leaf senescence in a non-yellowing mutant of *Festuca pratensis* Huds. Oxidative chlorophyll bleaching by thylakoid membranes during senescence. *Planta* **164:** 400–405.

Ueda, J. & Kato, J. (1982): Abscisic acid and C_{18}-unsaturated fatty acids as senescence-promoting substances from oat plants. *J. Plant Growth Regul.* **1:** 195–203.

Ueda, J., Morita, Y. & Kato, J. (1991): Promotive effect of C_{18}-unsaturated fatty acids on the abscission of bean petiole explants. *Plant Cell Physiol.* **32:** 983–987.

Van Rensen, J.J.S. (1975): Lipid peroxidation and chlorophyll destruction caused by diquat during photosynthesis in *Scenedesmus*. *Physiol. Plant.* **33:** 42–46.