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Introgression, tagging and expression of a leaf senescence gene in *Festulolium*

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SUMMARY

A mutation of a gene (discovered in *Festuca pratensis* Huds. and designated *sid*) confers indefinite greenness on senescing leaves. Via intergeneric hybrids with *Lolium multiflorum* L. and *Lolium perenne* L., the mutant gene (*sid*^{y}) has been introgressed into a range of *Lolium* backgrounds. Using genomic *in situ* hybridization we have identified segments carrying *sid*^{y} in recombinant chromosomes of *Lolium–Festuca* introgression lines. We also used *L. perenne* lines segregating 1:1 for the *staygreen* character to tag the gene with molecular markers. In two mapping populations a total of 84 genotypes were screened with isoenzymes, RAPD primers, RFLP probes and AFLP primer pairs. Over 180 polymorphic loci were identified, representing 10 linkage groups spanning 600 cM. Two AFLP markers are linked to *sid* at 4·6 and 14·9 cM, close enough to be usable for marker-assisted selection. Introgression of *sid*^{y} into *Lolium temulentum* L. resulted in the production of near-isogenic inbreeding lines suitable for comparative studies of gene expression. Using a variation of the method of representational difference analysis a very small number of cDNAs have been identified as promising candidates for *sid*, or genes directly regulated by *sid*.

Key words: *Festuca pratensis*, *Lolium multiflorum*, *perenne* and *temulentum*, leaf senescence, *staygreen* mutation, cDNA.

INTRODUCTION

The prominent visible symptom of leaf senescence is yellowing. The biochemistry of chlorophyll degradation is at last beginning to yield to experimental investigation (Matile *et al.*, 1996). Mutants in which yellowing is disabled have played an important role in revealing the catabolic pathway for leaf pigments. In mutants of three species, *Festuca pratensis*, *Phaseolus vulgaris* and *Pisum sativum*, there is convincing evidence that the enzymic step which destroys the green colour by opening the tetrapyrrole macrocycle is inoperative (Bachmann *et al.*, 1994; Vicentini *et al.*, 1995; Thomas *et al.*, 1996). Such *staygreen* genotypes give interesting insights into an aspect of the physiology of leaf senescence, but might also have unusual agronomic and ecological properties related particularly to disturbed nitrogen nutrition (Bakken *et al.*, 1997; Hauck *et al.*, 1997; Thomas, 1997). Because the genes for the chlorophyll catabolism pathway have yet to be cloned, and since the *staygreen* trait is of considerable practical value (Thomas & Smart, 1993), the variant loci of senescence mutants are important targets for tagging, mapping and isolation. The best-characterized *staygreen* mutation is the *sid*^{y} locus of *Festuca pratensis* (Thomas, 1987*a*). Genes can be freely introgressed across the range of species in the forage grass genera *Festuca* and *Lolium*, allowing the construction of populations for physical and genetic mapping in which the gene or gene complex of interest is an alien recombinant (see Humphreys *et al.*, 1997). In this paper we describe the chromosomal location, marker linkage and expression pattern of *sid*^{y} transferred from *Festuca pratensis* to *Lolium* backgrounds.

MATERIALS AND METHODS

Plant material

The source of the recessive mutant allele of the locus *sid* was a population of *Festuca pratensis* from the

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grass breeding programme of the former Welsh Plant Breeding Station (now IGER Aberystwyth) and the history of derived *F. pratensis* lines has been described by Thomas (1987*a*, *b*). Details of the scheme for intergeneric transfer of *sid*^{y} will be published elsewhere, but briefly the crossing sequence is as follows. Diploid *F. pratensis* line Bf993 (homozygous for *y*) was crossed with tetraploid *Lolium multiflorum* (homozygous *wildtype Y*) to produce a triploid hybrid (*YYy*). Backcrossing with diploid *wildtype L. multiflorum* gave segregating populations used for genomic *in situ* hybridization (GISH). Linkage analysis was carried out on two backcross populations derived from the F1 hybrid between diploid *F. pratensis* (*yy*) and *L. perenne* (*YY*). Crosses were also made between diploid phenotypically *staygreen* individuals from a segregating *Festulolium* population and the inbreeding annual species *Lolium temulentum*. We used the 'Ceres' strain of *L. temulentum* because of its experimental convenience as a classic model species for physiological studies (Périlleux, 1995). Gene expression was investigated in homozygous *yy* and *YY L. temulentum* plants from a backcross 3 (BC3) line.

Induction of senescence

The *staygreen* trait determined by the *y* allele of the *sid* locus is exhibited whenever yellowing would normally occur in the life-cycle or in response to artificially imposed treatments such as leaf detachment. Thus incubating detached laminae under moist conditions in darkness is a convenient screen for the phenotype (Thomas, 1987*a*, *b*). Emerging, mature and senescing leaves of intact *L. temulentum* plants (Thomas, 1990) were used for gene expression studies.

Genomic in situ *hybridization*

GISH was carried out as described by Thomas *et al.* (1994) on mitotic chromosome preparations from eight *L. multiflorum* introgression lines, each with a *staygreen* phenotype and therefore homozygous for *sid*^{y}. Total genomic DNA from *Festuca pratensis* was labelled with rhodamine-dUTP by nick translation, and 100 ng was applied to each slide. Sonicated *L. multiflorum* DNA at 4 μg per slide was used as blocker. All preparations were counterstained with DAPI. Fluorescence microscopy images were captured and processed using a CoolView® CCD camera attached to an Apple Macintosh® system running software from Improvision®.

Molecular markers

The two *L. perenne* populations segregating for *y* and *Y* employed for marker studies were designated P151/70 and P151/71. Polymorphisms in loci encoding four isoenzymes (*PGI/2*, *GOT/2*, *GOT/3*, and *SOD/1*) were evaluated according to Hayward & McAdam (1976). In addition, nine 10-mer primers (Operon Technologies, USA) for RAPD analysis (Williams *et al.*, 1990) were screened on both populations, as were a total of 45 *Lolium perenne*, oat, barley and wheat genomic and cDNA digoxigenin-dUTP and fluorescein-dUTP labelled RFLP probes (hybridization and chemiluminescent detection procedures were performed according to Leblanc *et al.*, 1997). For AFLP analysis only population P151/71 was used. *Eco*R1/*Mse*1 templates were prepared according to Keygene® (Netherlands) and AFLP amplification products were visualised either by incorporating 1% digoxigenin-dUTP into the amplification reactions followed by chemiluminescent DNA detection (Leblanc *et al.*, 1997) or by using the Promega (UK) Silver Sequence DNA Staining procedure according to the manufacturer's instructions.

Subtractive cDNA analysis

Total RNA was extracted according to Schünmann, Ougham & Turk (1994) and mRNA purified by poly-A^{+} selection using Dynabeads® (Dynal). Synthesis of cDNA was carried out with a Pharmacia kit. Messages present in yellowing leaf tissue of *L. temulentum* line BC3/F3 (*wildtype*) and absent from, or much reduced in, comparable tissue of *staygreen* introgression line BC3/F5 were isolated by cDNA subtraction using a modification of the representational difference analysis (RDA) procedure of Lisitsyn, Lisitsyn & Wigler (1993). *Wildtype* (tester) and *staygreen* (driver) cDNAs were cut with DpnII and homologous adaptors were ligated to the fragments. Three rounds of hybridization of tester to an increasing excess of driver were made, following the original Lisitsyn protocol: hybridization 1, $\times 100$; hybridization 2, $\times 1000$; hybridization 3, $\times 100000$. After each subtraction, the resulting fragments were PCR-amplified. A control subtraction was also made, comprising *staygreen* cDNA as tester and *wildtype* cDNA as driver. Subtraction products were cloned into pBluescript® plasmid (Stratagene) digested with *Bam*H1. After transformation, subtraction products were PCR-amplified directly from the plasmid, separated by gel electrophoresis and Southern blotted by standard techniques (Sambrook, Fritsch & Maniatis, 1989). The blots were probed with Dig-labelled (Boehringer Mannheim) control subtraction product. Clones which are not specific to *wildtype* plants, including any PCR artefacts, cross-hybridize between the two cDNA populations and were rejected. Plasmid DNA was isolated for individual clones which did not hybridize to the control subtraction.

Subtraction products and DpnII-digested, PCR-amplified cDNA from senescing leaves of *wildtype* and *staygreen* plants were Southern blotted. Total leaf RNA was also northern blotted. The blots were probed with plasmid-derived DNA from putative *sid* or *sid*-regulated clones random prime-labelled with ^{32}P (Stratagene).

RESULTS AND DISCUSSION

Introgression of sid^y *from* Festuca *into* Lolium

Humphreys (1989) showed that genes from the hexaploid species *Festuca arundinacea* could be exchanged with their homologues in *Lolium mutliflorum* by intergeneric hybridization followed by backcrossing. The readiness with which such introgressions can be made to occur in crosses between species in the *Lolium/Festuca* complex has been called 'promiscuous recombination'. In the present experiments *Festuca pratensis* line Bf993 was the source of the mutant *y* allele of the locus *sid*. Leaves of a *wildtype* line of *Festuca pratensis* turn yellow during the normal course of intact plant development, or when subjected to an inductive treatment such as transfer to darkness for several days; Bf993 leaves, on the other hand, remain green under these conditions (Thomas, 1987*a*). Segregation of *y* and *Y* in *Festulolium* populations was screened by incubating detached leaves in darkness for 6 d (Fig. 1*a*).

Physical mapping of sid^y

In intergeneric hybrids between *F. pratensis yy* and tetraploid (*YYYY*) *L. multiflorum*, the *multiflorum* background came through strongly in the backcross (BC) 1 families and selection led to the isolation of lines where *staygreen* was the only significant *Festuca*-like character. These introgression lines were subjected to GISH. Figure 1*b* is a representative example of the pattern of hybridization observed in this material. In this case three of the fourteen chromosomes of the diploid *L. multiflorum* set were identified as recombinant, each carrying a segment strongly labelled with rhodamine-tagged DNA from *F. pratensis*. Two of the hybridization sites are in matching regions, located at the distal end of the long arm of each of a pair of morphologically similar chromosomes. The remaining segment occupies a median position, spanning the centromere, and is unmatched. Since *y* is recessive, the introgressed segment carrying the mutant allele must be present in two doses in the diploid chromosome set of a *Lolium* with a *staygreen* phenotype. We conclude that *sid* maps to the end of the chromosome corresponding to the matched pair of recombinants. Based on current work to establish a detailed *Festulolium* karyotype, this is tentatively identified as chromosome 6, which carries the *PGI/2* locus on its short arm (Humphreys *et al.*, 1997) and coincides with linkage group 6 of the published *Lolium* genetic map (Hayward *et al.*, 1994).

Identifying linked molecular markers

The *staygreen* allele of *sid* was introduced from *F. pratensis* into *L. perenne* and two populations segregating 1:1 for green:yellow (see Fig. 1*a*) were screened with isoenzyme and molecular markers. Table 1 summarizes the number of polymorphisms observed in the mapping families. Of particular interest is the effectiveness of AFLP screening. A total of 6 primer pair combinations revealed 93

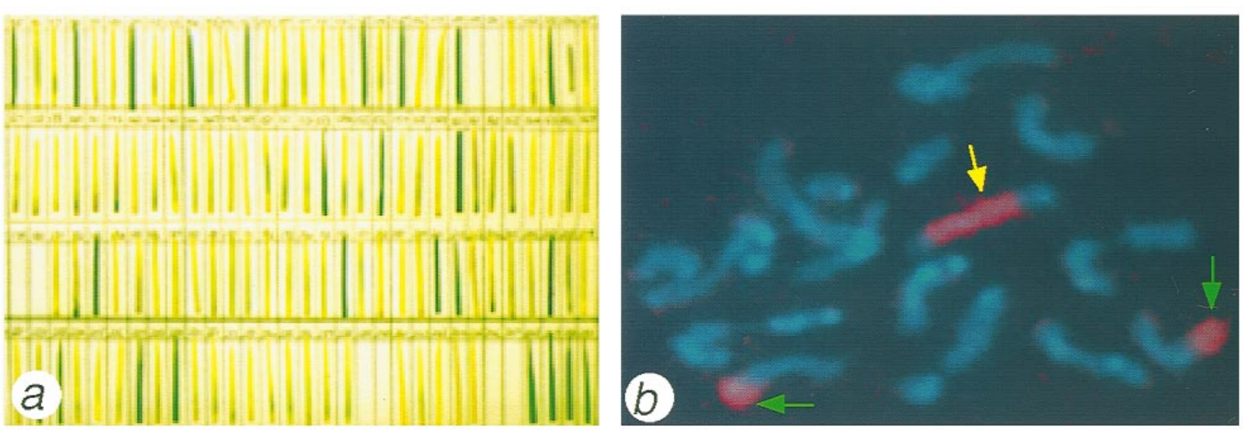

Figure 1. (*a*) Screening for the *staygreen* phenotype in *Lolium temulentum* introgression lines by incubating detached leaves of seedlings on moist chromatography paper in darkness for up to 6 d. Chlorophyll stability in homozygous recessive individuals is essentially indefinite so that scoring for the character is on an all-or-nothing basis. (*b*) Genomic *in situ* hybridization reveals *Festuca pratensis* segments (red) in the chromosome set (blue) of *staygreen Lolium multiflorum*. The green arrows indicate recombinant segments carrying the *sid* locus in a matching pair of chromosomes. The yellow arrow points to a single additional introgression, unrelated to the *staygreen* character.

Table 1. *Molecular markers screened in* Festulolium *introgression lines*

	Isoenzyme	RAPD	RFLP	AFLP	Total
Population P151/70					
Loci scored	4	9	45	0	58
Polymorphisms	4	3	44	0	51
Population P151/71					
Loci scored	4	9	44	6	63
Polymorphisms	4	2	34	93	133

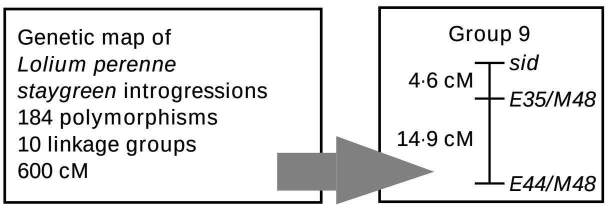

Figure 2. AFLP tags on the *sid* locus in relation to a genetic map of *Lolium perenne* introgression lines based on isoenzyme, RAPD, RFLP and AFLP markers.

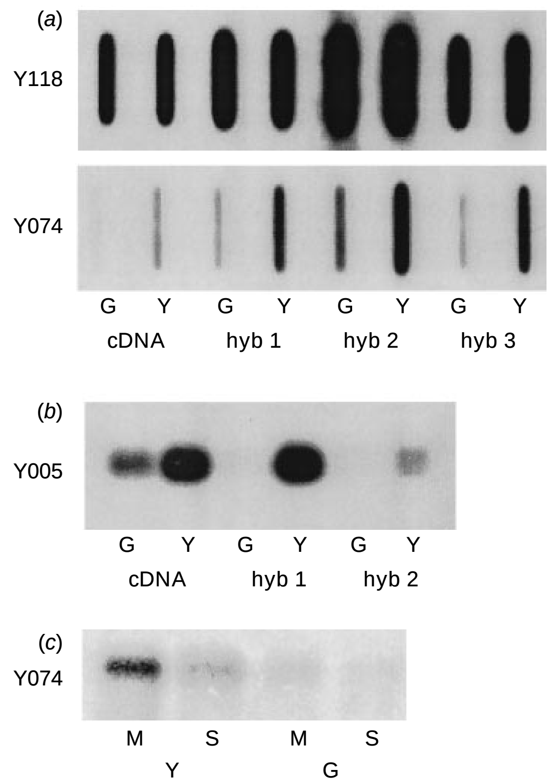

Figure 3. (*a*) Southern slot blots showing the original cDNA from *Staygreen* (G) and *wildtype* (Y) *Lolium temulentum* introgression lines, and the products of three rounds of the RDA hybridization procedure, probed with fragment Y118 (control) and Y074 (strongly differential). (*b*) Two rounds of RDA hybridization probed with fragment Y005. (*c*) Northern blot of RNA (10 μg loaded in each track) from mature (M) and senescing (S) *L. temulentum* leaf tissue, probed with fragment Y074.

polymorphic AFLP loci in population P151/71. Amongst these were two combinations, E35/M48 and E44/M48, each of which detected a polymorphism segregating with *sid*. The programme Joinmap® (Stam, 1993) was used to combine AFLP and other marker data into a map which comprises 10 linkage groups covering *c*. 600 cM. The nearest AFLP locus to *sid* is within 5 cM and the second tag is *c*. 15 cM distal to the first (Fig. 2). Even though the *staygreen* trait is very easy to score (Fig. 1 *a*), a molecular marker for sid^y, which is recessive, is useful for breeding purposes, as well as for tagging recombinant chromosome segments (Fig. 1 *b*).

Gene expression in a Lolium temulentum *introgression line*

To identify senescence up-regulated cDNAs specific to *wildtype L. temulentum*, but which are missing in *staygreen* introgression lines, we employed a modified version of representational difference analysis (Lisitsyn *et al*., 1993). RDA uses PCR to amplify DNA sequences that are polymorphic between two populations. The technique has proved to be ideal for the study of complex plant genomes (Donnison *et al*., 1996). A simple modification to the technique allows its use in comparing different stages of plant development. Fragments have been confirmed as true difference products by probing original RNA from the two genotypes on northern blots.

Figure 3 *a* presents Southern slot-blots of Y118, a fragment that showed no differential expression between *staygreen* (G) and *wildtype* (Y) *L. temulentum* and hence was useful as a control, and Y074 which gave a much stronger signal with Y than G. The most dramatically differential pattern was observed with fragment Y005 over two rounds of hybridization (Fig. 3 *b*). The weak and declining signal in the G tracks of Figure 3 *a*, *b* from cDNA through successive cycles of hybridization may be explained in terms of a multigene family, one member of which has a polymorphism that becomes exploited during the subtraction sequence. Figure 3 *c* is a northern blot of RNA from mature (M) and senescing (S) leaves of *staygreen* and *wildtype L.*

temulentum, probed with fragment Y074. The length of the clone was about 230 bp, and of the detected message 1·6–1·7 kb. On the indirect evidence of inhibitor experiments, it is thought that *sid* is normally unexpressed until induced at the initiation of senescence (Vicentini *et al.*, 1995; Matile *et al.*, 1996). If this is correct then the gene encoding the mRNA detected by Y074 is unlikely to be *sid* itself, since it was clearly active in mature (M) *wildtype* tissue, well before detectable senescence (S) has commenced (Fig. 3*c*). It would perhaps be surprising if mutation of *sid* did not have repercussions for expression of other genes, and indeed the *staygreen* trait in *Festulolium* is pleiotropic, at least in terms of the physiological consequences of impaired chlorophyll metabolism (Bakken *et al.*, 1997; Hauck *et al.*, 1997; Thomas, 1997). Y074 might correspond to such a downstream gene.

The high sensitivity of subtractive methods of isolating cDNAs for differentially-expressed genes can yield large numbers of clones (for a discussion of the application of this approach to leaf senescence, see Buchanan-Wollaston, 1997). In our laboratory both maize leaf senescence (I. Donnison, unpublished) and floral induction (S. D. Knott, I. Donnison & T. W. A. Jones, unpublished) have been analysed using the RDA technique, and many up- and down-regulated clones obtained. It is encouraging, therefore, that comparison of *L. temulentum* lines differing in the expression of just one major gene has provided such a very small number of clones, and that they are down-regulated in *staygreen*. Screening continues, and so far as well as Y005 and Y074 we have one other candidate, MI27. Preliminary sequence data from these fragments has not revealed homologies to genes currently in the database, but since the metabolism of chlorophyll and interacting components during leaf senescence has yet to be characterized in molecular biology terms, the lack of precedents is not surprising.

CONCLUSIONS

Establishing the complete sequence of molecular events from genotype to phenotype is still difficult, even for quite simple heritable traits. After all, it took 130 yr to define the molecular and biochemical basis of round/wrinkled and green/yellow in peas as originally described by Mendel (Thomas *et al.*, 1996). The *sid* locus in *Festulolium* is a single nuclear gene with a well defined physiological role, but to understand its function it has been necessary to describe a completely new metabolic pathway (Matile *et al.*, 1996) and to introgress the gene into new backgrounds to facilitate molecular analysis. Integrating physical and linkage mapping of recombinant chromosomes carrying a mutation of the locus with analysis of differential gene expression is a direct way of bridging the most recalcitrant gap between genotype and phenotype. The approach is suitable for the study of single qualitative loci such as *sid*, but also has great promise for investigating more complex quantitative characters such as drought response (Humphreys *et al.*, 1997).

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