*Phytochemistry*, Vol. 27, No. 2, pp. 345–348, 1988.
Printed in Great Britain.

0031–9422/88 $3.00 + 0.00


# PHOTOBLEACHING OF CHLOROPLAST PIGMENTS IN LEAVES OF A NON-YELLOWING MUTANT GENOTYPE OF *FESTUCA PRATENSIS*

HOWARD THOMAS* and PHILIPPE MATILE†

Plant and Cell Biology Department, Welsh Plant Breeding Station, Plas Gogerddan, Aberystwyth, Dyfed, SY23 3EB, U.K.; †Institut für Pflanzenbiologie der Universität, Zollikerstrasse 107, CH-8008 Zürich, Switzerland

(*Received* 22 *June* 1987)

**Key Word Index**—*Festuca pratensis*; Gramineae; meadow fescue; leaf senescence; chlorophyll; carotenoid; photobleaching.

**Abstract**—Chloroplast pigment contents in a normal (cv Rossa) and a non-yellowing mutant (Bf993) genotype of *Festuca pratensis* were compared during senescence of excised leaf tissue in darkness and under photobleaching conditions. Methylviologen-treated leaves of both genotypes were bleached to the same extent when illuminated. Feeding δ-aminolaevulinic acid in the dark for 24 hr followed by exposure to light also resulted in photodestruction of chlorophylls and carotenoids in both Rossa and Bf993. Mutant leaf tissue retained more than 92% of chlorophyll during eight days in continuous darkness, but the pigment became progressively more susceptible to photobleaching as senescence proceeded. The $t_{1/2}$ for loss of capacity to withstand photobleaching was 2.00 days, significantly less than that for chlorophyll degradation in senescing Rossa tissue (2.94–3.14 days). The ratios of green to yellow pigments decreased greatly during senescence of the normal genotype, but remained the same or increased during all photobleaching treatments. It is concluded that pigment degradation during senescence normally proceeds by a pathway quite separate from non-enzymic, free-radical-mediated routes exemplified by the photobleaching reaction.

## INTRODUCTION

The mechanism whereby chlorophyll is degraded *in vivo* during senescence and ripening has remained elusive despite at least seventy years of biochemical investigation. Among the enzymic activities suggested to bring about pigment breakdown are chlorophyllase [1], lipoxygenase [2], peroxidase [3], fatty acid-dependent oxidase [3, 4], Mg dechelatase [5] and Mg-tetrapyrrole oxidoreductase [6]. Alternatively, the pigments may be degraded by a non-enzymic process mediated by free radicals (or singlet oxygen) [7, 8]; here the onset of pigment degradation is envisaged as resulting from failure of the scavenging processes that normally protect against free-radical attack. The existence of a senescence mutant of *Festuca pratensis*, in which degradation of the chlorophyll-proteolipid complexes of thylakoid membranes is impaired [9], allows critical examination of the latter route. By generating free radicals in *Festuca* leaf tissue using light and chemical treatments, we sought to establish: (i) whether the non-yellowing lesion is associated with the possession of enhanced radical-scavenging capacities and (ii) how mechanisms which defend against photobleaching change during leaf senescence.

## RESULTS AND DISCUSSION

Fresh, non-senescent leaf tissue of Bf993, a non-yellowing mutant genotype of *Festuca pratensis*, was essentially identical in pigment complement to tissue of the normal variety Rossa (Table 1). Leaf segments excised at this time (0 days of senescence) and incubated for 48 hr on water under constant illumination exhibited little change in chlorophyll or carotenoid content. On the other hand, in the presence of 50 μM methylviologen, rapid loss of chlorophylls and carotenoids occurred on illumination (Table 1). Methylviologen accepts electrons from Photosystem 1 and is spontaneously re-oxidised by molecular oxygen with the release of superoxide. The superoxide may attack and bleach photosynthetic pigments and other cell components. This is one of the ways in which the dipyridylium herbicides, of which methylviologen (Paraquat) is the commonest in use, express their phytotoxicity. The effectiveness of methylviologen in bleaching chlorophylls and carotenoids is dependent on a continued flow of electrons from Photosystem 1, which in turn must be constantly supplied with electrons by Photosystem 2. Thus leaves in which Photosystem 2 had been poisoned with dimethyl-3-(3,4-dichlorophenyl)urea (DCMU) exhibited greatly reduced bleaching when treated with methylviologen, although DCMU alone had little or no influence on the pigment complement either of the mutant or of the normal genotype of *Festuca* (Table 1). It has been suggested [10] that the response of leaf tissue to Paraquat simulates the process of leaf senescence. On the other hand, there is no indication that Paraquat-tolerant grass varieties [11] have grossly altered patterns of foliar yellowing [Thomas, H., unpublished observations].

Figure 1 illustrates the results of an alternative method of evoking the photobleaching response in *Festuca* leaf tissue. Leaf segments were incubated in darkness for 24 hr with 5 mM δ-aminolaevulinic acid (ALA) before exposure to light for a further 24 hr. In darkness, ALA is metabolized as far as the light-dependent step of chlorophyll biosynthesis (protochlorophyllide oxidoreductase). Photoconversion of the enlarged pool of protochloro-

*Author to whom correspondence should be addressed.

Table 1. Chlorophyll and carotenoid contents of normal (cv Rossa) and non-yellowing senescence mutant (Bf993) *Festuca pratensis* leaf tissue before and after illumination in the presence of water (control), 50 $\mu$M methylviologen (MeVi) or 50 $\mu$M DCMU

| Genotype | Treatment | Carotenoids | mg/g Fr. wt ± s.e. chl. *a* | chl. *b* |
|---|---|---|---|---|
| Rossa | Fresh tissue | 0.31 ± 0.02 | 1.49 ± 0.11 | 0.70 ± 0.05 |
| | Control | 0.34 ± 0.02 | 1.59 ± 0.07 | 0.75 ± 0.03 |
| | MeVi | 0.08 ± 0.02 | 0.41 ± 0.10 | 0.26 ± 0.06 |
| | DCMU | 0.36 ± 0.01 | 1.54 ± 0.03 | 0.78 ± 0.04 |
| | MeVi + DCMU | 0.19 ± 0.06 | 1.31 ± 0.26 | 0.69 ± 0.11 |
| Bf993 | Fresh tissue | 0.34 ± 0.05 | 1.53 ± 0.13 | 0.71 ± 0.07 |
| | Control | 0.31 ± 0.02 | 1.56 ± 0.04 | 0.71 ± 0.03 |
| | MeVi | 0.07 ± 0.02 | 0.31 ± 0.14 | 0.21 ± 0.07 |
| | DCMU | 0.34 ± 0.02 | 1.48 ± 0.14 | 0.76 ± 0.06 |
| | MeVi + DCMU | 0.19 ± 0.06 | 1.22 ± 0.15 | 0.66 ± 0.02 |

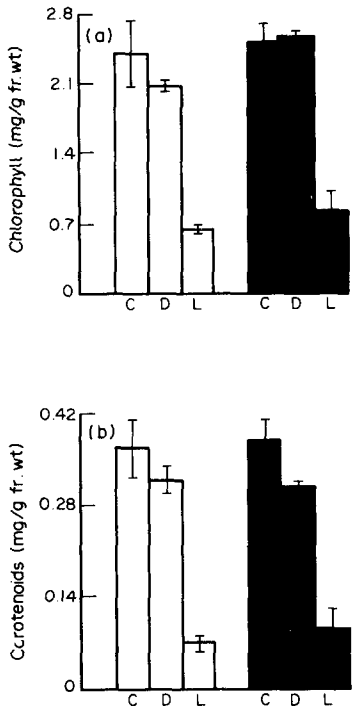


Fig. 1. Levels of (a) chlorophylls and (b) carotenoids in leaf segments of *Festuca pratensis* cv Rossa (normal genotype: □) and Bf993 (senescence mutant: ■). Non-senescent tissue was excised (day 0 control = C) and incubated with 5 mM $\delta$-aminolaevulinic acid for two days in darkness (D) or one day in darkness and one day in light (L). Vertical bars represent standard errors.

phyllide overloads the capacity of the photosynthetic apparatus to incorporate the newly-formed chlorophyllide into stabilized membrane complexes and the consequent uncontrolled formation of free radicals results in wholesale destruction of chloroplast pigments [12]. Mutant leaf tissue treated with ALA and illuminated behaved in an identical fashion to that of Rossa, losing about 70% of the total chlorophyll and 80% of the carotenoids present in the non-senescent leaf.

Hence, the mutation carried by Bf993 clearly does not endow that genotype with a significantly greater degree of resistance to free radical damage than is possessed by Rossa. Is the retention of photosynthetic pigments during foliar senescence in the mutant accompanied by an equivalent durability in its protective mechanisms? We tried to answer this question by allowing excised leaves of both genotypes to senesce in darkness and, at daily intervals, transferring tissue to continuous light. Pigments were extracted two days later. Figure 2 presents curves of chlorophyll content in light-treated tissue superimposed on the data for leaves senescing in continuous darkness for both genotypes. The slight increase in chlorophyll levels over the first day in darkness is a commonly-observed feature in this and other species and seems to be a manifestation of a high capacity for light-independent chlorophyll biosynthesis in *Festuca* leaves [13, Adamson, H., personal communication). After this initial increase, chlorophyll began to decline in dark-incubated Rossa leaves so that by day 8 levels were less than 14% of the value for pre-senescent tissue. By contrast, the marked stability of chlorophyll in the mutant was clearly demonstrated, more than 92% of total chlorophyll being retained over the same period. Interestingly, when pigments of Bf993 were determined by high performance liquid chromatography, rather than by spectrophotometry in crude acetone solution as here, a pronounced decline in chl. *a* and *b* was observed during senescence [14].

This suggests that some conversion of chlorophylls to more polar, though still green, derivatives (chlorophyllides, for example) occurs in the mutant. Illuminating Rossa tissue at intervals during the senescence sequence generally had the effect of inhibiting further pigment loss; that is, leaves transferred from darkness on a given day tended to contain higher levels of pigment after the 48 hr light treatment than did leaves remaining in darkness for two days from the time of transfer. On the other hand, from day 6 onwards (that is, from 4 days of darkness prior to illumination) mutant leaves rapidly lost their capacity to withstand the bleaching effects of continuous illumination. Half-times for degradation (± s.e.), calculated according to [15] were 2.94 ± 0.31 days for chlorophyll in senescing Rossa leaves and 33.0 ± 9.1 days for Bf993; the

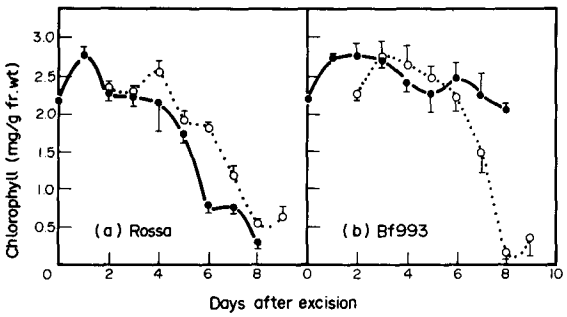


Fig. 2. Chlorophyll $a+b$ in excised leaf tissue of (a) Rossa (normal) and (b) Bf993 (mutant) *Festuca* kept in continuous darkness (●) or in darkness followed by transfer to light for two days immediately before extraction (○). Vertical bars represent standard errors.

Table 2. Ratios of chlorophyll to carotenoid (determined as mg/g fr. wt) in normal (Rossa) and mutant (Bf993) *Festuca pratensis* leaf tissue under conditions of senescence and photobleaching

| Treatment | Ratio of chl. $(a+b)$:carotenoids ± s.e. Rossa | Bf993 |
|---|---|---|
| Non-senescent tissue | 6.99 ± 0.02 | 6.70 ± 0.31 |
| Eight days D | 0.68 ± 0.09 | 9.27 ± 0.13 |
| Six days, D, two days L | 1.87 ± 0.25 | 6.61 ± 1.13 |
| MeVi | 8.12 ± 0.42 | 7.87 ± 1.50 |
| ALA + L | 9.60 ± 1.05 | 9.36 ± 0.72 |

L = light, D = dark, MeVi = methylviologen, ALA = δ-aminolaevulinic acid.

values for chlorophyll in photobleached tissue were 3.14 ± 0.31 and 2.00 ± 0.29 days for Rossa and Bf993 respectively.

We conclude that the genetic lesion in Bf993 is expressed through cellular activities which do not include bleaching reactions and their protective mechanisms. If this is correct then the curve for illuminated tissue in Fig. 2b may be considered to represent the declining potency of these mechanisms during normal senescence. Furthermore, the fact that pigments in the mutant are stable during senescence despite the complete loss of scavenging capacity towards the end of the senescence period argues strongly against such a loss being the primary cause of chlorophyll degradation in aging tissues. A further reason for rejecting failure of protection as a physiological mechanism for pigment breakdown in senescence concerns the indiscriminate nature of free radical attack *in vivo*. Photobleached tissue is white, because all pigments are degraded, but normal senescent leaf tissue is usually yellow in colour. This is customarily interpreted as indicating that carotenoids are less labile than chlorophylls and become unmasked as senescence proceeds. In the present study yellow pigments in the crude acetone extract were estimated spectrophotometrically as carotenoids using the coefficients of ref. [16], but no attempt was made to assess the contributions of alkaloids such as perloline and other compounds, which previous work suggests make a significant contribution to the colour of senescent leaf tissue [14, Düggelin, T., unpublished observations]. Table 2 presents ratios of chlorophyll to 'carotenoids' for leaves of the two genotypes under conditions of senescence in darkness compared with the various photobleaching treatments described previously. Yellowing of Rossa leaves over eight days in darkness was evident in the reduction of the ratio to one-tenth of its day 0 value; by contrast, incubation with methylviologen or ALA destroyed chlorophyll and yellow pigments more or less equally, maintaining the ratio at or above the original value. All three photobleaching treatments also altered chlorophyll and carotenoids to the same degree in the mutant.

This study provides strong arguments against the non-enzymic route for pigment breakdown in senescent tissue. The catabolic pathway itself remains as obscure as ever. It would be helpful if products of chlorophyll degradation further along the pathway of catabolism than the various well-recognized porphyrin derivatives could be identified. A series of coloured compounds with some of the chemical properties of bile pigments have been isolated from senescing *Hordeum* and *Festuca* (normal but not mutant genotype) leaf tissue [17]; but no extractable activity capable of forming them from chlorophyll has yet been found. Establishing this pathway is necessary for the full understanding of chlorophyll turnover processes [18]. Such processes clearly play a major part in the regulation of chloroplast biogenesis and disassembly [19, 20].

## EXPERIMENTAL

Plants of *Festuca pratensis* variety Bf993 (non-yellowing mutant) and cv Rossa (normal senescence) were grown to the fifth or sixth leaf stage as described in ref. [21]. Youngest fully-expanded leaves were removed, cut ends were immediately immersed in $H_2O$ and a further 1 cm of tissue from the cut surface was excised to prevent embolisms. Senescence was promoted by maintaining detached leaves in continuous darkness at 20° for up to 8 days. Chemical treatments were applied by incubating leaf segments 1 cm in length, cut from the middle portion of the lamina, at 20° in Petri dishes containing filter paper moistened with aq. solns of test compounds. Illumination was provided by a fluorescent source of intensity 300–400 $\mu mol/m^2/sec$ (after passing through $H_2O$ acting as a heat filter). Tissue was weighed, frozen in liquid $N_2$, thawed and extracted by grinding with $Me_2CO$ (1 ml per 1 cm of leaf). The homogenate was centrifuged at 8° for 10 min at 10 000 *g*. Chlorophylls and carotenoids were quantified as described in [20]. All values presented are the means of at least three replicate extractions.

*Acknowledgements*—Mair Lloyd Evans and Ben Thomas provided invaluable technical assistance. This work was funded by the UK Agricultural and Food Research Council and the Swiss National Science Foundation.

## REFERENCES

1. Amir, D., Goldschmidt, E. E. and Altman, A. (1986) *Plant Sci.* **43**, 201.
2. Holden, M. (1965) *J. Sci. Food Agric.* **16**, 312.
3. Martinoia, E., Dalling, M. J. and Matile, Ph. (1982) *Z. Pflanzenphysiol.* **107**, 269.
4. Lüthy, B., Martinoia, E., Matile, Ph. and Thomas, H. (1984) *Z. Pflanzenphysiol.* **113**, 423.
5. Schoch, S. and Vielwerth, F. X. (1983) *Z. Pflanzenphysiol.* **110**, 309.

6. Gassman, M. and Ramanujam, P. (1986) in *Regulation of Chloroplast Differentiation* (Akoyungolou, G. and Senger, H., eds) pp. 115–123. Alan R. Liss, New York.
7. Leshem, Y. Y., Halevy, A. H. and Frenkel, C. (1985) *Processes and Control of Plant Senescence*. Elsevier, Amsterdam.
8. DuPont, J. and Siegenthaler, P. A. (1986) *Plant Cell Physiol.* **27**, 473.
9. Thomas, H. (1987) in *Developmental Mutants in Higher Plants* (Thomas, H. and Grierson, D., eds) pp. 245–265. Cambridge University Press, Cambridge.
10. Chia, L. S., Thompson, J. E. and Dumbroff, E. B. (1981) *Plant Physiol.* **67**, 415.
11. Harper, D. B. and Harvey, B. M. R. (1978) *Plant Cell Environ.* **1**, 211.
12. Rebeiz, C. A., Montazer-Zouhoor, A., Hopen, H. J. and Wu, S. M. (1984) *Enz. Microbiol. Technol.* **6**, 390.
13. Adamson, H. Y., Griffiths, T., Packer, N. and Sutherland, M. (1985) *Physiol. Plant.* **64**, 345.
14. Gut, H., Rutz, C., Matile, Ph. and Thomas, H. (1987) *Physiol. Plant.* (in press).
15. Thomas, H., Lüthy, B. and Matile, Ph. (1985) *Planta* **164**, 400.
16. Shoemaker, R. C., Cody, A. M. and Palmer, R. G. (1985) *Theor. Appl. Genet.* **69**, 279.
17. Matile, Ph., Ginsburg, S., Schellenberg, M. and Thomas, H. (1987) *J. Plant Physiol.* **129**, 219.
18. Hendry, G. A. F. and Stobart, A. K. (1986) *Phytochemistry* **25**, 2735.
19. Thomas, H. (1986) *J. Plant Physiol.* **123**, 97.
20. Thomas, H. (1987) *Theor. Appl. Genet.* **73**, 551.
21. Pollock, C. J. (1976) *Plant Sci. Letters* **7**, 27.