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Sid: a Mendelian locus controlling thylakoid membrane disassembly in senescing leaves of *Festuca pratensis*

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Summary. A spontaneous mutation arising in *Festuca pratensis* has the effect of stabilizing the pigment-proteolipid complexes of thylakoid membranes so that leaf tissue does not turn yellow during senescence. Inheritance of the non-yellowing character was analysed in crosses between the wild-type cultivar Rossa and a mutant line Bf 993. Electrophoretic variants of cytoplasmic phosphoglucoisomerase coded by alleles of the nuclear gene *Pgi-2* were used to identify hybrids during intercrossing. About 96% of the F_1 progeny were heterozygous and all were phenotypically yellowing. In the F_2 generation yellow : green segregated in a ratio of 2.14 : 1, not significantly different from 3 : 1. In the backcross between F_1 and Bf 993 the ratio was 1 : 1 yellow : green. There was no indication of linkage to *Pgi-2*. Senescence of detached Bf 993 and Rossa leaves was compared with that of the F_1 hybrid. The hybrid behaved in an essentially identical fashion to the wild-type parent, and in marked contrast to the mutant, in all aspects of the senescence syndrome investigated, including loss of chlorophyll, carotenoids and the light-harvesting chlorophyll-protein of thylakoid membranes, and elevation of the particulate protein : chlorophyll ratio in the terminal stages. It is concluded that there exists in *Festuca pratensis* a nuclear gene, designated *Sid* (senescence-induced degradation) which regulates turnover of hydrophobic components of photosynthetic membranes in ageing leaf tissue and which occurs in at least two allelic forms, *y* (yellow) dominant over *g* (green).

Abbreviations: PGI = phosphoglucoisomerase, *Pgi* = nuclear gene coding for PGI, TSH = tris buffer containing 2-mercaptoethanol, SDS = sodium dodecyl sulphate, Chl = chlorophyll, LHCP = light-harvesting chlorophyll *a/b* binding protein, ELISA = enzyme-linked immunosorbent assay

Key words: *Festuca pratensis* Huds. – Meadow fescue – Gramineae – Leaf senescence – Mutation – Chloroplast – Phosphoglucoisomerase isoenzymes – Membrane turnover

Introduction

The sedentary way of life adopted by terrestrial plants presents special problems of survival. It is characteristic of higher plant species that they respond to transient, aleatory stresses or to longer-term, predictable seasonal variations by a process of morphological remodelling: existing organs are abandoned, usually after the nutrients they contain have been recovered and stored or utilised to make new, better-fitted parts.

The adaptive and highly integrated nature of cell and organ senescence as it occurs at many points in the plant life-cycle has been taken to signify that the process must be genetically programmed (Thomas and Stoddart 1980; Woolhouse 1984). There have been, however, relatively few studies of the formal genetic basis of plant senescence. Heritable variations in the timing of the onset of foliar senescence, its rate when underway and its relationship with other events in plant development such as seed maturation have been described for a number of crop species, including *Glycine* (Pierce et al. 1984; Phillips et al. 1984), *Zea* (Swank et al. 1982) and *Sorghum* (Duncan et al. 1981). In very few cases has it been possible to dissect genetically the leaf senescence syndrome in the same way as fruit senescence (ripening) has been analysed (e.g. Tigchelaar et al. 1978). *Festuca pratensis* is an exception: Thomas and Stoddart (1975) described a mutant line in which the complex of events occurring in foliar senescence has apparently been partially disabled. Several subsequent publications describe the characteristics of senescence in non-yellowing *Festuca* mutants (Thomas 1977, 1982, 1983a; Harwood et al. 1982; Hilditch et al. 1986). The mutation has marked pleiotropic effects on the disassembly of thylakoids, which survive to an advanced stage of senescence as ultra-

structurally recognisable, though functionally inactivated, pigment-proteolipid membrane figures.

Although it became increasingly clear from observations during variety production trials that the yellowing phenotype is dominant over the mutant, no definitive inheritance study has been presented hitherto. Formal demonstration that the locus behaves as a single nuclear gene in a standard crossing programme is given here, together with a comparison of senescence in leaves of wild-type and mutant lines with a hybrid between them.

Materials and methods

Plant material

Parental material for inheritance studies was selected from normal (cv. Rossa) and non-yellowing mutant (Bf 993) populations of *Festuca pratensis* Huds. Plants used in the crossing programme were grown from seed in John Innes No. 3 compost in a glasshouse with natural heat and light supplemented as necessary to give 20 °C and a 16 h photoperiod. Plants were vernalised by transfer from glasshouse to outdoor areas in late October. Vernalised material was returned to the glasshouse in early March the following year. Flowering occurred in May. Inflorescences at matched states of maturity were made inaccessible to foreign pollen by enclosing in a cellophane bag. Seeds were harvested in July and stored at room temperature for a few weeks to overcome any residual after-ripening dormancy. For senescence studies of parents and hybrids, plants were germinated and grown in vermiculite on a nutrient medium under controlled environment conditions as described by Thomas (1982). Senescence was induced by detaching youngest fully-expanded leaves of plants at the 5–6 leaf stage and incubating in darkness (Thomas et al. 1985).

Isoenzyme analyses

Small pieces of leaf tissue were crushed in 50 mmol l^{-1} tris pH 7.2 containing 0.05% 2-mercaptoethanol at 4 °C and the liquid absorbed onto small filter paper wicks. Wicks were applied to starch gels and phosphoglucoisomerase (PGI) isoenzymes separated electrophoretically and visualised as described by Jones et al. (1980). Extracts of a tetraploid hybrid *Lolium* in which the four major *Pgi-2* alleles (*a, b, c, d*) are expressed (Jones et al. 1980) were routinely included for reference purposes.

Isolation of proteins and pigments from senescing tissue

At each sample point three detached leaves of each genotype were selected, base and tip regions discarded and the remaining central portions cut into segments 5 mm long. Each replicate sample consisted of ten segments taken at random. Four replicated extractions were made at each point. The sample was weighed and homogenized at 4 °C in 500 µl TSH buffer (50 mmol l^{-1} tris pH 7.5 containing 0.04% 2-mercaptoethanol). After centrifugation for 6 min at 12,000 × g, the pellet was washed with a further 500 µl of TSH and the supernatants combined to give a soluble extract. The pellet was incubated for 1 h at 40 °C with 500 µl TSH containing 1% sodium dodecyl sulphate (SDS), centrifuged 6 min at 12,000 × g, extracted with a further 500 µl of TSH-SDS and supernatants combined to give a particulate extract. Soluble proteins were precipitated for 1 h at 4 °C with an equal volume of 10% trichloroacetic acid and collected by centrifugation for 6 min at 12,000 × g. Particulate proteins were precipitated by incubation for 1 h at −20 °C with 4 volumes of acetone and centrifuged for 6 min at 12,000 × g. The acetone-soluble supernatant was retained for pigment determination

Protein and pigment determination

Soluble and particulate protein pellets were solubilised for 20 h at 40 °C in 50 mmol l^{-1} Na phosphate pH 7.5 containing 1% SDS and estimated by the procedure of Lowry et al. (1951). Bovine serum albumin was used as the standard. Chlorophylls (Chl) *a* and *b* were quantified using the coefficients of Strain et al. (1971) and total carotenoids determined according to the relation

$$C_c = (4.75\ A_{452}) - (0.226\ C_p) \quad (1)$$

where C_c and C_p are the concentrations (mg l^{-1}) of carotenoid and Chl $a+b$, respectively, (Shoemaker et al. 1985).

Immunological measurement of light harvesting protein

The apoprotein of the light-harvesting Chl *a/b* complex of the thylakoid membrane (LHCP) was determined in the particulate extract by the enzyme-linked immunosorbent assay (ELISA) procedure of Hilditch (1986) using authentic purified *Festuca* LHCP as a standard.

Results and discussion

Origin of the mutation

The non-yellowing mutation arose apparently spontaneously in a population selected from S.215, a cultivar of *Festuca pratensis:* a single individual displaying the character was identified in a selection growing at the Welsh Plant Breeding Station in 1969. An F_2 line derived from the original plant was designated Bf 986 in 1972. The genotype used in the present studies, Bf 993, was derived from a cross between Bf 986 and the cultivar Rossa. The latter was used as the wild-type comparison in the work described here.

Selection of PGI genotypes

It became apparent as the above lines were developed that the green character is not expressed in hybrids with normal yellowing varieties. Formal genetic analysis of the mutation was undertaken using isoenzymes of PGI to monitor the crossing process. At least four allelic variants (designated *a, b, c, d* – Jones et al. 1980) of the *Pgi-2* locus in species of the *Lolium-Festuca* complex have been identified and hybrids between individuals possessing different isoforms of PGI-2 are readily identified by starch gel electrophoresis. Populations of the mutant line Bf 993 and the wild-type cultivar Rossa were screened for PGI phenotype. As is common in the predominantly outbreeding forage grass species, considerable heterogeneity at the *Pgi-2* locus was observed, even in the apparently uniform com-

mercial variety Rossa. By surveying only a relatively small number of individuals, normal and mutant lines with distinct PGI phenotypes were identified (Fig. 1). PGI is a dimeric enzyme. Subunits coded by different alleles of *Pgi-2* associate in pairs to produce isoforms of the holoenzyme: a single isoenzyme band indicates homozygosity at the corresponding *Pgi* locus. Bf993 and Rossa individuals with genotype $Pgi\text{-}2^{b/b}$ and $Pgi\text{-}2^{c/c}$ (Fig. 1) were selected from populations growing in the summer of 1982.

Inheritance studies

In the 1983 season, after vernalisation, Bf993 $Pgi\text{-}2^{b/b}$ and Bf993 $Pgi\text{-}2^{c/c}$ were crossed with Rossa $Pgi\text{-}2^{c/c}$ and Rossa $Pgi\text{-}2^{b/b}$, respectively. Initially seeds from each of the two inflorescences of each isolation were harvested and the plants grown from them analysed separately, but because there was no evidence of a maternal influence on the inheritance of either PGI or the green mutation, the results presented are for combined progeny of each cross. A total of 217 F_1 seedlings from $Pgi\text{-}2^{b/b} \times Pgi\text{-}2^{c/c}$ crosses were screened for PGI pattern. Three $Pgi\text{-}2^{b/b}$ and 6 $Pgi\text{-}2^{c/c}$ individuals were identified, the remainder being heterozygous. This is equivalent to about 4% self-fertility, not atypical for this species. Each of the homozygous F_1 individuals was identical in yellowing phenotype with the corresponding parent; all F_1 hybrids were yellowing. In 1984 hybrids were backcrossed with parents. Others were intercrossed to produce the F_2 generation. The progenies were analysed for PGI-2 and senescence phenotype as before. The results are summarised in Fig. 2. $Pgi\text{-}2^{b/b}$, $Pgi\text{-}2^{b/c}$ and $Pgi\text{-}2^{c/c}$ segregated in the F_2 generation in a ratio very close to 1 : 2 : 1 (χ^2 significant $P < 0.05\%$). The ratio of 2.14 : 1 for yellow and green phenotypes is not significantly different from 3 : 1 (χ^2 significant $P < 0.05\%$). The backcross between F_1 and the recessive parent Bf993 gave a 1 : 1 ratio. Interestingly, the crosses $Pgi\text{-}2^{c/c}$ (Bf993 or Rossa) $\times$ $Pgi\text{-}2^{b/c}$ produced virtually no $Pgi\text{-}2^{b/c}$ progeny. The explanation for this may reside in the influence of an incompatibility (*S*) locus which, in the related genus *Lolium,* is known to be linked to *Pgi-2* (Cornish et al. 1980). The general picture is clear, however: the green locus is inherited as a classical Mendelian recessive. Moreover, there is no linkage between non-yellowing and *Pgi-2* in *Festuca pratensis.*

Senescence in green × yellow hybrids

During 1985 crosses were again made between Bf993 $Pgi\text{-}2^{b/b}$ and Rossa $Pgi\text{-}2^{c/c}$. Out of 34 individual progeny analysed for PGI isoenzymes, 33 were $Pgi\text{-}2^{b/c}$ and one $Pgi\text{-}2^{c/c}$. Ten *b/c* seedlings were grown on under controlled conditions and senescence of detached leaves was compared with that of Bf993 and Rossa tissue after 0, 3 and 6 days incubation in darkness. By day 6 the hybrid had lost 83% of its Chl *a,* 87% of its Chl b and 77% of its carotenoids, and Rossa a similar amount (Table 1). By contrast, pigments in the mutant were highly stable, in agreement with previous reports (Thomas and Stoddart 1975; Gut et al. 1985). From the data of Fig. 3a for total Chl and Table 1 for carotenoids, half-times of degradation may be calculated as described by Thomas et al. (1985). Values of 13.9 and 12.2 days for Bf993 Chl and carotenoids, respectively, compare with figures of 1.8 and 3.1 days for Rossa and 2.2 and 2.9 days for the hybrid. A prima facie explanation for the green lesion is a defect in the pigment catabolising system in ageing leaf tissue; but Thomas et al. (1985) found that levels of the Chl oxidising activity presumed to initiate pigment degradation (Thomas 1986) were, if anything, higher in Bf993 than in Rossa.

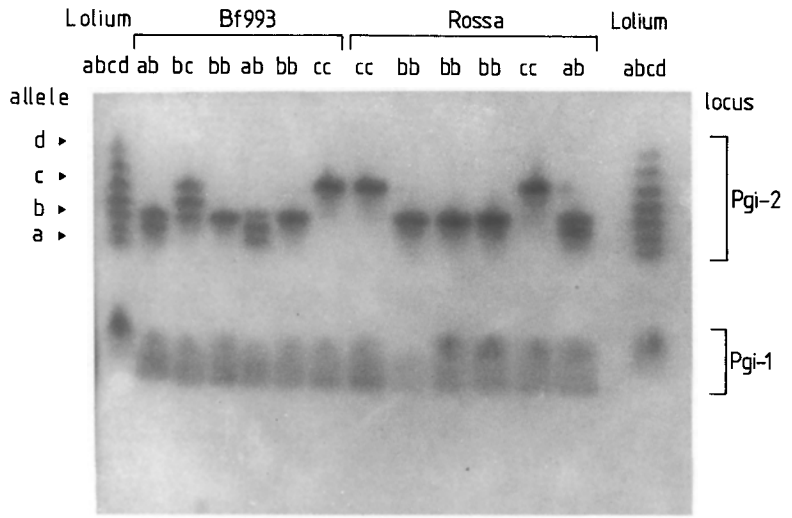

Fig. 1. Starch gel electrophoretogram of leaf phosphoglucoisomerase isoenzymes isolated from representatives of a population of wild-type (cv. Rossa) and non-yellowing mutant (Bf993) *Festuca pratensis.* Allelic forms of PGI-2 are identified by reference to the isoenzyme pattern of a heterozygous *Lolium* tetraploid (outer lanes)

The pleiotropic nature of the mutation is further emphasized by the protein data presented in Table 2 and Fig. 3 b. The major particulate protein, LHCP, is comparable in stability to its prosthetic group, declining to a low level in the hybrid and in Rossa while Bf 993 retains an almost unchanged measure (Fig. 3 b). In contrast to previous studies (Thomas 1983 a; Thomas and Stoddart 1975; Thomas et al. 1985) there was some evidence for a reduced rate of soluble protein breakdown in Bf 993 (Table 2). Nevertheless, the difference between half-times for degradation of normal and mutant soluble fractions (6.9 and 5.0 days, respectively) is of only exiguous statistical significance and, of more relevance to the present study, the hybrid behaved more like Rossa than Bf 993 in this respect. Contrasts between the genotypes are emphasised when proportions of intrinsic components of thylakoid membrane are compared. Thus from about 60% of total particulate protein, LHCP of Rossa and the hybrid declined to about 25% by day 6, whereas the LHCP of Bf 993 increased to over 70% in the same time (Table 2). Similar modulations in the LHCP of normal and mutant *Festuca* lines were observed by Hilditch (1986). An equally striking increase in the particulate protein : Chl ratio occurred in hybrid and Rossa leaves by 6 days (Fig. 2). By contrast, Bf 993 maintained a more or less constant ratio over the period of the experiment. Thomas (1983 b) reported that elevated protein : Chl ratios are characteristic of normal *Festuca* leaf tissue during the later stages of senescence whereas in the green mutant particulate protein and Chl remained in constant proportion over the same interval.

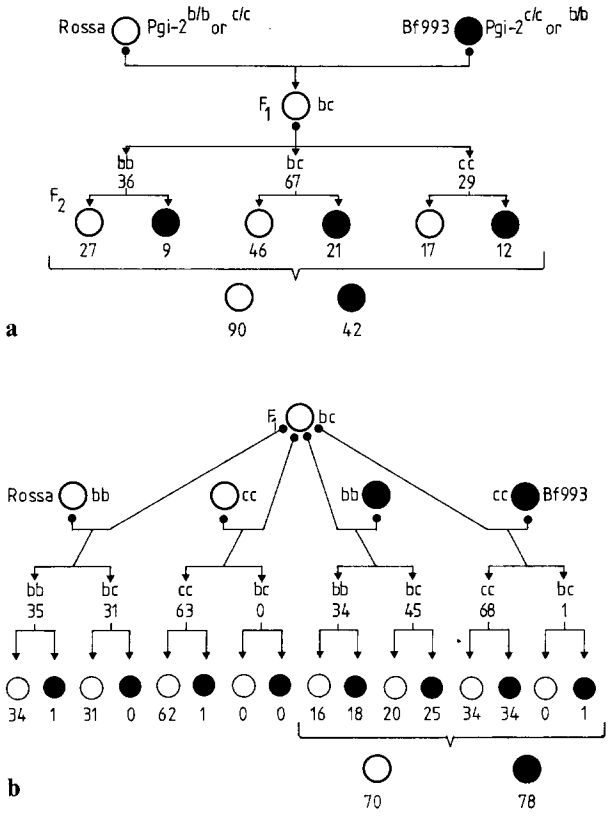

Fig. 2 a, b. Summary of the inheritance figures for the non-yellowing character and for the *b* and *c* alleles of *Pgi-2* in *Festuca pratensis*. **a** From P to F_2; **b** Backcrosses between F_1 and parents. (○ : yellow phenotype: ● : mutant phenotype)

Conclusion

Hybrids between wild-type *Festuca* and the non-yellowing mutant line behave in an essentially identical fashion to the normal parent in senescence tests. The

Table 1. Chlorophyll and carotenoid contents of normal (cv. Rossa), non-yellowing mutant (Bf 993) and normal × mutant hybrid *Festuca pratensis* leaf tissue during senescence in darkness

Days	Genotype	mg g^{-1} FW ± SE		
		Chl *a*	Chl *b*	Carotenoids
0	Bf 993	0.95 ± 0.09	0.41 ± 0.04	0.48 ± 0.05
	Hybrid	1.23 ± 0.13	0.47 ± 0.12	0.65 ± 0.06
	Rossa	1.01 ± 0.06	0.42 ± 0.02	0.53 ± 0.04
3	Bf 993	1.14 ± 0.14	0.45 ± 0.05	0.54 ± 0.06
	Hybrid	0.78 ± 0.11	0.27 ± 0.06	0.42 ± 0.06
	Rossa	0.67 ± 0.11	0.27 ± 0.04	0.36 ± 0.05
6	Bf 993	0.76 ± 0.12	0.25 ± 0.01	0.34 ± 0.05
	Hybrid	0.21 ± 0.05	0.06 ± 0.01	0.15 ± 0.03
	Rossa	0.12 ± 0.05	0.04 ± 0.02	0.11 ± 0.02

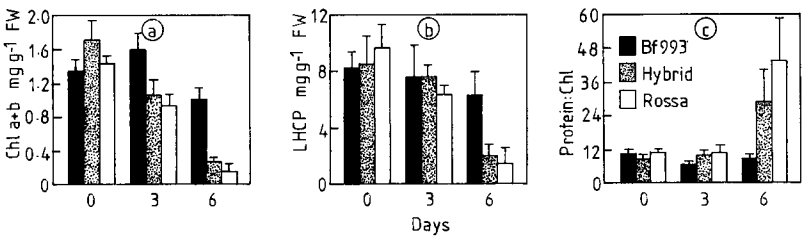

Fig. 3. (**a**) Total chlorophyll; (**b**) light-harvesting chlorophyll *a*/*b* binding protein and (**c**) particulate protein:chlorophyll ratio, with standard error in each case, of senescing leaf tissue from normal, mutant and hybrid *Festuca pratensis*

Table 2. Soluble protein, particulate protein and light-harvesting chlorophyll protein as a proportion of particulate protein in senescing leaf tissue of wild-type (cv. Rossa), mutant (Bf 993) and hybrid *Festuca pratensis*

Days	Genotype	Protein mg g^{-1} FW ± SE		LHCP (%)
		Soluble	Particulate	
0	Bf 993	21.4 ± 1.0	14.4 ± 0.7	58.5
	Hybrid	25.8 ± 1.8	14.9 ± 1.1	57.5
	Rossa	18.5 ± 2.2	15.1 ± 0.7	64.8
3	Bf 993	17.0 ± 5.2	10.9 ± 1.9	71.0
	Hybrid	13.3 ± 2.3	10.1 ± 1.8	76.4
	Rossa	13.4 ± 2.2	9.3 ± 0.9	69.2
6	Bf 993	11.8 ± 1.3	8.8 ± 0.7	72.6
	Hybrid	7.9 ± 1.4	7.5 ± 2.9	26.5
	Rossa	8.1 ± 2.4	6.1 ± 1.1	24.8

mutation is inherited as a nuclear locus and is unlinked to another known nuclear gene, *Pgi-2*. The results of a number of previous studies have established that the mutation has a range of phenotypic consequences, resulting in an extreme persistence of components of the hydrophobic core of thylakoid membranes during natural or induced senescence (Thomas 1977, 1982, 1983a; Harwood et al. 1982). It is proposed, therefore, that there is a nuclear locus, designated *Sid* (senescence-induced degradation), which regulates the turnover of chloroplast membranes during the ageing process in *Festuca* and which exists in at least two allelic forms, wild-type *y* (yellow) and recessive mutant *g* (green). The precise biochemical location of the lesion in mutant ($Sid^{g/g}$) individuals is not entirely clear, but there are reasons to believe that it is sited in the pathway of thylakoid lipid catabolism (Thomas et al. 1985; Thomas 1986). The existence of genetic variability at this locus has implications, both practical and theoretical, for studies of the programming of senescence processes in higher plants. In particular, molecular biological approaches to a fuller understanding of the senescence syndrome and its control become feasible.

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