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Construction of a *Festuca pratensis* BAC library for map-based cloning in *Festulolium* substitution lines

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Abstract Introgression in *Festulolium* is a potentially powerful tool to isolate genes for a large number of traits which differ between *Festuca pratensis* Huds. and *Lolium perenne* L. Not only are hybrids between the two species fertile, but the two genomes can be distinguished by genomic in situ hybridisation and a high frequency of recombination occurs between homoeologous chromosomes and chromosome segments. By a programme of introgression and a series of backcrosses, *L. perenne* lines have been produced which contain small *F. pratensis* substitutions. This material is a rich source of polymorphic markers targeted towards any trait carried on the *F. pratensis* substitution not observed in the *L. perenne* background. We describe here the construction of an *F. pratensis* BAC library, which establishes the basis of a map-based cloning strategy in *L. perenne*. The library contains 49,152 clones, with an average insert size of 112 kbp, providing coverage of 2.5 haploid genome equivalents. We have screened the library for eight amplified fragment length polymorphism (AFLP) derived markers known to be linked to an *F. pratensis* gene introgressed into *L. perenne* and conferring a staygreen phenotype as a consequence of a mutation in primary chlorophyll catabolism. While for four of the markers it was possible to identify bacterial artificial chromosome (BAC) clones, the other four AFLPs were too repetitive to enable reliable identification of locus-specific BACs. Moreover, when the four BACs were partially sequenced, no obvious coding regions could be identified. This contrasted to BACs identified using cDNA sequences, when multiple genes were identified on the same BAC.

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Introduction

This paper describes the construction of a large-insert genomic library of the forage grass *Festuca pratensis*. A number of hybrids of *Festuca* and *Lolium* species (*Festulolium* complex) exhibit a range of features which, as far as we are aware, are unique compared to other crop species: not only are these hybrids fertile, but the two parent genomes can be distinguished by genomic in situ hybridisation (GISH), and recombination between homoeologous chromosomes and chromosome segments occurs at high frequency (King et al. 1998a, 1998b, 2002a, 2002b; Armstead et al. 2001) A number of the species included in this complex, including *Lolium perenne* (perennial ryegrass), are economically important and, therefore, these hybrids provide a means of introgressing valuable traits into plant breeding material. For example *F. pratensis* is regarded as a major source of abiotic (Humphreys et al. 2004) and biotic stress tolerance, which can be used to improve *L. perenne*. More or less complex traits of fundamental biological importance, such as domestication, perenniality and ecological resilience, are also particularly amenable to genetic dissection by introgression analysis in *Festulolium*. By a programme of introgression and a series of backcrosses, a number of *L. perenne* lines have been produced that

contain small *F. pratensis* substitutions carrying a beneficial and scorable trait. Because these hybrids are the result of a wide cross and due to the fact that grasses are obligate outbreeders (therefore highly polymorphic), this material provides an ideal source from which to derive DNA-based polymorphic markers targeted towards the trait carried on the *F. pratensis* substitution. Not only are these markers valuable for marker-assisted breeding, thereby reducing the need for phenotypic screening in successive generations, but they also provide a tool in conjunction with a large-insert genomic DNA library for the map-based cloning of important agro-ecological genes such as those responsible for stress tolerance or interaction with herbivores and for non-food use. Bacterial artificial chromosomes (BACs) are the large-insert library tool of choice because compared to the main alternative, yeast artificial chromosomes (YACs), they are easy to handle and the clones are stable and less likely to be chimeric (Shizuya et al. 1992; Woo et al. 1994; Yu et al.2000). In addition to providing a route for map-based cloning, a BAC library is also useful for physical mapping, genomic structural analysis and comparisons of specific regions between species (Yu et al. 2000).

We report here on the construction of a BAC library from *F. pratensis* and the screening of this library using primers designed from amplified fragment length polymorphisms (AFLPs) linked to a trait (staygreen) transferred from *F. pratensis* to *L. perenne* (Thomas et al. 1997). The staygreen trait represents a mutation in primary chlorophyll catabolism (Vicentini et al. 1995) that has been introgressed into several *Lolium* species (Thomas et al. 1997; Roca et al. 2004). The identification of BACs which contain molecular markers derived from *Festuca* segments has established the principle of introgression-based cloning of genes specifying traits of practical and fundamental significance, some of which have already been transferred from *Festuca* into *Lolium*.

Materials and methods

Isolation of high-molecular-weight genomic DNA

High-molecular-weight (HMW) DNA was isolated from diploid *Festuca pratensis* [$2n = 2x = 14$; accession Bf 1183(1)], the same genotype being used to generate a chromosome substitution series in *Lolium perenne*). A total of 20 g of leaf material from replica plants of the same genotype was harvested from field-grown plants during the spring and frozen at −80°C. The leaf material was ground in liquid nitrogen and nuclei isolated according to the method of Zhang et al. (1995). The nuclei were embedded in agarose plugs and prior to digestion were subjected to a pre-electrophoresis step using a CHEF-DR II pulsed field gel electrophoresis (PFGE) apparatus (BioRad, Hercules, Calif.) as described by O'Sullivan et al. (2001).

Partial digestion and size selection of digested DNA

The *F. pratensis* HMW DNA was partially digested using *Hin*dIII and then separated in a single step by PFGE at 170 V for 16 h with a linear pulse ramp from 0.5 s to 40 s. Following staining of the flanking regions of the gel containing the HMW DNA ladder, two to three gel slices in the range of 100–150 kb were typically excised and the DNA recovered by dialysis as described by O'Sullivan et al. (2001).

Ligation and transformation

The partially digested DNA was ligated with *Hin*dIII-digested pBeloBAC11 vector using a vector/insert ratio of 10:1. Ligation and DNA transformation were carried out as described by O'Sullivan et al. (2001). BAC clones were ''picked'' into 96-well microtitre plates, incubated at 37°C overnight, glycerol added and stored at −80°C. A total of 139 BAC clones, selected to cover a range of size selections used to construct the library, were cultured overnight and the DNA then isolated and restricted using *Not*I. BAC insert sizes were estimated from these digests. In addition 25 of these BAC DNA digests were Southern-blotted and hybridised to a radiolabelled probe of *F. pratensis* genomic DNA according to standard procedures (Sambrook et al. 1989).

Setting up of DNA pools for PCR-based screening

DNA pools of microtitre plate-grown BAC clones were generated to enable a PCR-based screen of the library. Enough clones were generated to fill 512 microtitre plates, which would enable the use of a three-dimensional 8×8×8 microtitre plate grid as the basis for the screen. The entire library was replicated in microtitre plates with 200 µl of medium per well. Following an overnight incubation, all cultures from a single plate were pooled and these 'plate' pools arranged into superpools in three dimensions (X, Y, Z), with 6 ml of culture from each of eight plates (= 768 clones in 48 ml of LB) in each of 192 superpools. Thus, each plate was represented in three superpools so that, once DNA had been extracted, a PCR screen of 192 DNA pools should generate three ''hits'' per positive colony—two to identify the specific plate and the third as confirmation. Once the superpools had been created, the bacteria were pelleted and BAC DNA isolated.

Screening using staygreen-associated markers

The BAC library was screened using primers designed from eight AFLP markers known to map closely to a

staygreen trait introgressed from *F. pratensis* [accession Bf 993(17)] to *L. perenne* (Moore et al. unpublished data). After identification of the superpools yielding PCR fragments of the predicted size, we checked a matrix to confirm which plates made up each pool and, therefore, which plate(s) contained the AFLP fragment. Two methods were used to identify the individual BAC clone from the 96 clones in the plate: (1) the 96 clones were gridded onto a nylon membrane and following overnight growth and DNA transfer, they were hybridised; (2) a microtitre plate was inoculated and grown overnight, and the DNA pools generated from the eight rows and twelve columns were screened by PCR as before. Once identified, selected PCR-positive BACs were digested with *Bam*HI, the fragments "shot-gun" cloned into pBluescriptII-KS and the selected fragments partially sequenced. Longer fragments were sequenced by primer-walking. DNA sequences were compared to public DNA and protein databases using the FASTA and BLASTX programmes, respectively, of the GCG package (Genetics Computer Group, University of Wisconsin, Madison).

Arraying of BACs and hybridisation to chloroplast DNA

BAC clones were macro-arrayed onto Hybond NX membrane (Amersham, UK) using a Qiagen Bio-Robot 3000 (Qiagen, Valencia, Calif.) custom-programmed to array using a 96-pin replicating tool (VP Scientific, San Diego, Calif.). The robot arrayed onto a membrane supported on solidified medium. A range of spotting densities was assessed, and although higher densities were theoretically achievable, the one ultimately used was a 6×6 array. In addition, each clone was arrayed in duplicate, so that 2×1,728 BAC clones were arrayed onto each 80 mm×120-mm filter. After arraying, the BAC clones were incubated overnight and the colony DNA transfer performed according to standard procedures (Sambrook et al. 1989). To test for chloroplast contamination of the BAC library, we designed primers for three chloroplast genes (*ndhA3*, *psbA2* and *rbcL*) using wheat and barley sequences available in the public nucleotide databases. Partial gene sequences were PCR amplified from *F. pratensis* and yielded fragments of 1.5 kbp, 800 bp and 1.2 kbp, respectively. The gene fragments were pooled, radiolabelled and hybridised with three membranes using standard methods (Sambrook et al. 1989).

Results

BAC library construction and quality control

The BAC library was constructed on the basis of results from six size-selection experiments. A total of 49,152 BACs were "picked" into 512 96-well plates, with an estimated coverage of 5,491 Mbp; this equates to an average insert size of 111.7 kb per BAC clone. In general, larger inserts were observed for BACs derived from the higher molecular-weight gel slices (Fig. 1), but the number of colonies derived from higher molecular-weight gel slices was relatively small compared to the number derived from lower molecular-weight ones. However, in other experiments the reverse was observed, so that the average insert size was smaller in the higher molecular-weight gel slice than in the lower molecular-weight gel slice. Most of the BACs were therefore generated from gel slices that covered the region 100–140 kb.

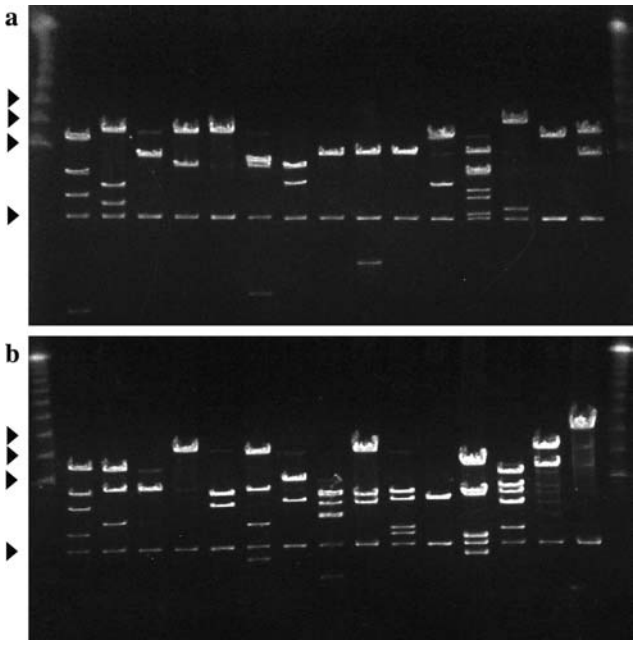

Fig. 1 DNA from selected *Festuca pratensis* bacteria artificial chromosome (BAC) clones was restricted using *Not*I and the fragments separated by pulse field gel electrophoresis (PFGE). BACs were generated by restricting *F. pratensis* genomic DNA with *Hin*dIII, followed by PFGE and cloning of the size-separated genomic DNA: **a** genomic DNA fragments of 100–130 kb, **b** genomic DNA fragments of 130–160 kb. The average insert sizes of the BAC inserts in this figure were estimated to be 109 kb (**a**) and 134 kb (**b**). The *arrowheads* to the *left* indicate (**a** and **b**, *from top to bottom*) the 145.5-, 97.0- and 48.5-kb bands of the lambda ladder (NEB, Beverly, Mass.) and the position of the 7.0-kb pBeloBAC11 *Not*I vector fragment (*lowermost arrowhead*)

The 139 BAC clones selected to cover the range of sizes used to construct the library were cultured overnight and their DNA isolated and restricted using *Not*I. All of these clones contained insert DNA in addition to the vector band. When 25 of these BAC DNA digests were Southern-blotted and hybridised to *F. pratensis* genomic DNA, at least one *Not*I fragment per clone cross-hybridised to the probe (Fig. 2). Moreover, of the 53 *F. pratensis* genomic fragments observed following *Not*I restriction of these 25 BACs, only three fragments, all 5–10 kb in size, failed to hybridise to the probe. Of

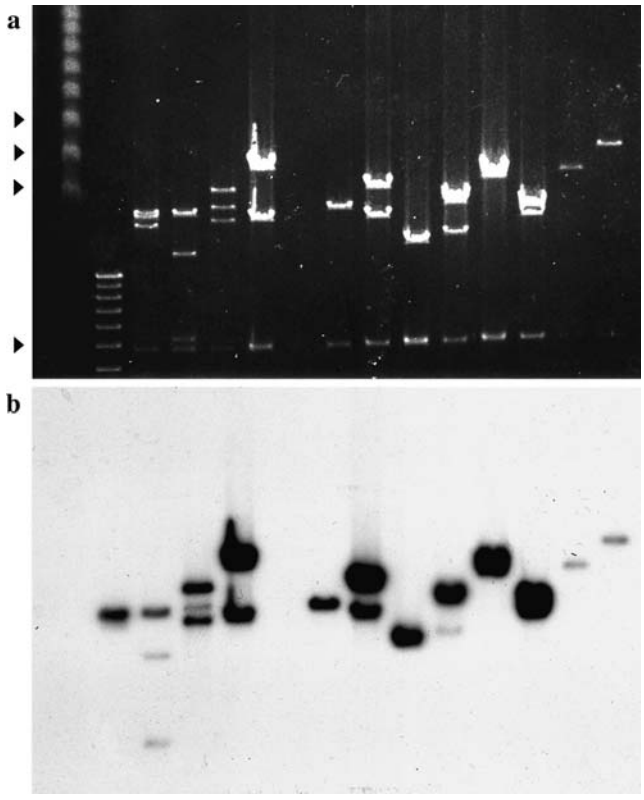

Fig. 2 DNA from selected *F. pratensis* BAC clones was restricted using *Not*I and, following PFGE (**a**), Southern-blotted and hybridised to [^{32}P]-radiolabelled *F. pratensis* genomic DNA (**b**). The *arrowheads* to the *left* indicate (**a**, *from top to bottom*) the 145.5-, 97.0- and 48.5-kb bands of the lambda ladder (NEB) and the position of the 7.0-kb pBeloBAC11 *Not*I vector fragment (*lowermost arrowhead*)

the 4,992 BAC clones macro-arrayed onto nylon the membrane and hybridised to the three chloroplast genome-derived genes, approximately 25 BACs hybridised strongly and a further 50 hybridised weakly-to-moderately.

PCR-based screen of BAC library with AFLP-derived primers

The 192 pools of BAC DNA were screened using primers designed from sequences of eight AFLP fragments that map close to a staygreen (SG) locus in *F. pratensis* (Table 1). Between one and seven clones were identified for six of these primer pairs, while two primer pairs appeared to amplify fragments distributed throughout the *F. pratensis* genome. Four BACs, identified using AFLP-derived primer sequences, were digested, and a number of the fragments were cloned and sequenced. In total, 200 sequencing reactions were run to yield approximately 74 kb of non-overlapping DNA sequence from the four BACs, but no unambiguous gene encoding regions, except reverse transcriptase, could be identified. A number of sequences were homologous, or highly homologous, to GAG-POL polyproteins of a range of long terminal repeats (LTRs) and non-LTR retro-elements, particularly those found in cereals. Of the fragments sequenced, 69% contained reverse-transcriptase or other retrotransposon-like sequence.

Discussion

Bennett et al. (1982) estimated the size of the *F. pratensis* haploid genome to be 2,181 Mbp , and with 49,152 BACs containing an estimated 5,491 Mbp of DNA we predict the library to contain the equivalent of 2.5 genomes. Screening with the AFLP fragments, which map close to a SG locus introgressed from *F. pratensis*, resulted in an average of 2.3 clones per screen (ignoring screens that gave six or more clones, which were presumably present in the genome at a frequency of more than one copy), a figure that validates the predicted library size. Screens for nine single-copy genes with primers designed from cDNA or sequence-tagged site (STS) sequences from the same library identified an average of 1.9 PCR-positive clones (Armstead et al. 2004 and unpublished data), again close to this estimate. With the average insert size being $I = 111.7$ kb, the number of clones (N) being 49,152 and the genome size (GS) being 2,181,000 kb, we deduce that the probability (P) for a specific single-copy marker to be represented in this library is $P = 1\text{-}e^{N[ln(1-I/GS)]} = 0.9193$ (Clarke and Carbon 1976).

All of the clones analysed contained inserts, and when hybridised to *F. pratensis* genomic DNA at least one fragment of each BAC clone tested cross-hybridised. That 94% of the BAC sub-fragments tested hybridised to a genomic DNA probe indicated that the majority of this DNA was repetitive. DNA sequencing from BACs

Table 1 Amplified fragment length polymorphism (AFLP) fragments used to screen the *F. pratensis* BAC library, their estimated distance in centiMorgans from the staygreen locus (SG), fragment size and number of BACs identified using a PCR-based screen

Code	AFLP Marker	Distance from SG	Approximate band size (bp)	Number of BACs identified
SGsts1	H44/M40/1	+0.6	390	7
SGsts2	H35/M43/1	+4.8	300	2
SGsts3	H43/M54/1	−4.4	200	3
SGsts4	E31/M53/1	−4.4	210	∞
SGsts5	E36/M50/1	−1.4	120	1
SGsts6	E37/M54/1	−1.4	330	∞
SGsts7	H39/M50/1	−4.4	150	6
SGsts8	H39/M47/1	−1.4	250	3

also confirmed this. Given the size of the *F. pratensis* genome at approximately 4.5-fold that of rice or 12-fold that of *Arabidopsis thaliana*, this ''repetitiveness'' is not too surprising as a smaller proportion of the genome will be expected to be taken up by gene sequences. In addition to our hybridising BAC clones to *F. pratensis* genomic DNA, we also hybridised BACs to chloroplast DNA and estimated that chloroplast contamination was in the region of 0.5–1.5%. The pre-electrophoresis step that we adopted appears to have been effective as very small fragments were not observed in the clones tested (Figs. 1 and 2). Chalhoub et al. (2004) describe a number of technical improvements to increase the quality and efficiency of cloning, and in future investigations it will probably be worthwhile to incorporate these into the protocol.

The PCR-based screening carried out on 192 superpools proved to be a rapid, efficient and reliable method of identifying BAC plates containing positive clones. From four of the eight AFLPs, it was possible to identify the corresponding BAC clones; the other four AFLPs tested were too repetitive to enable reliable identification of locus-specific BACs. When the four BACs identified were sequenced, no obvious coding regions could be identified. This contrasts to BACs identified using primers derived from cDNA sequences (Armstead et al. 2004; unpublished data), when multiple genes were identified on the same BAC. Thus, on the basis of our screening to date, it would appear that genomic DNA-derived AFLPs do not make very good markers for screening the BAC library. One interesting investigation to carry out the future would be to screen the library using AFLPs derived from methylation-sensitive restriction enzymes such as *Pst*I in order to determine if such BACs are more likely to contain gene sequences. However, despite the limitation of the *Eco*RI/*Mse*I AFLP fragments used in this study, we were still able to identify low-copy DNA sequences in the BAC fragments. Given that the internal sequence of most polymorphic AFLPs was not sufficiently polymorphic to distinguish between *L. perenne* and *F. pratensis* alleles, the BAC sequences provide highly useful additional sequence information to generate polymorphic PCR-based markers. Moreover, once markers sufficiently close to a trait of interest in a substitution line have been identified, it will be possible to use the *F. pratensis* BAC library as an important part of the toolkit to enable map-based cloning in this and related species. The *F. pratensis* BAC library will also provide a useful tool for grass comparative genomics to compliment the existing BAC libraries of cereal crops, including rice (Zhang et al. 1996), wheat (bread, Allouis et al. 2003; durum, Cenci et al. 2003), barley (Yu et al. 2000), Sorghum (Woo et al. 1994) and maize (flint, O'Sullivan et al. 2001; dent, Tomkins et al. 2002).

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