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Leaf senescence in a non-yellowing mutant of *Festuca pratensis*

III. Total acyl lipids of leaf tissue during senescence*

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Abstract. The lipid compositions of leaves from *Festuca pratensis* cv. Rossa (yellowing) were compared with those from a non-yellowing mutant, Bf 993. The leaves of Bf 993 contained a higher level of acyl lipids on both a fresh-weight and a dry-weight basis. Diacylgalactosylglycerol, diacylgalabiosylglycerol and phosphatidylinositol were relatively enriched in the Bf 993 mutant while phosphatidylcholine was relatively reduced. There were no differences in the fatty-acid compositions of individual lipids between the two varieties. During senescence, the lipids of cv. Rossa were progressively degraded over an 8-d period. In contrast little lipid degradation was observed in the Bf 993 mutant during the first 4 d. The results support the hypothesis that the slower senescence changes of the Bf 993 mutant may be due, in part, to an altered membrane lipid composition.

Key words: Chloroplast membranes – *Festuca* – Leaf senescence – Lipid (during senescence) – Mutant (*Festuca*).

Introduction

Although leaf senescence requires current protein biosynthesis (Thomas 1976; Thomas and Stoddart 1980) there is compelling evidence that the onset of chloroplast disassembly does not result from de novo synthesis of hydrolytic and oxidative enzymes but rather from the activation of pre-existing metabolic machinery present in the leaf in a latent form (Thomas and Stoddart 1980; Huffaker and Miller 1978; Pollock and Lloyd 1978; Meyer and Biehl 1981). The question therefore arises as to how the interaction between hydrolases and oxidases and their in vivo substrates is regulated. A mutant genotype of *Festuca pratensis* exists in which degradation of thylakoid membranes and their pigments during senescence is impaired (Thomas and Stoddart 1975; Thomas 1977). The stability of the hydrophobic proteins and other non-polar constituents of mutant chloroplast lamellae appears to be an expression of reduced accessibility to the proteinases and other enzymes that fully degrade stroma and extrinsic membrane components (Thomas 1982a, 1982b). These observations suggest that membrane lipids are a possible controlling factor in the dismantling of the chloroplast and that the non-yellowing mutation is a lesion of lipid metabolism.

Chloroplast membranes are characteristically rich in the galactosylglycerides, diacylgalactosylglycerol and diacylgalabiosylglycerol (digalactosyldiglyceride), which represent about 18% and 12% of the dry weight of the lamellae. The principal charged lipids are diacylsulphoquinovosylglycerol and phosphatidylglycerol (Harwood 1980). Although the exact distribution of the lipids in chloroplast membranes is unknown it seems likely that the galactosylglycerides form the basic bilayer structure while the charged lipids associate with specific proteins as 'boundary lipids' (Anderson 1975). Recent studies on spinach have suggested that diacylsulphoquinovosylglycerol and phosphatidylinositol are bound to the P-700-chlorophyll *a* protein complex while phosphatidylglycerol and diacylsulphoquinovosylglycerol associate with the light-harvesting complex (Rawyler et al. 1980). In agreement, phosphatidylglycerol is preferentially located on the stromal face of the thylakoids (where the light-harvesting complex may protrude,

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Carter and Staehelin 1980) while diacylgalactosylglycerol is more evenly distributed (Unitt and Harwood 1982) and is not associated with the chlorophyll-protein complexes (Heinz and Siefermann-Harms 1981).

A comparison of the lipid content and fatty-acid composition of the non-yellowing mutant with that of the normal genotype has revealed a number of differences between the two. These differences may account, at least in part, for the disparate stabilities of chloroplast membranes during senescence and the results are now reported.

Material and methods

Plant material. Seedlings of *Festuca pratensis* cv. Rossa (yellowing) and Bf 993 (non-yellowing) were grown in vermiculite on a nutrient medium as described by Thomas (1982a). The temperature was 20° C and daylength was 16 h. Fully expanded fourth or fifth leaves were cut into 1-cm sections, surface sterilized and incubated under aseptic conditions at 20° C on moist filter paper in darkness. In some experiments leaf tissue was freeze-dried before extraction. No significant difference was observed between the lipids of fresh and lyophilized material.

Lipid extraction and analysis. Total lipids were extracted from the plant tissue by homogenising the latter with boiling isopropanol (about 20 ml g^{-1} of tissue). Extraction was continued by the method of Garbus et al. (1963) with isopropanol replacing methanol. The lipids were separated into individual lipid classes by thin-layer chromatography on 0.15 mol l^{-1} ammonium sulphate-Silica Gel G plates using acetone:benzene:water (90:30:8, by vol.) as solvent (Khan and Williams 1977). The lipids were also separated by two-dimensional thin-layer chromatography on Silica Gel G plates using chloroform:methanol:water (65:25:4, by vol.) as solvent in the first dimension and chloroform: acetone:methanol:acetic acid:water (10:4:2:2:1, by vol.) in the second dimension. Lipids were routinely revealed by spraying the plates with 0.001% aqueous Rhodamine 6G or 0.2% 8-anilino-1-naphthalene sulphonic acid in methanol and exposing the plates to UV light. Lipids were routinely identified by comparing their mobility to authentic lipid standards. They were also completely identified as detailed previously (Wharfe and Harwood 1978).

Individual acyl lipids separated by thin-layer chromatography were quantified from their fatty-acid compositions. Each lipid zone was scraped from the thin-layer plate and transmethylated by treating the silica gel with 2.5% H_2SO_4-methanol. This method gave more than 98% recovery for all lipid types. The fatty-acid methyl esters were separated by gas-liquid chromatography in columns (1.5 m long, 0.4 mm diameter) of 15% diethyleneglycolsuccinate on Supelcoport, 100–120 mesh or 15% EGSS-X on Supelcoport 100–120 mesh (Supelco Inc. Bellefont, Penn., USA) at 185° C using a Perkin-Elmer F33 gas chromatograph (Perkin Elmer Ltd., Beaconsfield, Bucks., U.K.) coupled to a Varian CDS 101 integrator (Varian Associates, Inc., Palo Alto, Cal., USA). Individual peaks were routinely identified by their equivalent chain length (ECL) value and comparison with authentic standards. Confirmation of major components was provided by oxidation (Downing and Greene 1968) and reduction (Roehm and Privett 1969) of individual fatty acids and gas-liquid chromatography of their products. Quantification was provided by the inclusion of an internal standard of methylpentadecanoate.

Results and discussion

When the acyl-lipid content of young leaves of the non-yellowing mutant and normal genotype were examined a number of notable features were apparent (Table 1). The acyl-lipid composition of the normal genotype was similar to that of other leaf tissues (Harwood 1980). The two galactosylglycerides and phosphatidylcholine were major components while smaller amounts of neutral lipids (mainly triacylglycerols), diacylsulphoquinovosylglycerol, cardiolipin, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol were detected. In comparison with the normal genotype, Bf 993 contained higher levels of total acyl lipids, galactosylglycerides and phosphatidylinositol and a smaller relative amount of phosphatidylcholine.

The higher acyl-lipid content (on a fresh-weight basis) of Bf 993 means that while the major non-chloroplast lipid, phosphatidylcholine, and the mitochondrial lipid, diphosphatidylglycerol retain their absolute levels in the leaves, the amounts of other lipids such as the chloroplastic lipids (diacylgalactosylglycerol, diacylgalabiosylglycerol, diacylsulphoquinovosylglycerol, phosphatidylglycerol) are significantly increased. The chloroplasts of the non-yellowing mutant are similar in ultra-

Table 1. Comparison of the acyl-lipid composition of non-senescent (day 0) leaves of Bf 993 and Rossa genotypes of *Festuca pratensis*. *Abbreviations:* MGDG = diacylgalactosylglycerol; DGDG = diacylgalabiosylglycerol; SQDG = diacylsulphoquinovosylglycerol; DPG = diphosphatidylglycerol; PG = phosphatidylethanolamine; PG = phosphatidylglycerol; PC = phosphatidylcholine; PI = phosphatidylinositol. Figures represent means ± SE for five independent experiments carried out with total leaf tissue from plants aged 5–8 weeks. Statistical analysis was by Student's t test for paired samples. * $P<0.1$, ** $P<0.02$

	Rossa (Normal yellowing)	Bf 993 (Mutant non-yellowing)
Total lipid (mg g^{-1} FW)	7.9 ± 0.4	10.3 ± 1.2**
Lipid composition (% total acyl lipids)		
Neutral lipids	3.9 ± 1.8	3.6 ± 1.7
Non-esterified fatty acids	1.6 ± 0.4	0.6 ± 0.2
MGDG	30.6 ± 2.6	33.9 ± 3.4*
DGDG	22.4 ± 1.1	26.3 ± 0.7**
SQDG	5.7 ± 0.8	5.4 ± 1.1
DPG	4.2 ± 0.1	2.6 ± 1.0
PE	6.0 ± 0.9	5.6 ± 1.6
PG	6.1 ± 1.0	5.7 ± 0.4
PC	12.3 ± 0.8	8.6 ± 1.1**
PI	3.8 ± 0.1	7.0 ± 0.9**
Others	3.4 ± 1.2	0.7 ± 0.4

Table 2. Fatty-acid composition of the major acyl lipids of non-senescent leaves of non-yellowing and normal genotypes of *Festuca pratensis*. For lipid abbreviations see Table 1. nd = not detected; tr = trace (< 0.5%); 16:0 = palmitic acid; 16:1, 3-*trans* = *trans*-Δ3-hexadecenoic acid; 16:1, 9-*cis* = palmitoleic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; 18:3 = linolenic acid. Results are the mean ± SE for four independent experiments

Tissue	Lipid	Fatty acid composition (% total)							
		16:0	16:1 (3-*trans*)	16:1 (9-*cis*)	18:0	18:1	18:2	18:3	Others
Normal (Rossa)	MGDG	2.2±0.3	nd	0.6±0.1	0.7±0.2	1.6±0.2	5.2±0.4	89.0±1.1	0.7±0.1
	DGDG	12.2±0.8	nd	3.1±0.9	1.9±0.4	3.3±0.5	5.5±0.1	72.3±1.2	1.7±0.2
	SQDG	26.8±2.4	nd	4.6±0.9	6.5±1.4	6.3±1.4	9.0±0.7	45.9±5.4	0.9±0.2
	PC	26.3±0.9	nd	3.9±0.9	5.8±1.6	11.2±0.6	27.8±0.4	23.7±2.4	1.3±0.2
	PG	29.4±0.6	16.5±1.0	tr	4.0±0.8	5.6±0.5	10.3±0.4	33.2±1.4	1.0±0.2
Mutant (Bf 993)	MGDG	2.0±0.3	nd	0.4±tr	0.7±0.1	2.3±0.4	5.9±tr	88.1±0.9	0.6±0.1
	DGDG	12.6±1.2	nd	2.7±0.6	3.3±0.6	3.6±0.9	5.8±0.6	71.2±8.2	0.8±0.1
	SQDG	25.4±1.3	nd	5.8±2.9	7.4±1.1	4.9±0.1	8.1±0.1	47.4±7.3	1.0±0.2
	PC	27.0±0.3	nd	0.3±0.1	4.4±0.3	12.1±0.1	30.2±0.3	26.0±0.1	tr
	PG	28.7±1.2	16.7±1.7	tr	5.6±0.3	10.1±3.5	9.4±0.5	27.2±0.5	2.3±0.5

structure to those from the normal genotype (Thomas 1977), with no evidence of a more extensive lamella system or larger numbers of plastids per cell to account for the elevated levels of chloroplast lipids. Furthermore, an obvious morphological change in chloroplast structure, such as the level of granal stacking, might be expected to produce a significant difference in the chlorophyll contents of non-senescent mutant and normal leaves. Such a difference is not observed (Thomas and Stoddart 1975). A more detailed study of chloroplast ultrastructure and lipid distribution is in progress.

Differences between the genotypes in acyl-lipid levels without an obvious chloroplast morphological correlation, led us, naturally, to examine the fatty-acid components of individual lipids since the acids may be very important in the latter's function (Mazliak et al. 1980). In Table 2 the fatty-acid compositions of the major acyl lipids of both genotypes are shown. There were no significant differences in the fatty-acid compositions of individual lipids between the two genotypes. Both leaf types contained galactosylglycerides with high α-linolenate contents, the diacylgalacbiosylglycerol compound having a higher palmitate content. Diacylsulphoquinovosylglycerol was characterized by a high palmitate and α-linolenate content and phosphatidylcholine by the presence of significant oleate and linoleate as well. Phosphatidylglycerol contained about 17% of the unique trans Δ 3-hexadecanoate. These fatty-acid compositions were similar to those observed for other higher-plant leaves (cf. Harwood 1980). Thus, the differences in acyl lipids in the two genotypes did not include changes in the fatty-acid compositions also. It

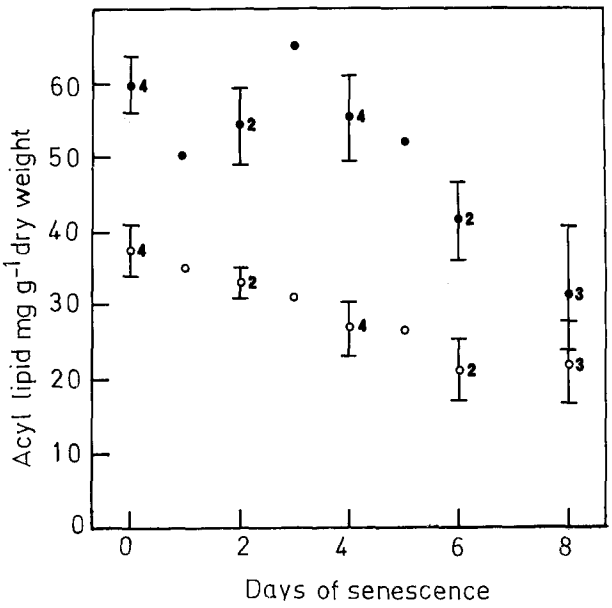

Fig. 1. Lipid content of leaves of *Festuca pratensis* during senescence. Analyses were performed on freeze-dried tissue. Results are the means ± SE of separate analyses as indicated. o, Rossa (yellowing); •, Bf 993 (non-yellowing)

seemed that the unique fatty-acid complement of each lipid was retained despite the increased demands for lipid accumulation in the non-yellowing genotype.

A characteristic of mutant chloroplasts is the small volume change they undergo during senescence when compared with those of the normal genotype. Furthermore, granal degradation and accumulation of plastoglobuli are markedly reduced in the mutant (Thomas 1977). The acyl

Table 3. Changes in the relative levels of acyl lipids during senescence of the leaves of non-yellowing mutant and normal genotypes of *Festuca pratensis*

Tissue	Senescence (d)	No. of experiments	Acyl Lipid Distribution (% total)							
			Neutrals[a]	MGDG	DGDG	SQDG	PE	PG	PC	Others
Normal (Rossa)	0	4	6.8	28.7	24.1	6.8	7.7	5.7	16.2	4.0
	2	2	8.9	25.9	21.9	7.5	8.5	6.3	18.7	2.3
	4	3	11.1	22.1	21.0	7.2	9.7	4.0	21.3	3.6
	6	2	14.7	16.7	21.4	7.5	10.4	6.0	20.0	3.3
	8	3	14.8	8.6	21.3	8.7	15.7	7.0	20.8	3.1
Mutant Bf 993	0	4	5.3	30.1[b]	29.3[b]	5.1	7.6	5.2	11.4[d]	8.0
	2	2	5.7	28.1[b]	27.8[b]	6.0	7.3	5.4	13.5[b]	6.3
	4	3	6.2	22.0	26.6[c]	5.6	7.6	8.0	18.4	5.6
	6	2	13.9	16.9	23.8	5.3	12.2	7.0	16.0	4.9
	8	3	18.2[b]	10.9	19.7	7.0	13.5	8.7	16.1	5.9

[a] Includes non-esterified fatty acids. Figures are means of the number of experiments indicated. Tissue was freeze-dried before extraction
[b] Statistically significant, $P<0.01$
[c] $P<0.02$
[d] $P<0.001$ (Statistical analysis was by Student's t test for paired samples)

lipids of such leaves were examined during 8 d of senescence (Fig. 1). The lipid content of Rossa leaves decreased progressively with time, whereas Bf 993 leaves maintained total lipid levels for 4 d before degradation became significant. Acyl-lipid levels in the mutant remained significantly higher than those of the normal genotype over the first 6 d of senescence.

The relative levels of acyl lipids in senescing leaves (Table 3) were very similar to those of non-senescent leaves (Table 1). Higher relative levels of galactosylglycerides and lower levels of phosphatidylcholine in the non-yellowing mutant were again observed. During senescence diacylgalactosylglycerol was degraded particularly quickly so that its relative level declined to about 10% of total lipids after 8 d in both genotypes. The relative percentages of the non-chloroplastic phosphatidylethanolamine and phosphatidylcholine increased during senescence in keeping with a preferential destruction of chloroplast membranes. Similar observations have been made on the lipids of senescing leaves of other species including bean (Fong and Heath 1977) and tobacco (Koiwai et al. 1981). An increase in non-esterified fatty acids which formed a major portion of the combined-neutrals fraction after 8 d was also observed (Table 3). These changes can be explained by the activity and substrate specificity of a non-specific leaf acyl hydrolase (Burns et al. 1979). The fact that this enzyme shows preference for mono-acyl lipids (Burns et al. 1979) explains why mono-acyl products did not accumulate in any great amount (Table 3). Since the total acyl-lipid content of both genotypes was approximately halved during 8 d of senescence, oxidation of liberated fatty acids must have been significant. Such degradative reactions are typical of senescing tissues (James 1953; Lloyd 1980, 1982) and may be important for carbon recovery or energy metabolism by the plant. After 8 d of senescence, in contrast to early stages of the process, there were no significant differences in lipid contents of the two genotypes (Fig. 1 and Table 3).

The fatty-acid composition of individual acyl lipids of senescent tissue (Table 4) was very similar to that of non-senescent tissue (Table 2) except for an increased palmitate and lowered linolenate content of certain lipids. During senescence there were no further significant changes in these fatty-acid compositions. This indicates that the acyl-hydrolase enzymes, in contrast to the highly specific enzymes of membrane biogenesis (Harwood 1976; Heinz and Harwood 1977) did not discriminate in any marked degree between the different molecular species available.

The results described here indicate that chloroplast membranes of the non-yellowing mutant of *Festuca pratensis* are different in lipid content from those of the normal genotype. Both genotypes contain comparable soluble acyl-hydrolase activities (unpublished data) which implies that impaired membrane degradation in the mutant is due to differences in substrate accessibility, probably as a consequence of the changed lipid character of the mutant's membranes. Our results suggest that lipids of the chloroplast membranes may be of partic-

Table 4. Fatty-acid composition of major lipids of the leaves of non-yellowing mutant and normal genotypes of *Festuca pratensis* determined at day 4 of senescence. Analysis was made of freeze-dried tissues (3 experiments). Means ± SE are shown. The fatty-acid compositions of individual lipids after 2, 6 or 8 d of senescence were not significantly different from those at 4 d. For lipid abbreviations see Table 1. nd = not detected; tr = trace (< 0.05%)

Tissue	Lipid	Fatty acid composition (% total)						
		16:0	16:1 (3-*trans*)	16:1 (9-*cis*)	18:0	18:1	18:2	18:3
Normal (Rossa)	MGDG	5.2±0.4	nd	1.0±0.4	1.0±0.4	4.1±1.2	9.7±1.2	77.5±1.0
	DGDG	21.1±1.8	nd	3.5±0.4	1.6±0.1	3.4±0.5	5.5±0.3	64.2±1.4
	SQDG	42.3±3.3	nd	3.5±1.5	3.3±1.9	7.1±1.7	7.0±0.5	34.6±5.3
	PE	33.7±4.8	nd	5.3±0.7	1.3±1.3	11.5±7.7	22.3±1.4	25.1±8.4
	PC	29.8±1.4	nd	5.5±2.7	1.8±0.2	7.7±1.3	26.3±1.3	26.4±2.5
	PG	21.0±3.1	12.3±2.3	tr	2.4±0.2	5.3±1.1	9.4±2.1	47.4±6.2
Mutant (Bf 993)	MGDG	7.7±56	nd	2.0±0.1	1.4±0.3	5.4±0.6	12.6±0.4	69.3±1.0
	DGDG	21.6±2.4	nd	4.1±1.7	2.6±0.5	6.2±1.3	6.4±0.2	58.4±2.8
	SQDG	46.7±0.7	nd	2.6±0.4	3.5±0.6	11.4±4.7	6.7±0.9	28.1±3.7
	PE	40.0±3.7	nd	5.4±1.8	3.0±0.5	10.4±2.2	21.7±3.0	18.0±1.8
	PC	30.1±3.5	nd	5.4±1.3	3.3±0.5	9.4±1.6	26.2±1.6	24.6±3.1
	PG	21.1±8.7	15.3±9.4	tr	1.8±0.8	11.5±5.9	11.5±5.9	39.3±6.1

ular importance in determining the course of senescence observed in the mutant. Further studies are now under way in order to examine the structure and functions of these membranes in more detail.

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