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Expression of the stay-green character introgressed into *Lolium temulentum* Ceres from a senescence mutant of *Festuca pratensis*

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Abstract A mutant allele at the nuclear locus *sid* confers indefinite greenness on senescing leaves of the pasture grass *Festuca pratensis*. Via the bridging species *Lolium multiflorum* and a programme of backcrossing and selfing, the mutant allele (designated sid^y) was introgressed into *Lolium temulentum* Ceres. The latter is a fast-growing, annual, inbreeding model grass with many advantages over the slower, perennial, genetically heterogeneous outbreeder *F. pratensis*. Analyses of photosynthetic pigments, total leaf proteins and individual plastid polypeptide components in senescing attached and detached leaves of *yy*, *yY* and *YY* plants confirmed that the stay-green phenotype of *yy F. pratensis* had been successfully introduced into the *L. temulentum* background.

Key words Leaf · Chlorophyll · Carotenoid · Protein · LHCPII · Cytochrome f · PSAD

Abbreviations *Chl* Chlorophyll · *L3*, *L4*, *L5*, *L7* leaf 3, leaf 4, leaf 5, leaf 7 · *LHCPII* light-harvesting Chl *a*/*b* binding protein of photosystem II · LSU large subunit · *PSAD* ferredoxin-binding protein of photosystem I

Introduction

At a conservative estimate, some 40 cloned genes of known or inferred function, and many more anonymous sequences, have expression patterns consistent with a role in leaf senescence (Smart 1994; Buchanan-Wollaston 1997). With the exception of 1 or 2 genes (notably those encoding cysteine endopeptidases – Griffiths et al. 1997), nothing approaching a consensus set of cDNAs for the core process of mesophyll senescence has emerged. Moreover, none of the genes so far isolated has been shown unequivocally to be essential for the initiation or progress of senescence. By contrast, several senescence mutants and genetic variants are known in a range of species (Thomas and Smart 1993; Noodén and Guiamét 1996; Oh et al. 1997), but none of the corresponding genes has yet been cloned. Progress in understanding the regulation of senescence will continue to be slow until these problems are resolved.

Perhaps the best characterised of the senescence mutations is the *y* allele of the *sid* (*senescence induced degradation*) locus in the grass *Festuca pratensis* (Thomas and Stoddart 1975). The mutation is inherited as a single recessive nuclear gene (Thomas 1987). Green tissues of the individuals homozygous for sid^y retain chlorophyll more or less indefinitely. This phenotype is an example of what Thomas and Smart (1993) refer to as a type C or "cosmetic" stay-green, since most of the senescence syndrome, including decrease in photosynthetic capacity and degradation of soluble leaf proteins, proceeds normally (Thomas and Stoddart 1975; Thomas 1982; 1987; Hauck et al. 1997). Similar phenotypes have been observed in *Phaseolus vulgaris* and *Pisum sativum* (the latter corresponding to the green cotyledon character originally described by Mendel). In all three cases, the biochemical lesion has been identified as a deficiency in the chlorophyll-degrading enzyme phaeophorbide *a* oxygenase (Bachmann et al. 1994; Vicentini et al. 1995; Thomas et al. 1996). The mutation is likely to be sited either in the structural gene for oxygenase itself or in a locus that regulates its induction at the onset of senescence.

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Festuca pratensis is a far from ideal subject for analysing the *sid*^{y} stay-green trait. It is a slow-establishing perennial and an obligate outbreeder that has to be handled as genetically heterogeneous populations rather than individual genotypes with uniform genetic backgrounds. Few molecular studies have been made on the species. But the occurrence of the stay-green mutation in *Festuca* has some advantages. Recombinant chromosomes resulting from the introgression of *F. pratensis* genes into species of the related genus *Lolium* are readily identified by genomic in situ hybridisation (GISH). This has allowed the stay-green locus to be physically mapped in the *Festuca-Lolium* genome (HM Thomas et al. 1994; H Thomas et al. 1997). Repeated backcrossing has reduced the size of the recombinant segment until it becomes feasible to identify the mutant locus, or at least genes tightly linked to it, by searching for *Festuca*-specific polymorphisms in genomic or cDNA clones.

Introgression into *Lolium* is a means of overcoming the limitations of *Festuca pratensis* as a subject for molecular studies of the *sid*^{y} gene. *Lolium temulentum* is a diploid inbreeder readily crossable with other species in the *Lolium-Festuca* complex. It is quick to establish, grows fast and has a short generation time, and it is easy to establish isogenic lines. Some molecular studies have already been carried out on this species (Winters et al. 1995; Thomas et al. 1997; Gallagher and Pollock 1998). Furthermore the genotype Ceres has long been a model system for flowering and source-sink studies (e.g. McDaniel and Hartnett 1996; Perilleux and Bernier 1997). This paper describes how we introduced *sid*^{y} into *L. temulentum* Ceres to generate experimentally convenient lines for biochemical and molecular analyses of the stay-green trait.

Materials and methods

Seed sources and pedigree recording

Seeds of the parents *Festuca pratensis* Bf993 [stay-green mutant homozygous for the *y* allele of the gene *sid* (Thomas 1987)], *Lolium multiflorum* and *L. temulentum* used in the crosses between *Festuca* and *Lolium* species were obtained from the Genetic Resources Unit at IGER. Thomas et al. (1994) have described the intergeneric transfer of the stay-green character from Bf993 into the *L. multiflorum* cultivar 'Trident' (homozygous for *Y*). Introgression of the *sid*^{y} gene from *L. multiflorum* homozygous for *y* into the Ceres strain of the inbreeding annual species *L. temulentum* was achieved at the diploid level through the backcrossing and selfing procedures described below. To maintain an inventory of F_1 hybrid and backcross plant material in the hybridisation programme, we assigned numbers commencing with the code P to each stage of crossing and selfing.

Growing conditions

All plant material (including parents, hybrids and backcross derivatives) used in the crossing programme was grown from seed. Crossing procedures were carried out in the summer under controlled glasshouse conditions at 20°C. Selfing of the backcross progeny took place under long daylength conditions in a growth cabinet (20°C, 16 h/8 h light/dark cycle). Seed populations from the backcrosses and selfing generations were harvested from each individual plant. About 50 seeds from each genotype were grown to the seedling stage, and detached leaves of individual plants were tested for the stay-green trait.

Production of F_1 hybrid and backcross generations

L. temulentum Ceres (homozygous for *Y*) was used as the female parent in a cross with a single genotype (homozygous for *y*) of *L. multiflorum* to produce an F_1 hybrid that was morphologically intermediate in character between the two species. The F_1 hybrid was rescued by removing immature embryos 15–18 days after pollination and germinating them on B5 medium (Gamborg et al. 1968) in an incubator at 20°C. The diploid F_1 hybrid was backcrossed to wildtype Ceres to produce the backcross BC_1 progeny of plants with a segregation ratio 1:1 (*YY*:*Yy*). An F_1 generation of plants with segregating populations of 1:2:1 (*YY*:*Yy*:*yy*) was obtained by selfing a range of BC_1 derivative plants to produce yellowing phenotypes (*YY* or *Yy*) and phenotypes that were homozygous (*yy*) stay-green in a 3:1 ratio. Further backcross and selfing procedures were undertaken with the intention of recovering essentially isogenic lines for all three populations from the same family. Throughout the programme detached laminae were used to identify *yy* plants by their stay-green phenotype as described by Thomas et al. (1997).

Senescence of attached and detached leaves

Seedlings were grown in a growth chamber at 20°C. Leaf tissue was harvested when the sixth leaf was fully expanded, 52 days after sowing. At this stage leaf 7 (L7) was still expanding, leaf 5 (L5) was mature, leaf 4 (L4) was at incipient senescence and leaf 3 (L3) was at the mid-senescence stage (to judge by its appearance in yellowing phenotypes). Fifth leaves were also detached and allowed to senesce in darkness for 4 and 8 days (4d and 8d) (Thomas et al. 1992). Thus, the L5 samples also represent 0 days (0d) in the excised leaf sequence. Harvested tissues were weighed, immediately frozen in liquid nitrogen and stored at −80°C until extracted. Four or five replicate samples of each age and treatment were taken.

Extraction and pigment determination

Leaves were extracted in phosphate-buffered lithium dodecyl sulphate (LiDS) as described by Mae et al. (1993). For pigment analyses, aliquots of 200 µl were removed from the homogenate, added to 800 µl of ice-cold acetone and incubated for 1 h at −20°C. After centrifugation at 11 000 g_{av} for 15 min at 4°C, chlorophylls (Chl) and carotenoids were determined spectrophotometrically, and amounts were calculated according to Hill et al. (1985) and Lichtenthaler and Wellburn (1983), respectively.

Electrophoresis and western blotting

LiDS-solubilised proteins were separated by SDS-polyacrylamide gel electrophoresis, and individual polypeptides were visualised by western immunoblotting as described by Thomas (1990). Preparation of antibodies to the light-harvesting Chl *a*/*b* binding protein of photosystem II (LHCPII) and to cytochrome *f* (both proteins from *F. pratensis*) is described in Hilditch (1986) and Davies et al. (1989), respectively. Richard Malkin kindly provided the antibody to maize

PSAD. Images of western blots and Coomassie-stained gels were captured using a Power Look II flatbed scanner (UMAX Data Systems, Hsinchu, Taiwan) and saved in TIFF file format. Band intensities were quantified using the PHORETIX 1D (version 3.0) software package (Phoretix International, Newcastle-upon-Tyne, UK).

Results

Introgression of *sid*^{y} into *Lolium temulentum*

The F_1 diploid hybrid P172/187, combining the haploid genomes of *L. multiflorum* (stay-green) and the wild-type *L. temulentum*, was recovered using embryo rescue. The hybrid, which was heterozygous (*Yy*) at the *sid* locus and displayed a yellowing (wildtype) senescence phenotype, was morphologically intermediate in character between the two species and completely male sterile. The BC_1 progeny plants (P175/98) gave the expected segregation ratio of 1:1 *YY*:*Yy* when the F_1 hybrid was crossed with diploid wildtype Ceres (*YY*). Since *Yy* and *YY* plants have identical (yellowing) senescence phenotypes, a further test cross with *yy* is necessary to distinguish the two genotypes before the crossing programme can proceed to the next generation.

Selfing BC_1 to the first generation, BC_1/F_1 (P175/98/3), produced stay-green phenotypes (homozygous for *y*) in the segregating population. One such stay-green line, P175/98/3/1, was crossed with Ceres to give a second backcross population, BC_2 (P176/1). The phenotype of all progeny was yellowing. A third backcross population was produced, BC_3 (P176/16), consisting of a 1:1 mixture of *YY* and *Yy* genotypes. The plants were morphologically indistinguishable from *L. temulentum* Ceres, indicating that repeated backcrossing and selfing in this material has removed a considerable amount of undesirable heterozygosity originating from *L. multiflorum* that was present in the initial F_1 hybrid. The crossing scheme (Fig. 1) is a rapid method for reconstituting the recipient *L. temulentum* parent and for controlled introgression of *sid*^{y} and other *Festuca* genes into *Lolium*.

L. temulentum-like phenotypes having been isolated in the BC_3 progeny, selfing to the fifth generation, BC_3/F_5, was employed to reduce heterozygosity further and to recombine away all but the minimal introgressed segment of the original *Festuca* genome carrying the stay-green allele. At this stage we introduced another backcross in order to recover stay-green and yellowing lines with a near-isogenic background. A homozygous (*yy*) genotype of BC_3/F_5 was hybridised with Ceres to produce the BC_4 (P185/76) generation. Further selfing of BC_4/F_2 produced the first segregating population (P185/76/12), from which two seed populations were derived: P185/76/12/19 (homozygous for *y*) and P185/76/12/11 (homozygous for *Y*). A third selfing generation produced seed populations P190/1, homozygous stay-green (*yy*), and P190/2, homozygous for yellowing (*YY*). Hybridising these two essentially isogenic lines gave the heterozygous population P190/3.

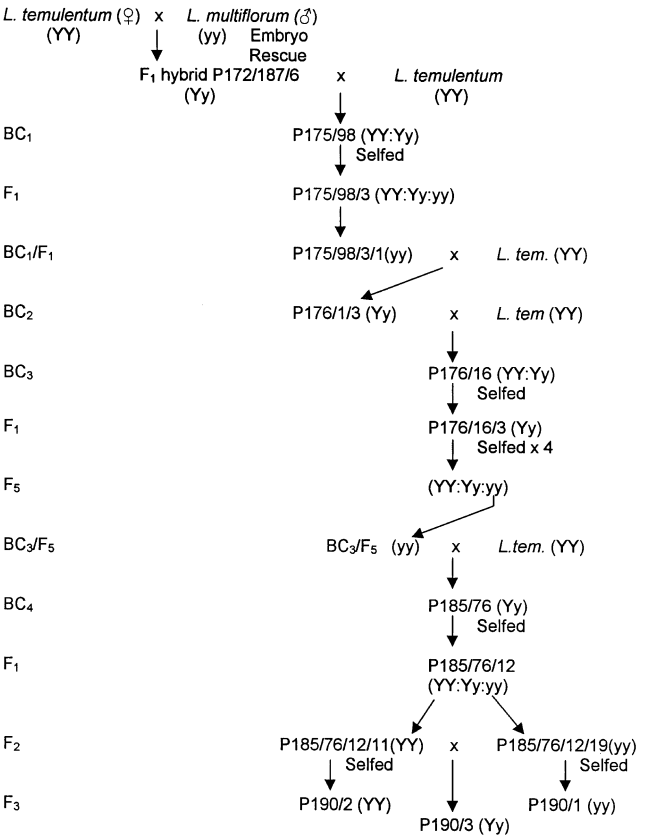

Fig. 1 Schematic diagram of the backcrossing and selfing procedures used in the transfer of the *Festuca pratensis* stay-green *sid*^{y}gene from *Lolium multiflorum* (homozygous *yy*) into wildtype *L. temulentum* Ceres (*YY*)

L. temulentum plants homozygous or heterozygous for *y* were morphologically and physiologically indistinguishable from *L. temulentum* Ceres. Leaf growth, shape and size were clearly of the fast-extending, broad, soft *temulentum* type rather than the slower, thinner, fibrous organs typical of *Festuca*. The inflorescence was a spike, with no trace of panicle morphology, and grains were large and awned, like those of Ceres. *L. temulentum* introgression lines needed no vernalisation and were florally induced by exposure to a small number of long days.

Pigments in senescing leaves of *sid*^{yy}, *sid*^{Yy} and *sid*^{YY} *L. temulentum*

To evaluate the senescence phenotypes of *yy*, *Yy* and *YY* *L. temulentum* lines, we compared Ceres with BC_3/F_5 heterozygotes and with *yy* plants from the

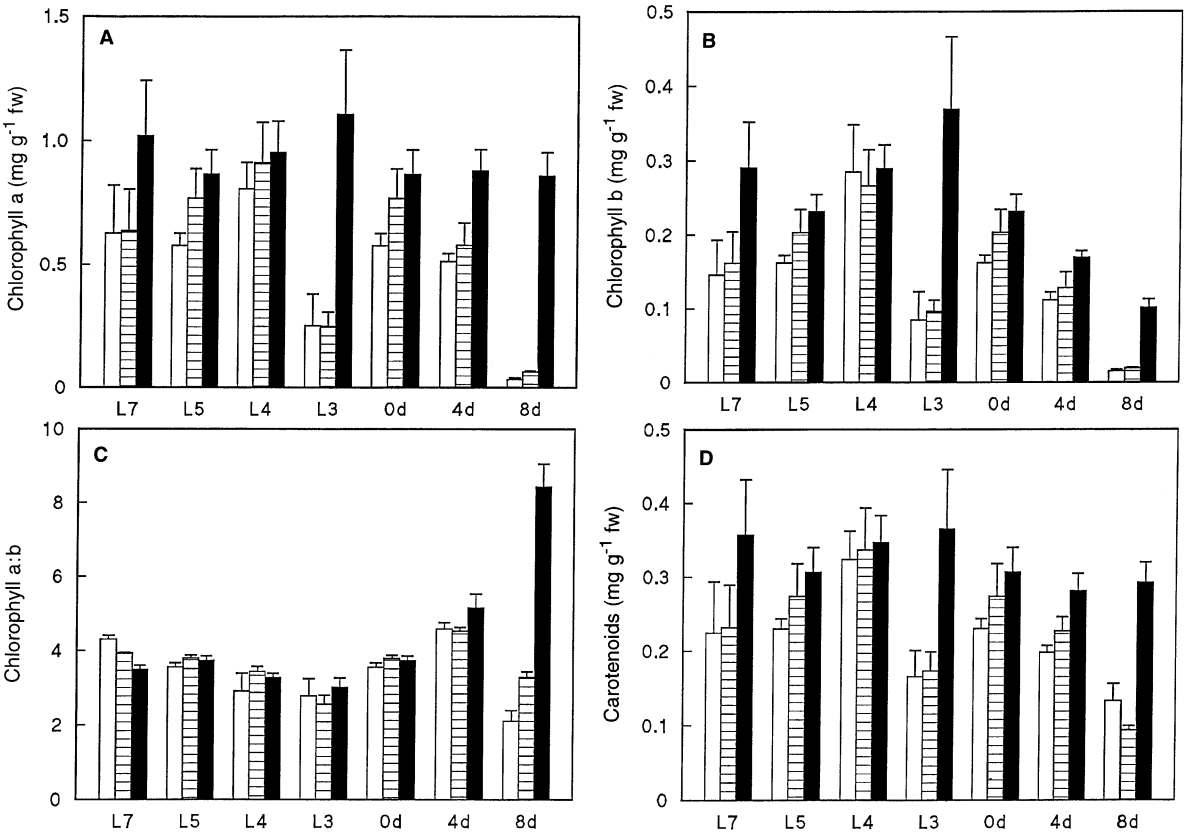

Fig. 2A–D Chlorophyll and carotenoid contents in leaves 3, 4, 5 and 7 (*L3–L7*) of intact *L. temulentum* plants and in detached L5 senescing for 0, 4 and 8 days (*d*) in darkness. *Open*, *shaded* and *black histograms* refer to *YY*, *Yy* and *yy* genotypes, respectively, and error bars are SEs ($n = 5$ for *L3–L7*, $n = 4$ for 4*d* and 8*d*)

BC_4/F_2 population. Ceres plants grown under short-day (non-floral inductive) conditions at 20°C carried six fully expanded leaves on the main tiller at the time of sampling. The lamina of leaf 7 was partly emerged at this time. Leaves 3, 4, 5 and 7 were harvested, and pigments and proteins were extracted. Some fifth leaves were also incubated in darkness for 4 and 8 days before extraction. Figure 2 presents pigment contents of ageing attached and detached leaves of *yy*, *Yy* and *YY* lines. Both Chl *a* (Fig. 2A) and Chl *b* (Fig. 2B) in leaf 3 taken from intact plants of *YY* and *Yy* had declined to a low level compared with amounts in mature and expanding leaves. By contrast, Chls in the oldest leaves of *yy* plants were maintained at pre-senescence levels. The Chl content of expanding *yy* leaves was significantly higher than that of the other two genotypes (Fig. 2A, B). Chl *a* was also stable in *yy* leaves induced to senesce by detachment and incubation in darkness (Fig. 2A). After 8 days detached leaves of the *YY* and *Yy* lines yielded less than 10% of the Chl *a* of tissue at day 0 (Fig. 2A). Chl *b* in *yy* declined significantly over the 8-day incubation period in darkness, but the decrease in the other two genotypes was greater (Fig. 2B). The large increase in the Chl *a*:*b* ratio (Fig. 2C) reflects the difference in stability of *a* and *b* in senescing detached leaves of *yy*. The pattern of loss and retention of Chl *a* in the three genotypes was closely followed by the behaviour of total carotenoids (Fig. 2D).

Proteins in senescing leaves of sid^{yy}, sid^{Yy} and sid^{YY} *L. temulentum*

Figure 3 shows electrophoretic separation of polypeptides extracted from the leaves described above. Tracks were loaded on an equal fresh weight basis, and gels were stained with Coomassie blue. Attached leaves 7, 5 and 4 gave similar polypeptide profiles in *YY*, *Yy* and *yy L. temulentum* lines, but whereas senescent L3 of *yy* plants showed a profile similar to those of the younger leaves, significant protein loss was apparent in L3 of the *YY* and *Yy* genotypes. Densitometric measurement of total Coomassie-stainable protein in each track showed that L3 in both *YY* and *Yy* plants contained less than 50% the protein per unit fresh weight than the same leaf in *yy* plants. After an 8-day senescence in the dark, detached leaf 5 of *yy* plants retained 65% of its original protein content, whereas 8d leaves of *YY* and

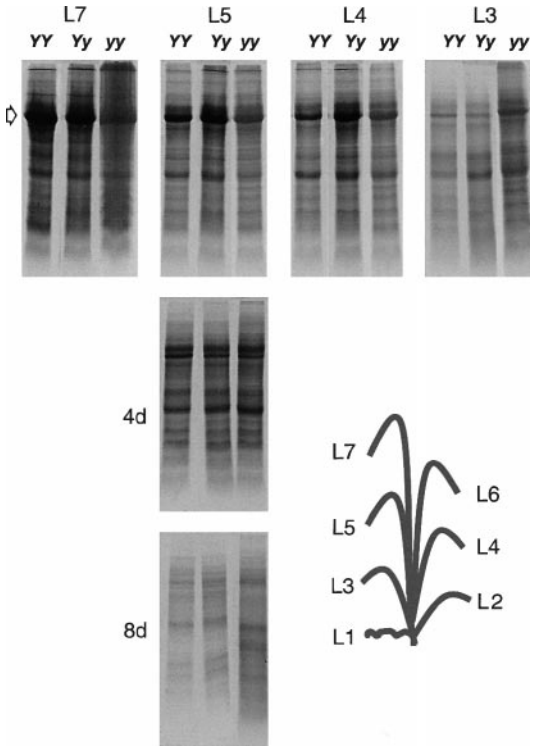

Fig. 3 Total proteins of *YY*, *Yy* and *yy L. temulentum* leaves separated by SDS gel electrophoresis and stained with Coomassie brilliant blue. Schematic diagram shows positions of leaves on the intact plant. Panels 4*d* and 8*d* refer to detached L5 senescing in the dark for 4 and 8 days, respectively. The large subunit of Rubisco is indicated

Yy plants had lost approximately 65% of their total protein. The behaviour of the large subunit (LSU) of Rubisco reflected that of total protein, although the differential loss during senescence on the plant was greater in *YY* and *Yy* plants, with *YY* L3 containing only 32% of the LSU content of L7 and *yy* L3 retaining 69% of its Rubisco. During senescence of detached leaves in the dark, however, degradation of Rubisco was not appreciably retarded in *yy* plants, with only about 20% of the original content remaining after the 8-day dark treatment in all three genotypes.

Western blotting

Figure 4 illustrates the contrasting behaviour of chloroplast polypeptides during senescence in stay-green (*yy*) and yellowing (*Yy*, *YY*) genotypes of *Lolium temulentum*. The LHCPII antibody detected a group of polypeptides at an M_r around 27×10^3. Anti-cytochrome *f* reacted with a band at around 37×10^3 and a group of two or more less distinct bands with slightly higher mobilities. Antibody against PSAD lit up a single band of M_r about 23×10^3. These patterns and molecular sizes are consistent with those already established for these proteins in grasses. Plants with the *yy* genotype retained a much greater proportion of the Chl binding protein LHCPII and the photosystem I polypeptide PSAD (both nuclear-encoded) than did *YY* and *Yy* plants, even after the 8-day senescence in the dark. In contrast, little or no preferential retention of cytochrome *f* (plastid-encoded) was apparent in *yy* plants, either during dark-induced senescence or in attached leaves on the plants.

Discussion

Intergeneric transfer between *Festuca* and diploid *Lolium* species is a convenient way of putting a gene into an alien background, thereby isolating its effects and enabling exploitation of any novel trait it may specify (Humphreys 1989). The amount of interspecific variation that will be available within hybrids between species of *Lolium* and *Festuca* will depend on the extent of recombination (chromosome partner exchanges) occurring between the two genomes. *L. temulentum* chromosomes are approximately 1.5 times the size of those in *L. multiflorum*, with a DNA content of 6.23 pg compared with 4.31 pg (Hutchinson et al. 1979). Nevertheless, studies on chromosome pairing relationships in interspecific hybrids have shown that the two genomes are closely related and not differentiated on the basis of chromosome size alone (Jauhar 1975). Recombinant chromosomes resulting from introgression of *F. pratensis* into *L. multiflorum* are, however, readily identified by GISH (HM Thomas et al. 1994). Preliminary studies of advanced introgression lines of *L. temulentum* with the stay-green phenotype indicate the presence of a single pair of recombinant chromosome segments that are presumed to carry the *sid* locus (Harper and Morgan, unpublished). The stay-green phenotype in *L. multiflorum* introgressions has been mapped to a recombinant segment of *F. pratensis* origin at the terminus of the long arm of a chromosome tentatively identified as 6 in the *Festulolium* complement (HM Thomas et al. 1994; H Thomas et al. 1997).

Senescing leaves of *L. temulentum* plants homozygous for the mutant allele of *sid* retained greenness indefinitely, both when attached to the plant and when induced to senesce by detachment and dark incubation (Fig. 2). As previously shown in *F. pratensis* (Thomas 1987), heterozygotes are phenotypically identical to the wildtype, confirming that *y* is fully recessive to *Y*. The metabolic lesion in stay-green *F. pratensis* was shown to be located in the Chl catabolism pathway, at the ring-opening reaction (Vicentini et al. 1995). The two preceding steps in pigment breakdown – dephytylation and dechelation – operate in the mutant, but buildup of polar products upstream of the biochemical blockage is limited because of an as-yet poorly understood feedback mechanism (Thomas et al. 1989). It is probable

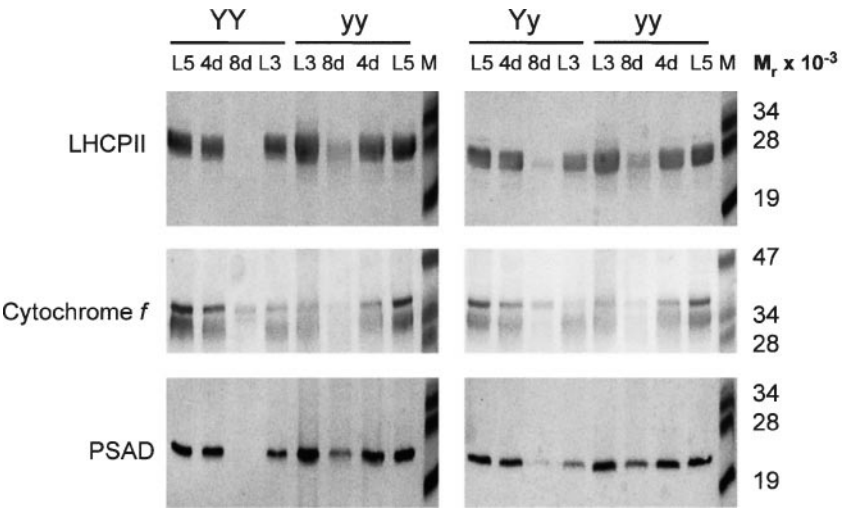

Fig. 4 Western immunoblot detection of chloroplast polypeptides in senescing attached (*L3*, *L5*) and detached (*4d*, *8d*) leaves of *L. temulentum* genotypes homozygous and heterozygous for the *y* and *Y* alleles of the senescence gene *sid*. *M* Molecular size standards. Gels are loaded to allow immediate side-by-side comparisons of samples from senescent tissues of contrasting genotypes

that Chl *b* is normally degraded during senescence by first being metabolised to Chl *a* (Matile et al. 1996), and there is no reason to believe that *b* to *a* conversion does not operate normally in *yy* plants. This may explain the decline in Chl *b* observed in detached *yy* leaves, leading to a marked increase in the Chl *b* : *a* ratio in such leaves. The differential stability of Chls *a* and *b* was not apparent when *yy* leaves senesced on the intact plant. It is known that the overall rate at which the Chl catabolism pathway runs and the tightness with which the different component processes are coordinated differ markedly between senescing intact and excised tissues (Rodoni et al. 1998). *L. temulentum* lines with the stay-green phenotype are of interest as physiological models to dissect the respective control processes.

Total protein content closely followed pigment content in leaves of the contrasting *L. temulentum* genotypes (Fig. 3). Senescent attached or detached leaves of *Yy* and *YY* individuals retained less than 50% of the total protein present in young expanding leaves, whereas more than 65% of the total protein was present in comparable leaves of stay-green *yy* plants. Several previous studies have shown an abnormal stability of total particulate protein during senescence of *yy F. pratensis* leaves (Thomas 1977, 1982, 1983, 1987). On the other hand, decrease in the amounts of conventionally extracted buffer-soluble proteins occurred at essentially wildtype rates in stay-green *F. pratensis* (Thomas and Stoddart 1975; Thomas 1983, 1987). The trends in total soluble protein during senescence of *F. pratensis* genotypes are reflected in the behaviour of the predominant component, Rubisco (Thomas 1977, 1982). Nevertheless, Davies et al. (1990) isolated Rubisco tightly bound to thylakoid membranes and found that this fraction was much less labile during senescence of stay-green *F. pratensis* than of the wildtype. In the present study we used an all-inclusive extraction procedure that did not distinguish between these two pools of Rubisco, but we assume that the clear persistence of the large subunit in *yy L. temulentum* (Fig. 3) is accounted for by the membrane-associated fraction.

Observations of stay-greens in several species, as well as in vitro studies of complex stability, convincingly demonstrate a role for Chl in regulating the assembly, resilience and dismantling of thylakoid membrane pigment-proteolipids during plastid differentiation and senescence (Thomas 1997). The higher abundance of LHCPII in the L3 and 8d samples of *yy L. temulentum* compared with those of the yellowing genotypes (Fig. 4) is consistent with this relationship between Chl and its associated proteins. It was noticeable that the surviving LHCPII in senescent leaves of *yy* had a fuzzy appearance, indicative of partial degradation. Immunelectron microscopy has shown that the LHCPII of senescent stay-green *F. pratensis* remains associated with recognisable membrane structures in the gerontoplast (Hilditch et al. 1989). It is likely that the proteases responsible for degrading Rubisco and other stroma proteins begin to attack accessible parts of pigment-stabilised proteins such as LHCPII when the supply of soluble substrates runs down. The partial stability conferred by Chl on the proteins with which it is complexed in the thylakoid also extends to polypeptides that are not themselves directly pigment-binding but which associate closely with proteins that are. Figure 4 presents evidence that the product of the gene *psaD*, the ferredoxin-docking protein of Photosystem I (Zilber and Malkin 1988), is such a thylakoid component, since it was comparable in stability to LHCPII in senescing leaves of *yy L. temulentum*.

The behaviour of cytochrome *f* (Fig. 4) was unexpected. Previously we found that the rate of degradation of this polypeptide during senescence in stay-green *F. pratensis*, whilst not reduced to the same extent as that of photosystem-associated and light-harvesting proteins, is appreciably slower than in the yellowing genotype (Davies et al. 1990). In the *L. temulentum*

background, *yy* did not have a stabilising effect on cytochrome *f*. We speculate that this contrast with *F. pratensis* is related to surprisingly large interspecific differences in proteolytic capacity. In general, *Lolium* species remobilise vegetative nitrogen more actively than do those of the *Festuca* genus, and *L. temulentum* is one of the most efficient recyclers amongst the *Lolium*s (Kingston-Smith, Thomas, Bortlik, unpublished observations). This may be a consequence of incidental selection of *L. temulentum* as a weed of cereals so that it has exaggerated, cereal-like qualities of a high harvest index, strong vegetative and reproductive sinks for nitrogen and a generally hyperactive remobilisation system. The predominantly luminal location of cytochrome *f* (Sczcepaniak et al. 1989) may provide partial inaccessibility to the relatively weak proteases of *F. pratensis* but not to the more aggressive mobilisation activity of *L. temulentum*.

In conclusion, the advanced near-isogenic lines developed in the breeding programme described in the present paper, where sid^y is a phenotypic landmark in an alien genomic background, enable experiments on the organisation and expression of the gene to be carried out with precision. Results of a preliminary investigation have already been presented (Thomas et al. 1997), and more detailed studies are in progress.

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