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 locus of Festuca pratensis (Thomas, 1987a). 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 transferred from Festuca pratensis to Lolium backgrounds.
grasp 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 (1987a, b). Details of the scheme for intergeneric transfer of *sid* will be published elsewhere, but briefly the crossing sequence is as follows. Diploid *F. pratensis* line B993 (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 *Festucaerum* 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. Thus incubating detached laminae under artificially imposed treatments such as leaf detachment normally occur in the life-cycle or in response to *staygreen* introgression 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, ×100; hybridization 2, ×1000; hybridization 3, ×100 000. 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 pBlue-script® plasmid (Stratagene) digested with BamHI and ligated to the fragments, 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.

**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*.

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 (*PG1/2, GOT/2, GOT/3*, and *SOD/I*) 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. *EcoR1/MseI 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, Ouigham & 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, ×100; hybridization 2, ×1000; hybridization 3, ×100 000. 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 pBlue-script® plasmid (Stratagene) digested with BamHI. 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* from *Festuca* into *Lolium***

Humphreys (1989) showed that genes from the hexaploid species *Festuca arundinacea* could be exchanged with their homologues in *Lolium multiflorum* 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, 1987a). Segregation of *y* and *Y* in *Festulolium* populations was screened by incubating detached leaves in darkness for 6 d (Fig. 1a).

**Physical mapping of *sid***

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 1b 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* 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. 1a) 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...
Table 1. Molecular markers screened in Festulolium introgression lines

<table>
<thead>
<tr>
<th>Population</th>
<th>Isoenzyme</th>
<th>RAPD</th>
<th>RFLP</th>
<th>AFLP</th>
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<td>P151/71</td>
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<td>Polymorphisms</td>
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<td>2</td>
<td>34</td>
<td>93</td>
<td>133</td>
</tr>
</tbody>
</table>

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.

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. 1a), a molecular marker for sid*, which is recessive, is useful for breeding purposes, as well as for tagging recombinant chromosome segments (Fig. 1b).

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 3a 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 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.
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. 3c). 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, M127. 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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