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# Construction of a BAC library of pearl millet, *Pennisetum glaucum*

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**Abstract** A bacterial artificial chromosome (BAC) library was constructed using nuclear DNA from pearl millet (*Pennisetum glaucum*), and used as a resource for the isolation of microsatellite sequences. The library contains a total of 159,100 clones with an average insert size of 90 kb, and corresponds to 5.8 haploid genome equivalents. The BAC library was pooled for screening by the polymerase chain reaction (PCR) as well as robotically gridded on high-density filters. PCR-based screening of a subset of the library (4.7 haploid genome equivalents) using five sequence-tagged site (STS) and six microsatellite markers identified between 2 and 11 positives superpools (5.4 on average). The frequency of BAC clones carrying inserts of chloroplast DNA was estimated to be less than 1% by hybridisation with a rice chloroplast probe.

**Keywords** Bacterial artificial chromosome (BAC) · Pearl millet · *Pennisetum*

## Introduction

Pearl millet, *Pennisetum glaucum* (L.) R. Br., is a monocotyledon belonging to the *Poaceae* family. It is the staple crop of some 90 million people mainly living in the semi-arid regions of India and Africa. Among the major cereals, pearl millet has the best adaptation to drought and heat. The crop suffers from a range of diseases of which downy mildew is the most important. Although heterogeneous landraces usually display reasonable levels of resistance, the introduction of genetically uniform pearl millet hybrids in India in the 1960s has resulted in devastating epidemics with yield losses of up to 30–40%. Not surprisingly, improved resistance to downy mildew, together with enhanced drought tolerance, is the main breeding target for pearl millet.

Pearl millet is a diploid, with a basic chromosome number of seven, and a haploid DNA content of 2.5 pg. Good genetic maps, encompassing the seven linkage groups are available (Liu et al. 1994; Devos et al. 1995), and their relationship to the maps of other grass species has been established (Devos et al. 2000). The genetic maps are being used to map traits of agronomic importance such as resistance to the fungus *Sclerospora graminicola*, causing downy mildew (Jones et al. 1995), and drought (C. Howarth, personal communication). A major downy mildew resistance gene, effective against Indian *Sclerospora* pathotypes and located on the top of linkage group 1, is currently being targeted for fine-mapping and subsequent gene isolation. Achievement of this latter objective requires the availability of a large-insert library.

Following the development of bacterial artificial chromosomes (BACs) by Shizuya et al. (1992), BAC vectors have been widely used for the construction of large-insert libraries from plant genomic DNA (Woo et al. 1994; Wang et al. 1996; Frijters et al. 1997; Yang et al. 1997; Danesh et al. 1998; Vinatzer et al. 1998; Nam et al. 1999; Tomkins et al. 1999). A positive selection vector, pBAC/SACB1, was designed by Bendahmane (1999). The vector is based on pBeloBAC11 and carries the *SacB* gene, which encodes the enzyme levansucrase from *Bacillus amyloliquefaciens* instead of *LacZ*. Expression of the gene is controlled by the IPTG inducible *Lac* operator, which is separated from the *SacB* gene by a unique *Bam*HI cloning site. The presence of an insert will uncouple the gene from the promotor, and only recombinant clones will survive in the presence of both sucrose and IPTG. In addition to the easy identification of the recombinant clones, the advantages of a zero-background vector are that complete dephosphorylation of the vector and the ratio of vector : insert DNA are less critical for successful ligations (Bendahmane 1999). We report the construction of a 5.8 haploid genome equiva-

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lent pearl millet library using the zero-background plasmid vector pBAC/SACB1.

## Materials and methods

### Plant material

*Pennisetum glaucum* var. Tift 23DB, an inbred line used as the standard pearl millet genotype (Anonymous 1991), was grown in a controlled environment room under 14 h light and with a day/night temperature of 28°C/25°C. Leaves of 3- to 5-week-old plants were harvested, cut into small pieces, frozen in liquid nitrogen and either processed immediately or stored at –80°C.

### Preparation of pearl millet insert DNA

Pearl millet high-molecular-weight DNA was prepared from nuclei essentially as described by Zhang et al. (1995). Nuclei from 40 g (fresh weight) of leaf tissue were embedded in approximately thirty 80-µl agarose plugs. The plugs were cut into small pieces (about 1 mm square), and 50-µl aliquots were partially digested with *Bam*HI. Seven microliters of *Bam*HI restriction buffer (NBL Gene Sciences), 7 µl of 4 m*M* spermidine (Sigma) and 0.7 µl of 0.1 mg/ml acetylated bovine serum albumin (BSA) (Sigma) were added, and the mixture was placed on ice for 30 min. One to two units of *Bam*HI restriction enzyme (NBL Gene Sciences) were added and allowed to diffuse within the agarose for a further 30 min on ice. The digestion was carried out at 37°C for 30 min and stopped by the addition of 0.7 µl of 0.5 *M* EDTA. Chopped plugs were immediately loaded onto a 1% agarose gel, and the wells were sealed. DNA fragments were fractionated by pulse field gel electrophoresis (PFGE) in 1× TAE buffer (11°C) in a 2015 Pulsaphor Plus (Pharmacia LBK) using a field strength of 6 V/cm and a reorientation angle of 120°. Three different pulse regimes were used. DNA separated using a ramped switch time of 1–40 s for 20 h was used in ligation 1. The application of a second size selection using a switch pulse time of 5 s for 20 h provided the DNA for ligation 2. The third ligation was carried out with DNA separated under a combined pulse regime of 90 s for 5 h, 2 s for 22 h and 5 s for 10 h. These latter conditions resulted in a well-defined separation of DNA fragments ≤50 kb and DNA fragments ≥100 kb. After size separation, fragments were cut from the gel, and the DNA was recovered by electro-elution (Strong et al. 1997).

### Preparation of vector DNA

DNA from the vector pBAC/SACB1 was isolated from 4 l of overnight culture as described (Bendahmane 1999) and digested with *Bam*HI. Vector ends were dephosphorylated with two units of shrimp alkaline phosphatase (USB, USA) per microgram of linearised vector for 90 min at 37°C, after which the phosphatase was heat-inactivated at 65°C for 30 min.

### Ligation and transformation

Twenty-five to fifty nanograms of size-selected pearl millet DNA was ligated to 50–80 ng of *Bam*HI-digested pBAC/SACB1 vector for 24 h at 16°C in a total volume of 50 µl and using 1–2 units of T4 DNA ligase (Boehringer Mannheim). The ligation products were dialysed against 0.1× TE on a 0.025-µm Millipore membrane using the ''drop dialysis'' method (Maruzyk and Sergeant 1980), and 0.5–3 µl was added to 20 µl of E. coli DH10B ElectroMAX ultra-competent cells (Gibco-BRL). Transformation was carried out by electroporation at 280 V (capacitance: 330 µF; resistance: 4000 Ω; impedance: low; charge rate: fast) in a pre-chilled 0.15-cm cuvette using a Gibco BRL cell porator apparatus. Recombinant clones were selected on LB (Luria-Bertani) medium containing 12.5 µg/ml chloramphenicol, 146 m*M* sucrose and 0.12 mg/ml isopropylthiogalactoside (IPTG), and stored at –80°C in 50 µl LB freezing media (Woo et al. 1994) in 384-well microtitre plates.

### Evaluation of the BAC library

To obtain an estimation of the average insert size, we extracted plasmid DNA from 188 randomly selected single colonies using the alkaline lysis method (Sambrook et al. 1989). The DNA was digested with *Not*I and separated by PFGE on a 1% agarose gel in 0.5× TBE (11°C, 6 V/cm, 5–15 s ramped switch times for 16 h).

For polymerase chain reaction (PCR) screening, DNA was extracted from single-plate pools. Ten single-plate pools formed a superpool. Five pearl millet sequence-tagged-site (STS) and six microsatellite (SSR) markers were used to evaluate the coverage of the pearl millet BAC library by determining the number of superpools that contained each marker. The STS markers PSMP461 and PSMP567 were chosen because they are linked to the downy mildew resistance gene at the top of linkage group 1. The other markers represented each of the other linkage groups. PSMP758 and PSMP2059, PSMP305, PSMP2001 and PSMP2064, and PSMP2018 map to the middle of linkage groups 2, 4, 5 and 6, respectively, while PSMP2056, PSMP2048 and PSMP834 are located distally on linkage groups 3, 6 and 7. Furthermore, high-density colony filters for hybridisation-based screening of the library were prepared using a BioGrid robot (Biorobotics, Cambridge, UK). Colony filters were processed and hybridised using standard techniques (Sambrook et al. 1989). The filters were hybridised to a further probe from the top of linkage group 1, PSM858. Screening for chloroplast (cp) DNA content was performed using a probe derived from the large subunit of rice ribulose 1,5-bisphosphate carboxylase (Nishizawa and Hirai 1987).

### Isolation of microsatellite sequences from BAC clones

The PCR-based isolation of microsatellite sequences, primer design and microsatellite analysis is described in Qi et al. (2001)

## Results and discussion

### Methodology

Compared to the method published for monocot BAC library construction by Zhang et al. (1995), the following modifications were carried out.

Firstly, embedding the pearl millet nuclei in agarose plugs rather than beads increased both the quality and concentration of the high-molecular-weight DNA. To facilitate diffusion of the restriction enzyme into the agarose, we sliced plugs prior to partial digestion. Mechanical shearing due to slicing was negligible.

Secondly, modified PFGE conditions resulted in a well-defined separation of large (≥100 kb) and small (≤50 kb) DNA fragments. The pulse regime that was employed initially, a ramped switch time from 1 to 40 s for 20 h, did not efficiently separate high- and low-molecular-weight fragments. Thirty percent of the recombinant clones contained inserts ≤60 kb following transformation with size-selected DNA in the range 100–300 kb (ligation 1; Fig. 1). Although smaller DNA fragments could be removed successfully by the application of a second size selection (switch pulse time of 5 s for 20 h) (ligation 2; Fig. 1), we aimed to keep the number of manipu-

lations to a minimum. Therefore, a combination pulse regime with switch times of 90 s for 5 h, 2 s for 22 h and 5 s for 10 h was introduced. This regime provided a good one-step separation of high- and low-molecular-weight DNA. The number of clones with insert sizes ≤60 kb dropped to 20.6% (ligation 3; Fig. 1).

Finally, the electroporation field strength was reduced from 400 V to 280 V, as this had been shown to increase the efficiency with which large DNA fragments could be introduced into *E. coli* (Frijters et al. 1997).

Using the combination pulse regime and 280 V electroporation strength, we obtained the highest transformation efficiency and average insert sizes when size-selected DNA in the range 150–250 kb was used for library construction.Transformation with DNA fragments in the 250 to 300-kb range and higher resulted in fewer transformants containing, on average, inserts that were smaller than those obtained with DNA in the 150 to 250-kb range.

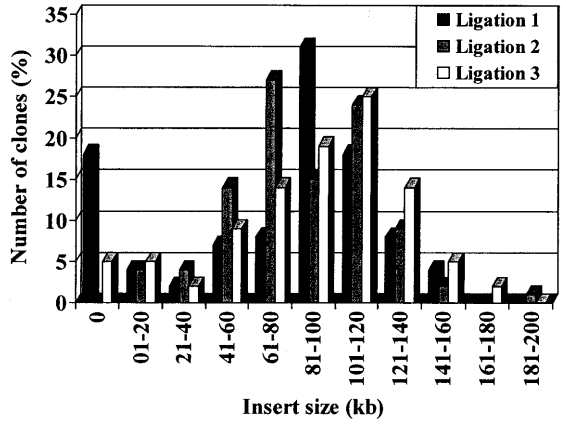


**Fig. 1** Insert size distribution in the library. Forty-nine, eighty-one and fifty-eight clones were randomly selected from three independent ligations and their insert sizes determined after *Not*I digestion and PFGE separation

## The BAC library

The pearl millet genomic library presented here is the result of three ligations, providing 18000, 92300 and 48800 clones with average insert sizes of 60 kb, 90 kb and 100 kb, respectively (Fig. 1). The average insert size of the first ligation, which contained a relatively high percentage of small clones due to the inefficient separation of high- and low-molecular-weight DNA, was further lowered by the relatively large number of clones lacking inserts (18%). No vector self-ligation control was carried out, but we believe that survival of the non-recombinant clones may have been due to vector damage incurred during dephosphorylation which prohibited expression of the *SacB* gene. Use of different vector preparates in the second and third ligation, giving 0 and 12 colonies/ng dephosphorylated and self-ligated vector, respectively, reduced the number of non-recombinant clones to 0 and 5%, respectively.

The number of *Not*I sites per clone varied from zero to seven, with an average of about one *Not*I site every 46 kb (Fig. 2). This is comparable to the frequency of *Not*I sites in other monocot species (Woo et al. 1994; Wang et

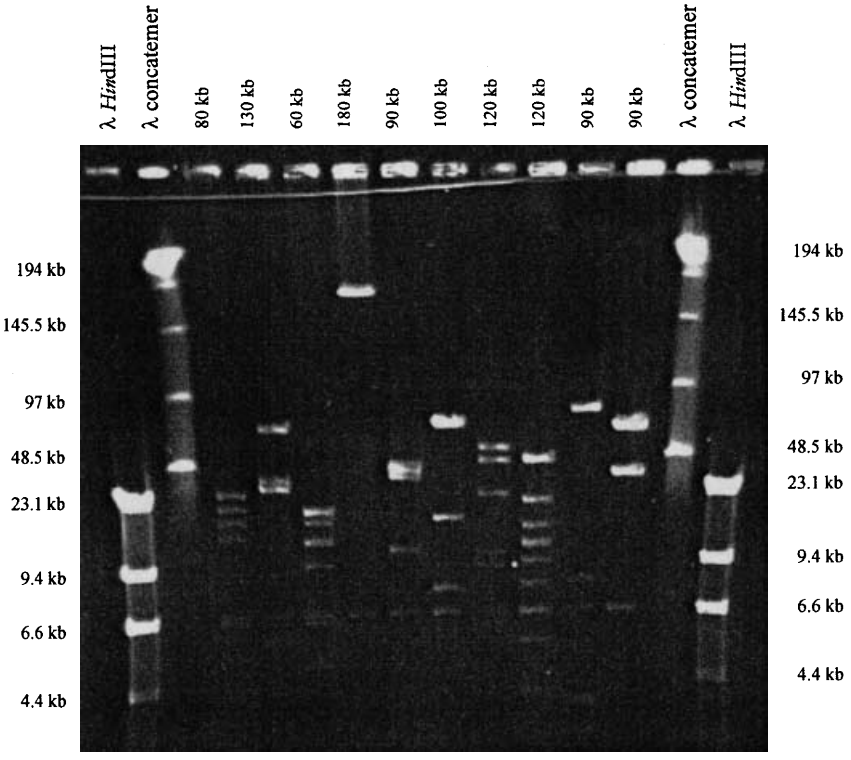


**Fig. 2** *Not*I restriction patterns of ten BAC clones (*lanes 3–12*). Insert sizes were calculated against λ *Hind*III (*lanes 1, 14*) and λ concatemer (*lanes 2, 13*) molecular-weight standards.The 6.8-kb fragment is the linearised pBAC/SACB1 vector

**Table 1** Origin and primer sequences for SSR markers isolated from BAC clones

| Marker | BAC origin | Primer sequences | Length (bp) | Repeat unit | PIC value[a] |
|---|---|---|---|---|---|
| PSMP2227 | B320A16 | ACACCAAACACCAACCATAAAG<br>TCGTCAGCAATCACTAATGACC | 197 | $(GT)_7$ | 0.21 |
| PSMP2229 | B320B03 | CCACTACCTTCGTCTTCCTCCATTC<br>GTCCGTTCCGTTAGTTGTTGCC | 241 | $(GT)_5$ | 0.74 |
| PSMP2231 | B320D03 | TTGCCTGAAGACGTGCAATCGTCC<br>CTTAATGCGTCTAGAGAGTTAAGTTG | 229 | $(TG)_{12}GG(TA)_4$ | 0.65 |
| PSMP2232 | B320D05 | TGTTGTTGGGAGAGGGTATGAG<br>CTCTCGCCATTCTTCAAGTTCA | 233 | $(TG)_8$ | 0.68 |
| PSMP2233 | B320E09 | TGTTTTCTCCTCTTAGGCTTCGTTC<br>ACCTTCTCCGCCACTAAACAACT | 258 | $(TG)_9$ | 0.64 |
| PSMP2235 | B320E13 | GCTTTTCTGCTTCTCCGTAGAC<br>CCCAACAATAGCCACCAATAAAGA | 192 | $(TG)_9$ | 0.19 |
| PSMP2236 | B320E19 | ATAAGTGGGACCCACATGCAGCAC<br>CGAAAGACTAGCAAAATTGCGCCTTC | 265 | $(TG)_4(GT)_4$ | 0.10 |
| PSMP2237 | B320F05 | TGGCCTTGGCCTTTCCACGCTT<br>CAATCAGTCCGTAGTCCACACCCCA | 233 | $(GT)_8$ | 0.60 |
| PSMP2240 | B320G08 | AGCCCAAAAGAAGTGGTCTAAC<br>CAACCACTAAAGTCTTACTGAACC | 147 | $(TG)_5$ | 0 |
| PSMP2242 | B320G17 | TAGCCTAATTGAACATTTTACGGAG<br>GCTTATCCAGGACATGCAATAC | 192 | $(TG)_9$ imp[b] | 0 |
| PSMP2246 | B320H20 | CGGATGCTAAATTAACCGAAGC<br>CCAGCTTGCTTCTGTTGCGTTC | 262 | $(TG)_7$ imp | 0.55 |
| PSMP2247 | B320I14 | CCAAACCGTAACCTGAAAAGCTACTG<br>GTGTCGGTTTGCTTCGTTCCTT | 203 | $(TG)_6$ imp | 0.60 |
| PSMP2248 | B320J15 | TCTGTTTGTTTGGGTCAGGTCCTTC<br>CGAATACGTATGGAGAACTGCGCATC | 167 | $(TG)_{10}$ | 0.49 |
| PSMP2249 | B320K09 | CAGTCTCTAACAAACAAACACGGC<br>GACAGCAACCAACTCCAAACTCCA | 153 | $(GT)_7$ imp | 0.36 |
| PSMP2251 | B320M06 | TCAAACATAGATATGCCGTGCCTCC<br>CAGCAAGTCGTGAGGTTCGGATA | 162 | $(TG)_6$ | 0.50 |
| PSMP2253 | B320M13 | CAGGTGATCTGTCTGGTTTCCTAATC<br>TAGCCACTGGAGTGCTACTGAA | 159 | $(TG)_{11}$ | 0.76 |
| PSMP2255 | B320N02 | CATCTAAACACAACCAATCTTGAAC<br>TGGCACTCTTAAATTGACGCAT | 264 | $(TG)_{34}$ | 0.86 |
| PSMP2261 | B320B11 | AATGAAAATCCATCCCATTTCGCC<br>CGAGGACGAGGAGGGCGATT | 193 | $(GA)_{16}$ | 0.78 |
| PSMP2263 | B320D16 | AAAGTGAATACGATACAGGAGCTGAG<br>CATTTCAGCCGTTAAGTGAGACAA | 238 | $(AG)_{33}$ | 0.72 |
| PSMP2266 | B320H09 | CAAGGATGGCTGAAGGGCTATG<br>TTTCCAGCCCACACCAGTAATC | 181 | $(GA)_{17}$ | 0.66 |
| PSMP2267 | B320K05 | GGAAGGCGTAGGGATCAATCTCAC<br>ATCCACCCGACGAAGGAAACGA | 241 | $(GA)_{16}$ | 0.79 |
| PSMP2270 | B320N06 | AACCAGAGAAGTACATGGCCCG<br>CGACGAACAAATTAAGGCTCTC | 153 | $(GA)_{26}$ imp | 0.80 |
| PSMP2271 | B320N18 | CCTTATATTGGACCGACTGCTGAC<br>CTCCCCCATACACGAGCGAGAA | 184 | $(GA)_{11}$ | 0.66 |
| PSMP2273 | B320P11 | AACCCCACCAGTAAGTTGTGCTGC<br>GATGACGACAAGACCTTCTCTCC | 169 | $(GA)_{12}$ | 0.75 |
| PSMP2274 | B320P12 | CACCTAGACTCTACACAATGCAAC<br>AATATCAAGTGATCCACCTCCCAA | 265 | $(GA)_{13}$ | 0.85 |

[a] The polymorphism information content (PIC) value was calculated according to Anderson et al. (1993):

$$PIC_i = 1 - \sum_{j=1}^{n} p_{ij}^2$$

where $p_{ij}$ is the frequency of the $j$th pattern of marker $i$, and the sum is made over $n$ patterns, assuming homozygosity of the pearl millet inbred lines

[b] Imperfect repeat

al. 1995; Zang et al. 1996; Moullet et al. 1999; Tomkins et al. 1999) and in marked contrast with *Not*I site distribution (<1 site/230 kb) in dicots (Choi et al. 1995; Frijters et al. 1997; Danesh et al. 1998; Nam et al. 1999). Monocots have a relatively higher GC content than dicot species [45% for millet (Martel et al. 1997) and 44% for rice (Wu and Wu 1992) compared to 38.6% for *M. truncatula* and 40.3% for *Arabidopsis thaliana* (Marie and Brown 1993)].This difference is even more pronounced (approx. 20%) when the comparison is restricted to coding sequences (Salinas et al. 1988). The relatively high GC content of genes, which have been shown to be clustered in gene islands in the genomes of many monocots (Devos and Gale 2000), may account for the higher frequency of *Not*I restriction sites in some parts of the genome. Further analysis is needed to confirm the expected

positive relationship between the number of *Not*I restriction sites and gene content in the pearl millet BAC clones.

## Genome representation

The 159100 clones contained an estimated total of 14200 Mb of pearl millet genomic DNA. Considering that pearl millet has a haploid genome size of 2450 Mb (Martel et al. 1997), the library contains 5.8 haploid genome equivalents. The probability of detecting any one sequence in the library is thus 99.6%. To evaluate the genome representation of the library, we tested 35 superpools, corresponding to 4.7 genome equivalents, for the presence of 11 pearl millet markers. It should be noted that the employed microsatellite markers had been isolated from an SSR-enriched small-insert genomic library (X. Qi and K.M. Devos, unpublished) and not from the BAC library. Two to eleven superpools were identified with each primer set, with an average of 5.4 superpools. Screening of the BAC colony filters (2.7 genome equivalents) with the restriction fragment length polymorphism (RFLP) probe PSM858 revealed six positives. The 12 probes tested are thus estimated to be represented, on average, 7.2 times in the complete library. This is consistent with the expected value of 5.8.

Screening of a subset of the library with a chloroplast sequence identified approximately 0.38% of the library sequences as cp DNA. Considering that the average chloroplast genome is 135 kb and that the average insert size of the clones is 90 kb, we can estimate that approximately 0.6% of the library was derived from cpDNA.

## Application of the library

The BAC library was built initially to provide a resource for map-based cloning of downy mildew resistance genes. However, another application has emerged from our more immediate need to develop microsatellite markers in pearl millet as tags in practical breeding programmes. Using the method described by Qi et al. (2001), so far, 42 $(GT)_n$ and 8 $(CT)_n$ microsatellites have been isolated from BAC clones pooled from a single 384-well microtiter plate. The primer sequences, BAC origin, SSR repeat and PIC values obtained in a panel of 20 pearl millet inbred lines are given in Qi et al. (2001) for SSR markers PSMP2201–PSMP2225 and in Table 1 for markers PSMP2226–PSMP2274. The SSR profiles of the pearl millet varieties are available from MilletGenes (http://jic-bioinfo.bbsrc.ac.uk/cereals/millet.html).

## Conclusion

A pearl millet BAC library covering 5.8 haploid genome equivalents and with a chloroplast content of about 0.6% was constructed. This library will be a valuable tool for map-based gene isolation in pearl millet and is available upon request as BAC filters. In addition, the library was shown to be a valuable resource in the isolation of SSR markers.

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