Molecular tagging of a senescence gene by introgression mapping of a stay-green mutation from *Festuca pratensis*

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Summary

- Intergeneric hybrids between *Lolium multiflorum* and *Festuca pratensis* (*Lm/Fp*) and their derivatives exhibit a unique combination of genetic and cytogenetic characteristics: chromosomes undergo a high frequency of homoeologous recombination at meiosis; the chromosomes of the two species can easily be discriminated by genomic *in situ* hybridization (GISH); recombination occurs along the entire length of homoeologous bivalents; a high frequency of marker polymorphism is observed between the two species.
- This combination of characters has been used to transfer and isolate a *F. pratensis* chromosome segment carrying a mutant 'stay-green' gene conferring a disrupted leaf senescence phenotype into *L. multiflorum*.
- The genetic location within the introgressed *F. pratensis* segment of the senescence gene has been mapped using amplified fragment length polymorphisms (AFLPs), and *F. pratensis*-specific AFLP markers closely flanking the green gene have been cloned.
- The use of these cloned sequences as markers for the stay-green locus in marker-assisted selection programmes has been tested. The potential application of *Lm/Fp* introgressions as a tool for the map-based cloning of introgressed *Fp* genes is discussed.

Key words: BAC libraries, chlorophyll breakdown, cloning, introgression mapping, *Lolium/Festuca pratensis* interspecific hybrids, mapping, senescence.

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Introduction

The distinctive recombination behaviour of genomes in the *Lolium–Festuca* complex may be exploited for rapid and efficient gene mapping and isolation. This is possible because *Lolium/Festuca* hybrids exhibit a unique combination of characters not seen in other plant genera *Lolium multiflorum* and *Festuca pratensis* are both diploid $2n = 2x = (14)$, and their hybrids undergo normal levels of intraspecific recombination and produce fully fertile progeny. Moreover, the chromosomes of the two species can be distinguished using genomic *in situ* hybridization (GISH) (Thomas *et al.*, 1994; King *et al.*, 1998, 1999; Armstead *et al.*, 2001; King *et al.*, 2002a,b). This contrasts markedly with interspecific hybrids in other plant genera such as wheat where, although the chromosomes of this crop species and many of its wild relatives can be discriminated in hybrid material, the frequency of interspecific recombination is very low (King *et al.*, 1994). In addition, many interspecific hybrids, in which the chromosomes of the different species can be easily distinguished, are almost completely sterile (Kamstra *et al.*, 1999).

A detailed study of a *L. perenne/F. pratensis* monosomic substitution line (13 *L. perenne* chromosomes and 1 *F. pratensis* chromosome) in which the *F. pratensis* chromosome was genetically and physically mapped revealed a high frequency of marker polymorphism between the chromosomes of the two species. In addition, although the frequency of recombination varied, it occurred along the entire length of the homoeologous bivalent (King *et al.*, 2002a,b). This previous work indicated a promising prospect for introgression mapping of the 'stay-green' gene from *F. pratensis* into *L. multiflorum.*

The combination of characters exhibited by *L. multiflorum*/*F. pratensis* and *L. perenne*/*F. pratensis* hybrids and their derivatives makes an ideal model system for intergeneric introgression and gene isolation:

1 The high frequency of recombination facilitates the transfer of *F. pratensis* chromosome segments, carrying target genes, into *L. multiflorum* and *L. perenne*.

2 A GISH analysis allows identification and classification of *Lolium*/*F. pratensis* introgressions (i.e. confirmation of the introgression of a *F. pratensis* segments into *L. perenne* and *L. multiflorum*) and an estimation of their physical size.

3 The distribution of recombination along the entire length of *L. perenne*/*F. pratensis* and *L. multiflorum* bivalents permits the transfer of any *F. pratensis* gene into *L. perenne* and *L. multiflorum*.

4 The high frequency of marker polymorphism between *F. pratensis* and *L. multiflorum* and *L. perenne* aids in the mapping of target *F. pratensis* genes on introgressed *F. pratensis* segments.

5 The system also facilitates the rapid identification of markers located on an introgressed *F. pratensis* chromosome segment, by screening an *L. perenne*/*F. pratensis* or *L. multiflorum*/*F. pratensis* introgression and the parental and hybrid germplasm from which it was derived. Any polymorphic marker present in the *F. pratensis* parent, the *Lolium*/*F. pratensis* hybrid and the introgression line itself, but not the *L. perenne* or *L. multiflorum* parents, must be located within the introgressed *F. pratensis* chromosome segment.

The work presented in this paper demonstrates the potential of the *L. perenne*/*F. pratensis* system for plant improvement, based on the transfer of a *F. pratensis* segment, which carries a mutation of a gene normally required for leaf yellowing during senescence (Thomas, 1987 and Thomas *et al.*, 1997), into *L. multiflorum*. The stay-green character results from a recessive mutation in the gene, and only plants homozygous for the mutation express the stay-green phenotype. Leaf segments of plants homozygous for the mutation remain green, while plants heterozygous or homozygous for the wild-type gene turn yellow as chlorophyll is broken down. The lesion in the chlorophyll breakdown pathway in plants homozygous for the green gene appears to result from the inability of plants to break down pheophorbide to red-chlorophyll-catabolite (RCC) because of a deficiency in pheophorbide-a-oxygenase (PaO) activity (Vicentini *et al.*, 1995; Rodoni *et al.*, 1997; Thomas *et al.*, 2001). Thus, the stay-green phenotype is believed to result from a mutation in the gene responsible for the production of the PaO enzyme or a regulator gene that controls the expression or activation of the gene/protein (Roca *et al.*, 2004).

Materials and Methods

Plant material

The wild-type locus that determines yellowing in senescing leaves is designated as Y and the recessive mutant (stay-green) allele as y. An emasculated, synthetic autotetraploid *L. multiflorum* ($2n = 4x = 28$) plant carrying four doses of the wild type gene (YYYY) was pollinated with *F. pratensis* homozygous for the recessive gene (yy) (for crossing scheme see Fig. 1). The resulting triploid F_1 ($2n = 3x = 21$, *LmLmFp*, 14 *L. multiflorum* chromosomes and seven *F. pratensis* chromosomes, YYy), which exhibited normal senescence, was used as the pollen parent (Thomas *et al.*, 1988) in crosses to diploid *L. multiflorum* (YY, $2n = 2x = 14$) (King *et al.*, 1998 have demonstrated that the progeny from *Lolium*/*F. pratensis* crosses are mostly diploid and normally carry one or two *F. pratensis* chromosome segments). Eighty backcross 1 (BC_1) progeny were produced from this cross. These BC_1 individuals (the majority of which would have been diploid) were expected to have a genotypic constitution of either YY or Yy. The Yy plants would be expected to carry a *F. pratensis* segment, carrying the y allele, which has replaced the homoeologous region of the *Lolium* chromosome that carries the wild-type Y allele. In order to determine the genotype of the BC_1 individuals each plant was split into two cloned plants. One clone was then grown to flowering and test-crossed with diploid *L. multiflorum* lines previously isolated and shown to be homozygous recessive for the *F. pratensis* stay-green gene (yy) (Thomas *et al.*, 1994, 1997). Twenty seeds selected from each of the 80 test-cross families were germinated and the leaves of the seedlings removed and placed on moist filter paper in Petri dishes. These detached leaves were then placed in the dark at room temperature.

The leaves derived from 20 test-cross individuals from each of the 80 families were screened for the presence or absence of green leaves. Test-cross progeny derived from BC_1 plants homozygous for the wild-type senescence gene (YY) were expected to have the genetic constitution Yy. Thus, all individuals were expected to turn yellow. By contrast, test-cross progeny derived from BC_1 individuals heterozygous for the mutant phenotype (Yy) were expected to segregate for green and yellow senescent leaves (Yy + yy = Yy yellow to yy green in a ratio of 1 : 1). BC_1 individuals identified as being heterozygous for the stay-green gene (Yy) were selected while BC_1 plants homozygous for the wild-type allele (YY) were discarded.

GISH analysis

A GISH analysis was performed (as described by King *et al.*, 1998 and King *et al.*, 2002b) on BC_1 plants that were identified as being of the genetic constitution Yy. BC_1 plants with a single small *F. pratensis* introgression were identified. The second clone of the BC_1 plants was then crossed as the pollen parent to the recurrent diploid *L. multiflorum* parent (YY) to produce a BC_2 mapping population ($n = 100$).

Amplified fragment length polymorphisms (AFLPs) analysis

Amplified fragment length polymorphisms were used to generate a genetic map of the introgressed *F. pratensis* chromosome segment.

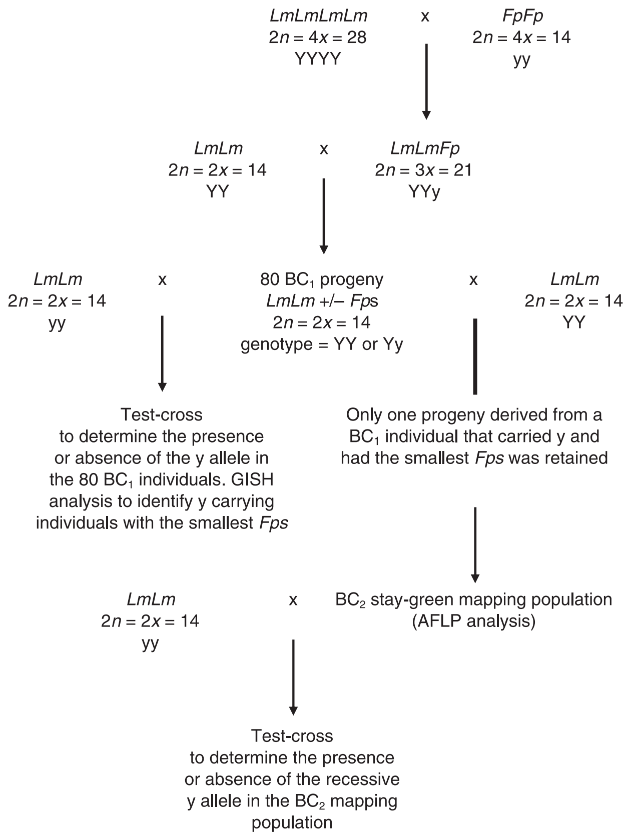

Fig. 1 Crossing scheme for producing the stay-green mapping population. *Lm*, *Lolium multiflorum* genome; *Fp*, *Festuca pratensis* genome; *Fps*, *F. pratensis* segment; Y, wild-type yellowing; y, recessive stay-green.

The AFLP analysis was as described by King *et al.* (1998) and King *et al.* (2002a), using the restriction enzyme pairs *Hin*dIII/*Tru* 91 and *Eco*RI/*Mse1*. Polymorphisms specific to the *F. pratensis* segment were identified by screening the parents (i.e. tetraploid *L. multiflorum*, diploid *F. pratensis*, diploid *L. multiflorum*, the *L. multiflorum*/*L.* multiflorum/*F. pratensis* triploid hybrid and the selected BC_1 genotype carrying a single small *F. pratensis* chromosome segment). Primer pairs which failed to give a *F. pratensis*-specific polymorphism or primer pairs which gave a *F. pratensis* specific polymorphism in the *F. pratensis* parent and *F. pratensis* hybrid but not in the selected BC_1 introgression genotype (i.e. those where the *F. pratensis* specific marker lay outside the introgressed *F. pratensis* chromosomes segment) were discarded.

Primer pairs which gave a *F. pratensis*-specific polymorphism in the *F. pratensis* diploid parent, the *L. multiflorum*/*L. multiflorum*/*F. pratensis* triploid and the selected BC_1 individuals were selected.

Genetic map

Selected AFLP primer pairs were used to screen the BC_2 population of a selected BC_1 individual. The segregation of the *F. pratensis* specific polymorphisms in the BC_2 was analysed using JOINMAP 2.0 (Stam, 1993) to generate a genetic map of the *F. pratensis* chromosome segment. Each of the individuals of the BC_2 mapping population was also test-crossed to the *L. multiflorum* genotype homozygous recessive for the stay-green gene (yy). Twenty progeny plants from each test cross were grown and their leaves removed and scored for the presence or absence of the stay-green phenotype as described above. Thus, this screen allowed the determination of the presence or absence of the *F. pratensis*-derived mutation (yy) in each of the BC_2 individuals. The data for the presence or absence of the stay-green gene were combined with the AFLP data in order that the senescence mutation could be mapped within the introgressed *F. pratensis* chromosome segment.

The AFLP bands located either side of the stay-green locus were selected. These AFLP bands were excised from silver-stained polyacrylamide gels and the DNA reamplified using the relevant AFLP primer pairs and reaction mix (King *et al.*, 1998; King *et al.*, 2002a). The DNA was then cloned into vector pGem T-Easy (Promega, Southampton, UK). Six clones derived from each of the excised AFLP bands were selected and sequenced using an ABI prism 3100 DNA analyser (Applied Biosystems, Warrington, UK). Where all six clones contained the same sequence, this was considered the polymorphic marker. When two or more sequences were identified, the polymorphic marker was identified by DIG-labelling DNA (Roche Diagnostics, Lewes, UK) from each clone type and hybridizing to Southern blots of AFLP reactions of *F. pratensis* and *Lolium* parents using the AFLP primers used to generate the original marker. Pairs of 21-mer primers were designed from the *F. pratensis* genomic DNA internal to the AFLP primers and used to screen DNA derived from the *L. multiflorum* and *F. pratensis* parents. Polymerase chain reaction (PCR) amplification products from both parents were sequenced and compared. Some PCR products were polymorphic in terms of size while others differed in internal sequence. Where the primers did not generate *F. pratensis*-specific polymorphisms they were used to screen a *F. pratensis* bacterial artificial chromosome (BAC) library (the BAC library represented 2.5*x* genome equivalents and was arranged in a format to enable PCR-based screening; Donnison *et al.* submitted). Any BAC clones identified were recultured, BAC DNA extracted and restricted using *Hin*dIII, *Kpn*I or *Sac*I. Selected BAC-fragments were cloned into suitably prepared pBluescript IIKS, sequenced and new primer pairs designed. These primers were again used to screen the DNA from the *L. multiflorum* and *F. pratensis* parents and those exhibiting *F. pratensis* polymorphisms were used as additional markers to map the BC_2 mapping population.

Results

Seven of the 80 test-cross progenies, derived from the test-crosses between the BC_1 individuals and *L. multiflorum* homozygous for the stay-green mutation (yy), showed segregation for green

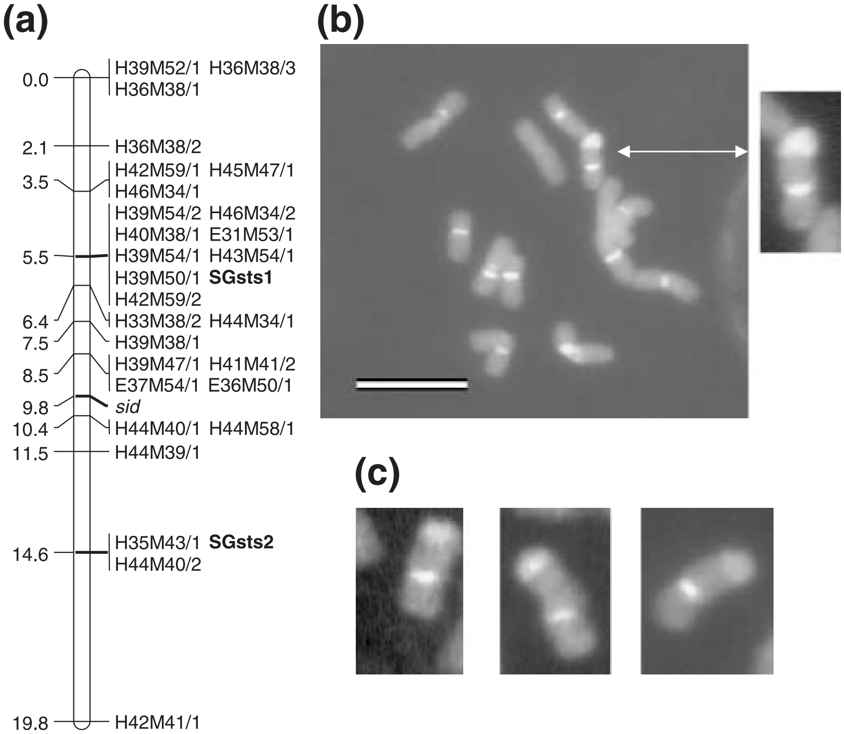

Fig. 2 (a) Linkage group of the introgressed *Festuca pratensis* segment containing the stay-green gene (*sid*). Genetic distances are indicated in centiMorgans. The positions of sequence-tagged-site (STS) markers derived from amplified fragment length polymorphism (AFLP)/BAC sequencing are indicated in bold-type; the positions of AFLP markers are indicated in normal-type. (b) The BC_1 plant with the smallest *F. pratensis* segment that carried the stay-green gene (*sid*). Bar, 10 μm. (c) BC_2 plants with reduced *F. pratensis* segments but still carrying the '*sid*' locus.

and yellow senescent leaves. Feulgen-stained chromosome counts of these plants revealed that two were aneuploid, comprising 15 chromosomes. These plants were discarded. The GISH analysis of the remaining five plants, each with the full complement of 14 chromosomes, identified two individuals that carried a single introgressed *F. pratensis* chromosome segment, one plant with two introgressions and two plants with three introgressions. Of the two plants that carried a single *F. pratensis* chromosome segment, the genotype that carried the smallest introgression was selected for AFLP analysis (Fig. 2). No further analysis was performed on the remaining genotypes.

A total of 266 AFLP primer pairs were used to screen the parental genotypes and the selected BC_1 individual carrying the smallest *F. pratensis* chromosome segment. Twenty-two primer pairs were used to generate 28 *F. pratensis*-specific polymorphisms that could be easily scored. One-hundred BC_2 individuals, generated by backcrossing the selected BC_1 individual with *L. multiflorum* homozygous for YY, were screened with the 22 AFLP primer pairs. These 100 BC_2 individuals were also test-crossed to the *L. multiflorum* homozygous genotype (yy). The analysis of these test-cross families revealed a ratio of 37 wild type YY genotypes to 50 heterozygous Yy genotypes (the test-cross analyses of 13 families were inconclusive and thus they were scored as missing data with regard to the stay-green trait). A χ^2 analysis of the data demonstrated that this result did not differ significantly from the expected 1 : 1 ratio.

The presence of the y mutation in a *F. pratensis* segment in the BC_2 was screened as a '+' while its absence was screened with a '–'. Similarly, the presence of a *F. pratensis* AFLP polymorphism on a *F. pratensis* chromosome segment in a BC_2 individual was screened as a '+' and its absence as a '–'.

The data was analysed to generate a genetic map of the *F. pratensis* chromosome segment. After the likeliest order of the markers within the linkage group was established, inspection of the genotype data set identified 16 apparent double recombination events around single markers (i.e. singletons (representing *c.* 0.5% of the total) in the 100 BC_2 individuals analysed). It is becoming generally accepted that the majority of singletons are an artefact of marker generation rather than real recombination events (Lincoln & Lander, 1992; Dib *et al.*, 1996; Broman *et al.*, 1998; King *et al.*, 2002a). Positive chiasma interference has been reported to prevent recombination closer than 15 cM in plants (Kearsey & Pooni, 1996) and the frequency of two chiasmata occurring in the same arm of a *L. pernne/F. pratensis* homoeologue has been shown to be extremely low (2%; King *et al.*, 2002a). The 16 singletons were therefore excluded from the data set. The final genetic distance of the *F. pratensis* chromosome segment between the terminal *F. pratensis*-derived AFLP markers was estimated to be 19.8 cM with the *sid* mutation located at 9.8 cM; the closest flanking markers to *sid* were at 0.6 cM and 1.3 cM (Fig. 2).

Twelve AFLP bands were excised, cloned and sequenced. Primers designed from one of these 12 AFLPs (SG1) which produced a *F. pratensis* specific fragment of 390 bp, immediately distinguished between the *Lolium* and *Festuca* genotypes, whereas the other 11 did not. Most of the internal sequences of the other markers were not polymorphic between the parents and therefore not useful for conversion to

STS markers. Therefore, a *F. pratensis* BAC library was screened with AFLP-derived primers from another two markers on the other side of the stay-green locus with the aim of identifying additional sequences suitable for designing new primers. One primer pair identified many BACs, indicating that the sequence was present in multiple locations in the genome. However, the other primer pair from SG2, which generated a *F. pratensis* AFLP fragment of 300 bp, identified two BACs and, given the 2.5*x* coverage of the BAC library, this fragment was considered to be present as a single copy sequence. An individual BAC was therefore identified for this marker, BAC DNA was extracted, digested and recloned. End-sequencing of these BAC subclones generated 7 kb of additional sequence. This sequence was used to generate an extra primer pair from a BAC subclone fragment which did not show homology to repetitive DNA such as retroelements of other monocot species. This primer pair was tested on *Lolium* and *Festuca* parental DNA and shown to be polymorphic. Primer pairs for this and the other polymorphic marker were then mapped back onto the genetic map and in both cases were found to map precisely to the same position as the original AFLP markers, on either side of the stay-green locus.

Discussion

This work describes the efficiency with which *F. pratensis* chromosome segments, carrying recessive target alleles such as the stay-green gene, can be transferred into *Lolium multiflorum.* Dominant *F. pratensis* alleles would require less effort to introgress into *Lolium* since test-crossing to determine the presence or absence of the phenotype they control would be unnecessary.

The ease of transfer of the *F. pratensis* segment, into *L. multiflorum* was facilitated by the high frequency of recombination exhibited between *L. multiflorum* and *F. pratensis.* Previous work (King *et al.*, 1998, 1999; King *et al.*, 2002a,b Armstead *et al.*, 2001) has demonstrated that a high degree of recombination occurs between *L. perenne* (a close relative of *L. multiflorum*) and *F. pratensis.* An in-depth analysis of *F. pratensis* chromosome 3, which shows a high degree of synteny with rice chromosome 1 and its *L. perenne* homoeologues, revealed that the highest frequency of recombination occurs between 15% and 20% from the end of either telomere (King *et al.*, 2002a, 2002b). However, although the frequency of recombination is lower over the rest of the *Festuca* chromosome (King *et al.*, 2002a,), and particularly at the centromere and nucleolar organizer region, it is sufficiently high for any *F. pratensis* chromosome segment to be introgressed into *Lolium.* A knowledge of the physical location of a gene is, however, of importance as it will give an indication of the number of BC_1 backcross progeny that need to be generated in order for a specific *F. pratensis* segment carrying a target gene to be introgressed into *L. multiflorum* or *L. perenne.* The introgression of *F. pratensis* genes located in areas of high recombination will require fewer BC_1 backcross progeny than for genes located in areas of low recombination.

The isolation and characterization of plants carrying a single *F. pratensis* segment, and subsequent selection of a single plant that carried the smallest *F. pratensis* introgression, was facilitated by using the *L. multiflorum/L. multiflorum/F. pratensis* hybrid as the pollen parent in crosses to the diploid *L. multiflorum* parent, and by the way in which ability of GISH can be used to discriminate between the parental genomes. The use of the triploid hybrid as the pollen parent has been shown to result in a high frequency of *Lolium/F. pratensis* introgressions. For example, 70% of the progeny from a cross between a *L. perenne/L. perenne/F. pratensis* triploid and diploid *L. perenne* carried *F. pratensis* introgressions. Of these, the majority were diploid and carried one or two *F. pratensis* introgressions (King *et al.*, 1998). As with *F. pratensis/L. perenne* introgression (King *et al.*, 1998; King *et al.*, 2002a,b), AFLP markers detecting *F. pratensis*-specific polymorphisms were readily identified in the *L. multiflorum* genotype carrying the smallest *F. pratensis* segment. Twenty-two selected primers, which gave 29 easily scored *F. pratensis* specific markers, were used to generate a genetic map of the *F. pratensis* segment. This led to the identification of AFLP markers that closely flanked the green gene at 0.6 cM and 1.3 cM.

Amplified fragment length polymorphisms provide a useful tool for mapping introgressed *F. pratensis/L. multiflorum* introgressions. However, AFLP analysis is a time-consuming process and routine marker-assisted selection in breeding programmes requires the development of simpler PCR-based assays. In the present study this was achieved by developing PCR-based markers directly, or indirectly, from closely linked AFLP bands. These PCR-based markers mapped, as expected, to the same position as the AFLP band from which they were derived. This study demonstrates that AFLP markers, derived from introgressed *F. pratensis* genes, can be converted into robust and simple to use PCR-based markers.

The work described above makes use of *F. pratensis*-specific AFLP polymorphisms to map an alien chromosome segment. An alternative strategy would be to derive markers from the sequenced rice genome since we have already demonstrated that rice RFLP markers can be mapped to *F. pratensis* chromosome segments (I. P. Armstead *et al.*, unpublished). Once the region of the rice genome that shows synteny with a *F. pratensis* chromosome segment has been identified, additional markers for the introgressed segment can be developed. This can be achieved by comparing a predicted coding sequence from rice with EST databases from other monocots. Primers can then be developed from regions that show very high conservation. Ninety per cent of such primers have been shown to generate an equivalent sequence in *Lolium* and *Festuca* and a high proportion show polymorphism between the two species (I. P. Armstead *et al.*, unpublished). The advantage of this strategy would be that it provides large numbers of markers for a specific region of the *F. pratensis* genome that is of interest, as well as possible information on gene function in the model monocot plant species. The potential of isolating *F. pratensis* genes via introgression mapping (i.e. the use of large numbers of rice markers and high resolution *Lolium*/*F. pratensis* mapping populations) is presently being investigated.

Sequence-tagged-site (STS) primer sequences linked to the stay-green mutation will be provided for research purposes upon request.

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