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Introgression mapping in the grasses

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Abstract

The unique properties of *Lolium*/*Festuca* hybrids and their derivatives provide an ideal system for intergeneric introgression. At IGER a focus on the *Lolium perenne*/*Festuca pratensis* system is being exploited to elucidate genome organization in the grasses, determination of the genetic control of target traits and the isolation of markers for marker-assisted selection in breeding programmes.

Introduction

Lolium perenne (*Lp*) and *Festuca pratensis* (*Fp*) hybrids and their derivatives exhibit a unique combination of characteristics, not seen in other plant genera, which makes the *Lp*/*Fp* system an ideal model for intergeneric introgression.

A high frequency of recombination between *Lp* and *Fp* chromosomes facilitates the transfer of *Fp* chromosome segments, carrying target genes, into *Lp*. Moreover, recombination occurs along the entire length of *Lp*/*Fp* bivalents, permitting the transfer of any *Fp* gene into *Lp*. However, despite this high recombination frequency at meiosis, the chromosomes of the two species can easily be distinguished by genomic *in-situ* hybridization (GISH). GISH analysis allows the identification and characterization of *Lp*/*Fp* introgressions, i.e. confirmation of the introgression of *Fp* segments into *Lp* and an estimation of their physical size. In addition, a high frequency of marker polymorphism is observed between *Fp* and *Lp* which aids in the mapping of target *Fp* genes on introgressed *Fp* segments.

The system therefore facilitates the rapid identification of markers located on an introgressed *Fp* chromosome segment by the screening of a *Lp*/*Fp* introgression together with the parental and hybrid germplasm from which it was derived. Any polymorphic marker present in the *Fp* parent, the *Lp*/*Fp* hybrid and the introgression line itself, but not the *Lp* parents, must be located within the introgressed *Fp* chromosome segment.

The work described in this paper is enabling: (1) the elucidation of the organization of genes along the *Lp*/*Fp* chromosomes; (2) the determination of the relationship between gene distribution and recombination, allowing comparisons to be made between genome organization in a small genome species, i.e. rice, and medium and large genome monocots such as grass, wheat and others; (3) the determination of the genetic control of key traits; and (4) providing a resource for gene isolation via a chromosome 'introgression' landing strategy.

Development of *Lp*/*Fp* substitution lines

The forage grass *Lp* ($2n = 2x = 14$) can be readily hybridized with *Fp* ($2n = 2x = 14$) to form a 14-chromosome hybrid which exhibits full pairing at metaphase

I of meiosis (Lewis 1966, Jauhar 1975). These diploid hybrids show nearly complete sterility (Lewis 1966, Jauhar 1975). However, *LpLpFp* triploids, derived by hybridizing synthetic tetraploid *Lp* (female parent) with diploid *Fp* (pollen parent), show both male and female fertility. When these triploids are backcrossed as the pollen parent to diploid *Lp* they give rise to BC_1 progeny with a complement of 14 chromosomes (Figure 1). Although the majority of the genome of these individuals is derived from the *Lp* parent, over 74% of them carry one or more *Fp* chromosome segments (King *et al.* 1998). Recombination, albeit at different frequencies, has been observed to occur along the entire length of the chromosomes (I. King *et al.* 1998, 1999, J. King *et al.* 2002a). In addition to individuals carrying *Lp*/*Fp* recombinant chromosomes, diploid plants carrying 13 *Lp* chromosomes and 1 *Fp* chromosome, i.e. monosomic substitutions, have been isolated (Figure 1). It should be noted that reciprocal introgressions to introduce *Lp* into *Fp* have been generated. The reciprocal introgressions are of interest with the particular aim of improving the forage quality of *Fp*. However, this review will concentrate on the former system.

A total of 550 BC_1 individuals were screened using restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs) (SSRs were derived from IGER's *Lp* genomic SSR library [manuscript in preparation] and from Vialactia's GeneThresher library [SSRs made available courtesy of Vialactia, NZ]). All seven monosomic substitution lines have now been isolated (manuscript in preparation). The SSR analysis revealed that at least at the macro-level synteny has been maintained between *Lp* and *Fp*.

Genetic and introgression mapping of *Fp* chromosome 3

A single *Lp*/*Fp* monosomic substitution carrying a *Fp* chromosome homoeologous to the Triticeae group 3 chromosomes and rice chromosome 1 (King *et al.* 2002a) was selected for further research.

Development of a genetic map of Fp chromosome 3

The *Lp*/*Fp* group 3 monosomic substitution, which carries a nucleolar organizer region (NOR), was backcrossed as the pollen parent to diploid *Lp* to produce a BC_2 mapping population (Figure 1). This population was analysed with AFLP using two enzyme combinations, *Eco*R1/*Tru*91 and *Hin*dIII/*Tru*91. The initial AFLPs analysis located markers specific to the *Fp* chromosome in the substitution (King *et al.* 2002a). DNA from the four plants involved in the production of the substitution, as well as the monosomic substitution itself, was screened. Markers found to be present in *Fp*, the triploid hybrid and the substitution, but absent from *Lp* (diploid and tetraploid genotypes) were classified as being specific to the *Fp* group 3 chromosome. Thirty-five primer pairs generating 104 markers

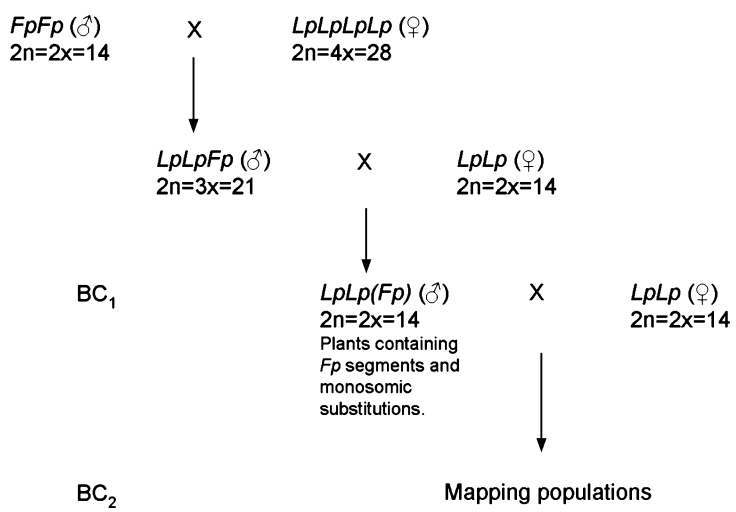

Figure 1. Crossing scheme for producing *Lolium*/*Festuca* introgressions and monosomic substitutions.

specific to *Fp* chromosome 3 were used to analyse 148 randomly selected plants. To keep scoring errors to a minimum all AFLP primer pairs/genotypes giving rise to singletons (genetic marker scores with an apparent recombination event on either side) and markers with low probability segregation patterns [as identified by the mapping programme JOINMAP 2.0 (Stam 1993)] were re-run and re-analysed at least once. This gave rise to a final genetic map of 81 cM.

Development of an introgression map of Fp chromosome 3

The genetic linkage map of the *Fp* chromosome was used to select 16 plants from the BC_2 mapping population for physical mapping (King *et al*. 2002b). These plants were chosen because they showed a relatively even spread of recombination points along the chromosome and, where possible, recombination points on either side of the centromere and NOR. Chromosome preparations were made from mitotic root tips and GISH was used to visualize the relative position and extent of *Fp* introgression into the *Lp* chromosome.

Fifteen of the 16 BC_2 plants used for the physical mapping involved single crossovers and a single BC_2 had resulted from two crossovers. All the *Fp* segments observed extended from one or other of the telomeres or, in the case of the double-crossover individual, from both telomeres. Thus two series of *Fp* segments were looked at using GISH: the first series increased in size from the telomere of the chromosome arm without the NOR, whilst the second increased in size from the telomere of the chromosome arm carrying the NOR. This resulted in the *Fp* chromosome being divided into 18 segments/bins (Figure 2). The physical sites of recombination appeared to occur along the whole length of the chromosome including regions close to the centromere and within the NOR (Figure 2).

Comparing the genetic and introgression maps of Festuca chromosome 3

By combining the genetic and physical maps it has been possible to assign each of the AFLPs used for genetic mapping of chromosome 3 to one of the 18 physical bins, allowing comparisons to be made between genetic and physical distance (King *et al*. 2002b).

Two gaps between crossover positions of greater than 10% of the chromosome length were observed on the physical map of the *Fp* chromosome. The distribution of recombination sites along the whole

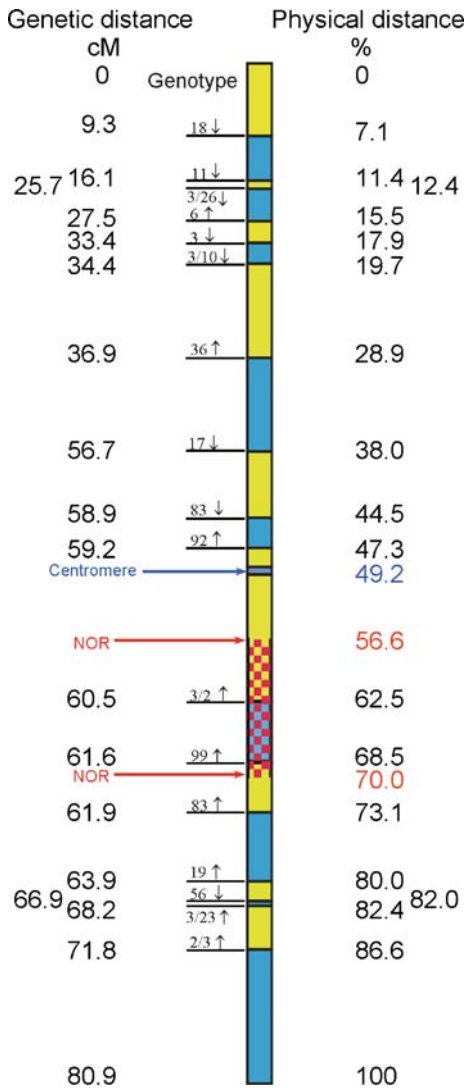

Figure 2. Physical map of *Lolium*/*Festuca* chromosome 3. Genetic distances for each segment are shown on the left, physical distances on the right. Horizontal black lines indicate sites of recombination between *Lolium* and *Festuca* (numbers over the lines show the genotype from which the segment was obtained). The physical positions of the centromere (blue arrow) and NOR (red arrows) are also shown on the left. Alternating blue and yellow colours are used only to aid discrimination between different recombinant lines carrying different-sized segments. For example, BC_2 18 carries a segment 9.3 cM in genetic length and 7.1% physical length while genotype BC_2 11 carries a segment 16.1 cM in genetic length representing 11.4% of the physical length.

length of the chromosome, however, shows that the present physical map has the potential to be broken down into much smaller sections by screening large populations for recombination between two markers that flank a specific region of the genome.

A comparison of the physical and genetic maps clearly shows how their inter-relationship varies from one part of the chromosome to another. The two largest gaps between crossover positions on the physical map do not coincide with gaps between markers on the genetic map; in fact the density of AFLP markers on the genetic map is such that the largest distance between markers is only 5.9 cM and there are only two other gaps of between 4 and 5 cM present. Of the two crossover position gaps on the physical map, the first (a gap of 15.2% of the chromosome length) contains 11 AFLP markers spread over a distance of just 1.3 cM, while the second (a gap of 13.4% of the chromosome length) contains eight AFLP markers spread over a distance of 9.1 cM.

Recombination frequencies were found to vary within, as well as between, arms. The highest frequency of recombination occurred at a physical distance of between 12% and 20% from the telomeres. The lowest frequency was found between 45% and 75% of the distance along the chromosome (the region of the chromosome containing both the centromere and the NOR).

Our results show that the centromere was physically mapped at 49.2% of the distance along the chromosome. The frequency of recombination started to increase at a distance of only about 5% from the centromere in the arm without the NOR. In contrast, it remained extremely low in the NOR arm for the whole of the region between the centromere and the NOR and including the NOR itself, but rose sharply after the end of the NOR. However, the peak in recombination frequency in the NOR arm was considerably smaller than the major peak in recombination frequency in the non-NOR arm. This result strongly suggests that the NOR, as well as the centromere, is associated with a reduction in the frequency of recombination. Similar evidence for little or no crossing over between the centromere and NOR has been reported for chromosomes 1B and 6B of wheat (Payne *et al*. 1984, Dvořák & Chen 1984, Snape *et al*. 1985), barley chromosomes 6 and 7 (Linde-Laursen 1979) and rye chromosome 1R (Lawrence & Appels 1986).

The distribution of recombination sites along the whole length of the chromosome includes sites very close to the centromere and within the NOR, although not between the two. Thus, although the region between the centromere and the NOR is associated with a reduction in the frequency of recombination (see above), recombination does take place within this general region.

The results obtained from this work are in agreement with data obtained from a range of other species where it has been shown that there is not a consistent relationship between genetic distance in cM and physical distance in base pairs, and that there is variation in this relationship from one part of the genome to another, e.g. Chen & Gustafson (1995), Gill *et al*. (1996a,b), Künzel *et al*. (2000), Erayman *et al*. (2004). Genetically close markers may be far apart in terms of base pairs (or vice-versa) due to differences in the frequency of recombination along the length of a chromosome. When considering the average length of DNA per unit of recombination, different segments of a chromosome should therefore be considered independently. In rice 1 cM is on average equal to 240 kb, although this figure actually varies from 120 to 1000 kb per cM (Kurata *et al*. 1994). In wheat the variation is even more extreme, with 1 cM equal to 118 kb in regions of high recombination but 22000 kb in regions of low recombination (Gill *et al*. 1996a,b). Regions corresponding to centromeres, and even some telomeres in tomato and potato, show a 10-fold decrease in recombination compared to other regions in the genome (Tanksley *et al*. 1992). Reduced recombination frequency in pericentric regions is also seen in many species including the grasses, e.g. wheat (Snape *et al*. 1985, Curtis & Lukaszewski 1991, Gill *et al*. 1996a,b, Erayman *et al*. 2004), barley (Leitch & Heslop-Harrison 1993, Pedersen *et al*. 1995, Künzel *et al*. 2000), rye (Wang *et al*. 1992), *Lolium* (Hayward *et al*. 1998, Bert *et al*. 1999). NORs may also cause a reduction in the frequency of crossing over, e.g. *Allium schoenophrasum* (J.S. Parker, personal communication). Recombination hotspots also occur (Künzel *et al*. 2000, Erayman *et al*. 2004).

Thus in the *Lp*/*Fp* work described here genes located on the *Fp* chromosome arm without the NOR will appear genetically much further apart than genes located on the chromosome arm carrying the NOR. However, the genetic distance between the genes on the

two chromosome arms will have very little relevance with regard to the physical distance between genes.

Development of an introgression map of the *Lp/Fp* genomes

Fp chromosome 3 has been used as a prototype for the development of an introgression map of the *Lp*/*Fp* genomes. Each of the six remaining *Lp*/*Fp* monosomic substitutions has been backcrossed to diploid *Lp* to generate BC_2 mapping populations. As with the monosomic substitution carrying *Fp* chromosome 3, the *Lp* and *Fp* homoeologous bivalents in the other substitution lines undergo high frequencies of recombination along the length of the chromosomes during meiosis, resulting in the generation of *Lp*/*Fp* recombinant chromosomes. The mapping populations derived from each of the remaining six substitutions are presently being screened with 500 SSR markers (IGER/Vialactia) which will enable the genetic mapping of the substituted *Fp* chromosomes. As for *Fp* chromosome 3, these genetic marker profiles are being used to identify individuals which carry *Lp*/*Fp* recombinant chromosomes with different-sized and overlapping *Fp* chromosome segments. The physical size and position of the overlapping *Fp* segments in each of the recombinant chromosomes will then be measured using GISH. These data will be assembled enabling each of the six remaining *Festuca* chromosomes to be divided into introgression bins; each will initially be divided into at least 20 bins composed of no more than 5% of the total chromosome length. However, the resolution of the system is such that it will be possible to divide the chromosomes into much smaller bins i.e. ≤0.4% of the total chromosome length.

Exploitation of the *Lolium/Festuca* introgression maps

A programme at IGER has recently been initiated to bin map single-nucleotide polymorphisms (SNPs) developed from every fifth BAC in the physical map of the rice genome. The bin mapping of rice BACs requires that a putative coding region is identified on each BAC to be mapped. This sequence is then used to screen other monocot databases. Primers are designed from conserved regions and these are used to PCR amplify the equivalent sequence in *Lp* and *Fp*. The products are sequenced and SNP markers that discriminate between *Lp* and *Fp* are designed. Once identified, SNPs can be mapped to a specific bin on a specific chromosome (a similar strategy will be applied to map the *Lp*/*Fp* orthologues of gene-based markers from other monocots such as barley). This strategy is proving successful for *Fp* chromosome 3. At the time of writing, sequences from 69 BACs from the rice 1 pseudomolecule (http://www.TIGR.org) have been mapped to *Fp* chromosome 3 (manuscript in preparation).

Bin mapping sequences from anchored rice BACs will enable the determination of the organization of genes in large genome plant species, i.e. *Lp* and *Fp*, and allow comparisons to be made with the small genome model species, rice. The work will allow the determination of the distribution of genes along the *Fp* chromosomes, i.e. are genes evenly distributed along the chromosome or present in clusters? If clustered, how are the clusters themselves organized: are they evenly distributed or are the clusters clustered? In addition, the work will allow the elucidation of the frequency and distribution of recombination relative to gene density, i.e. do peaks in recombination frequency coincide with gene-rich areas? Are some genes or clusters of genes located in areas of low recombination and, if so, has this led to the development of co-adapted gene complexes which confer a selective advantage? Knowledge of the relationship between recombination and the physical location of target genes is of importance since the success of both conventional breeding programmes, and gene isolation via forward genetics, is heavily dependent on the frequency of recombination in the region in which a target gene is located. Our initial results indicate that the majority of the markers derived from BACs from rice chromosome 1 are located in areas of low recombination.

Introgression mapping will also facilitate the exploitation of information and technology developed in the model plants. The rice genome project has provided the order and sequence of the genes on each of its 12 chromosomes. These data will enable the isolation of genes, via chromosome landing and walking strategies, from large genome monocot species. For example EST-based markers that flank a target gene in a large genome crop species can be used to identify the equivalent region, in terms of synteny, in the rice genome. Since the rice genome

has been sequenced every gene between the flanking markers in this species is known. These rice gene sequences can then be used to develop additional markers to isolate the target gene, via fine mapping and chromosome landing approaches, in the crop species itself. However, for this approach to be successful, the gene order in rice and other monocot crop species must be the same. The detailed bin mapping of physically mapped rice sequences in *Lp*/*Fp* will establish the extent to which this is true by providing an in-depth genome-wide assessment of the syntenic relationship between rice and *Lp*/*Fp*. In addition, the maintenance of synteny between rice and the grasses is likely to indicate a similar relationship between other monocots and rice. Thus the *Lp*/*Fp* introgression mapping described will benefit the whole monocot research community. In addition, strategies are presently being developed whereby introgression maps, in combination with other technologies, will provide a platform to directly, physically contig BAC from the *Lp*/*Fp* genomes.

The monosomic substitutions and the individuals making up the physical maps of each *Fp* chromosome will also provide a valuable resource for determining the genetic control of target traits and gene isolation. The seven monosomic substitutions will be screened for a specific trait. Once a *Fp* chromosome has been identified as carrying a gene(s) controlling the trait the relevant genotypes making up the physical map will also be screened. In this way it will be possible to physically map genes that control key traits. Furthermore, introgression mapping will have identified syntenic regions in rice, thus facilitating the isolation of gene(s) responsible for the control of a trait through map-based cloning techniques.

A strategy for the isolation of introgressed *Fp* genes

The unique combination of genetic and cytogenetic characteristics exhibited by *Lp*/*Fp* and, similarly, *L. multiflorum(Lm)*/*Fp* hybrids and their derivatives is being exploited to isolate markers tightly linked to target genes and to provide a springboard for gene isolation.

A *Fp* chromosome segment which carries a mutation of a gene normally required for leaf yellowing during senescence has been introgressed into *Lm* (Thomas 1987, Thomas *et al.* 1997) (The *Fp* segment has also been introgressed into *Lp* but the original introgression was used for the work described here). This stay-green character results from a recessive mutation in the gene, hence only plants homozygous for the mutation express the phenotype. Leaves 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). It was not initially known whether the stay-green phenotype resulted from a mutation in the gene encoding the PaO enzyme or from an alteration to a regulator gene that controls the expression or activation of the gene/protein (Roca *et al.* 2004).

A mapping family was generated from the *Lm*/*Fp* introgression line carrying the stay-green mutation and AFLPs were used to produce a genetic map of the *Fp* chromosome segment (Moore *et al.* 2005). AFLP analysis was performed, using the restriction enzyme pairs *Hin*dIII/*Tru*91 and *Eco*R1/*Tru*91. Polymorphisms specific to the *Fp* segment were identified by screening the parents and the selected BC_1 genotype carrying a single small *Fp* chromosome segment, i.e. primer pairs which gave a *Fp*-specific polymorphism in the *Fp* diploid parent, the *Lm*/*Lm*/*Fp* triploid and the selected BC_1 individuals.

Twenty-two selected AFLP primer pairs, giving 28 *Fp* specific polymorphisms (Figure 3A), were used to screen the mapping population. The segregation of the *Fp*-specific polymorphisms in the mapping population was analysed using JOINMAP™ 2.0 (Stam 1993) to generate a genetic map of the *Fp* chromosome segment (Figure 3A and B). Each individual from the BC_2 mapping population was also test-crossed to a *Lm* genotype homozygous for the recessive stay-green allele (*yy*) to determine the presence or absence of the stay-green allele in each of the individuals of the mapping population. The data for the presence or absence of the stay-green allele were combined with the AFLP data in order that the senescence mutation could be mapped within the introgressed *Fp* chromosome segment. The final genetic distance of the *Fp* chromosome segment containing the stay-green mutation was estimated to

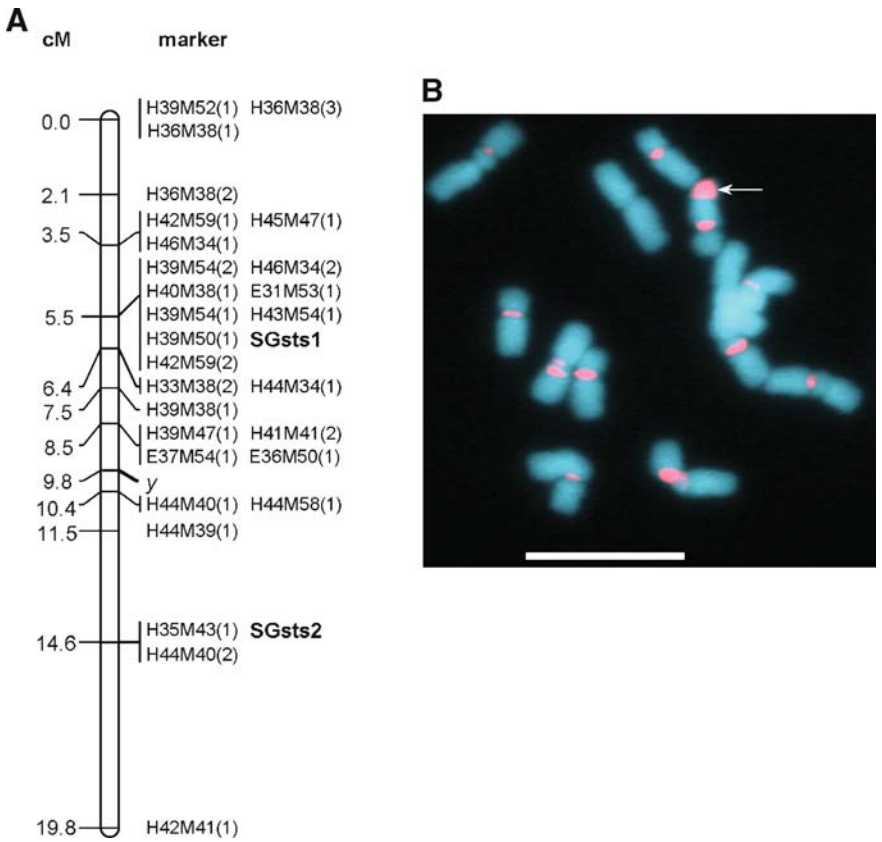

Figure 3. **A**: Linkage map of introgressed *Fp* segment (from *Fp* chromosome 5) containing the stay-green gene (*y*). The positions of STS markers derived from AFLP/BAC sequencing are indicated in bold type; the positions of AFLP markers are indicated in normal type. **B**: GISH image of BC_1 plant showing the *Fp* introgressed segment carrying the stay-green gene (*y*). The *Lm* chromosomes are shown in blue. The *Fp* introgressed segment (indicated by arrow) is shown in red (the remaining red signals highlight the position of the *Lm* centromeric regions). Bar = 10 μm. Modified from Moore *et al*. 2005 and reproduced by permission of *New Phytologist*.

be 19.8 cM with the stay-green *y* mutation located at 9.8 cM; the closest flanking markers to *y* were at 0.6 cM and 1.3 cM.

Twelve AFLP bands were excised, cloned and sequenced. Primers designed from one of these 12 AFLP (SGsts1) produced a *Fp*-specific fragment of 390 bp which distinguished between the *Lm* and *Fp* genotypes. The amplification products derived from the remaining 11 AFLP bands did not distinguish between the parents based upon either size or DNA sequence. Therefore, a *Fp* BAC library (Donnison *et al*. 2005) 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 amplified a sequence present on numerous BACs, indicating multiple locations in the genome. The other primer pair, which generated a *Fp* AFLP fragment of 300 bp, identified only two BACs; given the 2.5 × coverage of the BAC library, this fragment was considered to be likely to be present as a single copy sequence. Partial sequencing of this BAC allowed for the development of a further primer pair targeted to low-copy sequence which, when tested on *Lm* and *Fp* parental DNA, was shown to be polymorphic (SGsts2). Primer pairs for SGsts1 and 2 were then mapped and, in both cases, were found to locate precisely to the same position as the original AFLP markers from which they had been derived, on either side of the stay-green locus (Figure 3A).

The work described above makes use of *Fp*-specific AFLP polymorphisms to map an alien chromosome segment. An alternative strategy is to identify the region of the rice genome that shows synteny with a

particular *Fp* chromosome segment and to derive markers directly from the sequenced rice genome. 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 DNA sequence conservation (usually within exons) and which, preferably, span introns (to maximize the chance of identifying polymorphisms). 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 (manuscript in preparation). The advantage of this strategy is that it provides large numbers of markers for a specific region of the *Fp* genome that is of interest, as well as possible information on gene function in the model monocot plant species. The potential of isolating *Fp* genes through the use of rice-derived markers and a high-resolution *Lm*/*Fp* mapping population is presently being employed in the further analysis of the stay-green mutation. A large mapping population of about 1600 individuals has been screened with markers, derived from rice, that closely flank the green gene. This procedure, termed *introgression landing*, has identified a region of the rice genome that carries 30 predicted genes, one of which has now been identified as the candidate gene responsible for the stay-green phenotype (Armstead *et al*. 2006a,b). In addition to this, markers that discriminate between *Lp*, *Lm* and *Fp* and related species have been developed by similar strategies for use in IGER breeding programmes (Armstead *et al*. 2005, Humphreys *et al*. 2005).

Our initial research indicates that introgression landing, in combination with robust screening procedures for target traits, provides a fast and efficient method of isolating closely linked markers to target genes and provides a platform for gene isolation.

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