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Construction of bacterial artificial chromosome libraries and their application in developing PCR-based markers closely linked to a major locus conditioning bruchid resistance in mungbean (*Vigna radiata* L. Wilczek)

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Abstract Bacterial artificial chromosome (BAC) libraries have been widely used in different aspects of genome research. In this paper we report the construction of the first mungbean (*Vigna radiata* L. Wilczek) BAC libraries. These BAC clones were obtained from two ligations and represent an estimated 3.5 genome equivalents. This correlated well with the screening of nine random single-copy restriction fragment length polymorphism probes, which detected on average three BACs each. These mungbean clones were successfully used in the development of two PCR-based markers linked closely with a major locus conditioning bruchid (*Callosobruchus chinesis*) resistance. These markers will be invaluable in facilitating the introgression of bruchid resistance into breeding programmes as well as the further characterisation of the resistance locus.

Introduction

The value of large-insert libraries has long been recognised in genome analysis. They have been extensively exploited in physical mapping (Tao et al. 2001), map-based gene cloning (Zhang and Wing 1997), functional analysis of complex genomes (Antonarakis 2001) and the development of PCR-based molecular markers for targeted genome regions (Chen et al. 1995; Cregan et al. 1999). Due to their numerous advantages compared to cosmid and yeast artificial chromosome (YAC) libraries (Zhang et al. 1996a), bacterial artificial chromosomes (BACs) have become the system of choice for large-insert libraries. Although a recent development, BAC libraries have been constructed for many species. However, there are no reports of a BAC library for mungbean (*Vigna radiata* L. Wilczek).

Mungbean is widely grown in Asia and provides a major source of protein in cereal-based diets (Lawn and Ahn 1985). In Australia, mungbean is also a well-established crop, with producers aiming to supply the European and North American markets with premium quality sprouting seed (Lawn and Imrie 1994). Among the most serious pests which affect mungbean production and marketing are bruchids, belonging to the genus *Callosobruchus* (Coleoptera: Bruchidae). Two of the most damaging species are *C. chinesis* and *C. maculatus*, respectively. These insects attack stored seeds, and an infestation can result in major losses (Talekar 1988). No resistant commercial cultivars are available to date, and currently the insects are controlled by the use of insecticides. This type of preventive measure has two main disadvantages however: application of the insecticides adds extra costs to the mungbean industry, and residual insecticides are a major concern. For these reasons, bruchid resistance has been one of the main breeding objectives in mungbean improvement. To effectively breed a bruchid resistant cultivar, breeders have actively sought sources of bruchid resistance (Fuji et al. 1989). Resistance found in a wild relative of mungbean was mapped to a single locus that explained 87.5% of the phenotypic variation (Young et al. 1992). Kaga and Ishimoto (1998) studied the relationship between bru-

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chid resistance and vignatic acid production and found that these two traits were encoded by very closely linked genes.

Using a different source of resistance, we have also found a major locus for bruchid (*C. chinesis*) resistance, and a restriction fragment length polymorphism (RFLP) marker closely linked (1.3 cM) with this locus was identified (M. Humphry et al., unpublished). In this paper, we report the construction of mungbean BAC libraries and their application in developing PCR-based and locus-specific markers for this major locus conferring bruchid resistance.

Materials and methods

BAC library construction

pBeloBAC II was used as the cloning vector and two mungbean genotypes, ACC41 (a wild type belonging to *Vigna radiata* spp. *sublobata*) and ATF3640 (a cultivated type belonging to *V. radiata* spp. *radiata*), were used as DNA donors. High-molecular-weight (HMW) DNA was prepared from fresh leaf tissue (50 g) harvested from 4- to 6-week-old seedlings. Procedures for BAC library construction, including BAC vector preparation, isolation and partial digestion (using restriction enzymes *Hin*dIII and *Bam*HI) of HMW genomic DNA, separation and size fractionation of partially digested DNA by pulse field gel electrophoresis (PFGE), ligation and transformation of the recombinant DNA molecules into *Escherichia coli* by electroporation, were as described by Ma et al. (2000), with the exception that both single and double-size fractionated DNA was used for constructing the BAC clones.

Four DNA fractions—100–150 kb, 150–200 kb, 200–250 kb and 250–300 kb—were excised for each of the two genotypes and several ligations performed using the different DNA fractions and different ratios of insert:vector. Random clones (18–40) from each of the ligations were tested to determine insert sizes and ratios of empty clones. Single recombinant (white) colonies were inoculated in 5 ml of LB broth containing 12.5 μg/ml chloramphenicol and cultured at 37°C with shaking at 250 rpm for 16–20 h. Plasmid DNA was isolated from the overnight cultures as described by Zhang et al. (1996b). BAC inserts were released by *Not*I restriction and separated by PFGE in 0.5× TBE buffer at 6 V/cm, 11°C, with 5- to 15-s pulses for 18 h. Recombinant (white colonies) BAC clones from selected ligations were manually picked and stored in 384-well plates in duplicate.

Preparation and hybridisation of BAC colony filters

BAC clones were used to prepare colony filters using Hybond N^+ nylon membranes (Amersham, UK) as described by Zhang et al. (1996b). Each filter contained 384 single-spotted clones. To test the usefulness of the BAC libraries, we screened the colony filters with nine single-copy probes (Table 1). Methods for probe labelling and hybridisation were as described in Humphry et al. (2002). Insert DNA from positive BAC clones was purified as described by Zhang et al. (1996b).

Development of PCR-based markers for bruchid resistance

Of the nine single-copy probes used, Mgm213 is known to be closely linked (1.3 cM) to a major locus controlling bruchid resistance (M. Humphry et al., unpublished). Therefore, positive BAC clones identified by probing with Mgm213 were used to develop PCR-based markers for this locus. Purified BAC insert DNA from positive BAC clones was individually digested with *Sau*3AI (a four-base cutter) and ligated into *Bam*HI-restricted pBluescript II SK^+ vector. For each of the three BAC clones isolated, 1,152 subclones were individually picked and cultured in 384-well plates for 24 h, then dot-blotted onto Hybond N^+ nylon membranes. To isolate putative subclones containing simple sequence repeats (SSRs), we screened the membranes with $(AT)_{15}$, $(CT)_{15}$ and $(CTT)_{10}$ oligonucleotides as probes using the method described by Cregan et al. (1994). Inserts from positive subclones were purified and sequenced using the ABI Prism dRhodamine Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, Calif.) with the T3 primer. Sequence data were obtained from only one end for each of the positive subclones.

PCR primers were designed based on sequence data from seven different subclones using the primer selection programme SEQUENCER 3.1.1 (Gene Code, Ann Arbor, Mich.). These primers were tested against Berken and ACC41, the parents of the mapping population used to identify the major locus conditioning bruchid (*C. chinesis*) resistance (M. Humphry et al., unpublished). Primer pairs that detected polymorphism between the two parental genotypes were further analysed against a subset of 80 individuals from a recombinant inbred line (RIL, F_8) population derived from the two parents.

Table 1 RFLP clones tested and positive BAC clones identified

Probe	Positive BAC clones (estimated insert size in kilobases, if available)
VrCS13	8L2 (45), 8O14, 22C3 (75), 32N19 (120)
VrCS19	2H2 (110), 24O16 (60), 35E16 (145), 40G21 (155), 43I7 (155)
VrCS66	47H13 150), 49E5
VrCS126	39G18 (150), 39N21 (50)
VrCS176	38H14 (170), 38J13 (240)
VrCS198	26A4 (50), 31P13, 44A6
VrCS296	41O2 (110), 27L11(120), 50K15 (150)
VrCS343	16O12 (110), 26P13 (50), 43N4
Mgm213	27L7 (80), 29J3 (135), 41E23 (48)

Linkage between the newly developed PCR-markers and the original RFLP markers was determined visually.

Results

Construction of mungbean BAC libraries

Numerous ligations with different DNA fractions and different ratios of insert:vector were undertaken using either ACC41 or ATF3640 as the DNA source. Based on the results of random clone testing, two of the ligations were selected for generating BAC libraries. These ligations appeared to have larger inserts and fewer empty clones (data not shown). The first ligation was generated from *Hin*dIII partially-digested and double-size fractionated ACC41 DNA. From this ligation, a total of 6,912 clones were isolated (Fig. 1) of which 63 clones were randomly selected and tested. These clones contained inserts ranging from 45 kb to 180 kb with an average of 107 kb (Fig. 2). Two of the 63 clones (approximately 3.2%) did not contain inserts. The second ligation, using *Bam*HI-digested and single-size fractionated ATF3640 DNA, generated 11,904 clones. Inserts isolated from 205 randomly selected clones from this ligation ranged from 15 kb to 250 kb, with an average of 113 kb (Fig. 2). Three of the 205 random clones tested (1.5%) contained no inserts. In total, 18,816 BAC clones were picked from the two ligations, containing approximately 2,041 Mb of mungbean DNA.

To test the usefulness of the mungbean BAC libraries, we screened filters containing all of the18,816 BAC clones using nine single-copy RFLP probes, including one clone (Mgm213) known to generate a marker very closely linked (1.3 cM) to a major locus for bruchid resistance. Three of the nine RFLP probes detected two BAC clones each, four detected three BAC clones each, one detected four BAC clones and one detected five BAC clones (Table 1). Of the 27 positive BAC clones identified, 23 came from the *Bam*HI library and the remaining four from the *Hin*dIII library. On average, these nine RFLP probes detected three BAC clones each.

Isolation and characterisation of BAC subclones containing putative SSRs linked to bruchid resistance

The three BAC clones detected using Mgm213 were subcloned using the restriction enzyme *Sau*3AI and a total of 3,456 subclones isolated. Colony filters for these subclones were prepared and probed with $(AT)_{15}$, $(CT)_{15}$ and $(CTT)_{10}$. One positive subclone was identified with $(AT)_{15}$, 22 with $(CT)_{15}$ and 14 with $(CTT)_{10}$. Of the positive subclones identified, 20 were randomly selected and sequenced using the T3 primer. Only one of the 20 subclone sequences was found to contain a repeat long enough to be treated as an SSR; this repeat was $(AT)_{28}$.

Seven sets of PCR primers were designed, one based on flanking sequences of the SSR (SSRbr1) and the other six based on sequences of six random subclones (STSbr1-6) (STS, sequence-tagged-sites). Six of these PCR primer sets (Table 2) generated products from genomic DNA of ACC41 or Berken, but only two of the six sets of primers detected polymorphism between these two parental genotypes. SSRbr1 was not polymorphic between ACC41 and Berken. The two polymorphic sets of primers (STSbr1 and STSbr2) were further analysed against a RIL population between ACC41 and Berken (Humphry et al. 2002). STSbr1 generated a co-dominant marker, while STSbr2 generated a dominant marker. Both STSbr1 and STSbr2 co-segregated with the initial RFLP marker, Mgm213 (Fig. 3).

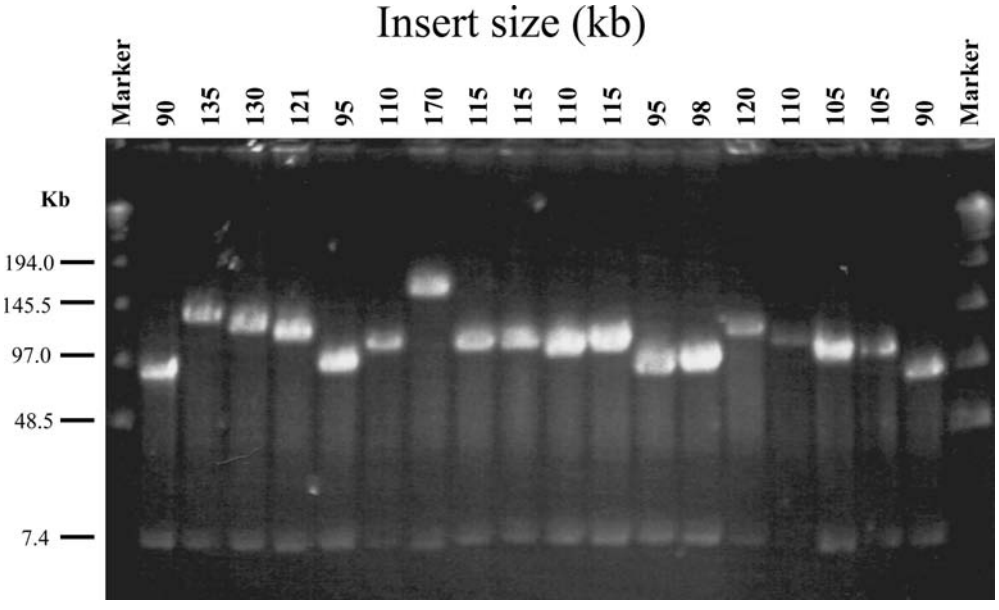

Fig. 1 PFGE analysis of ACC41 random BAC clones digested with *Not*I

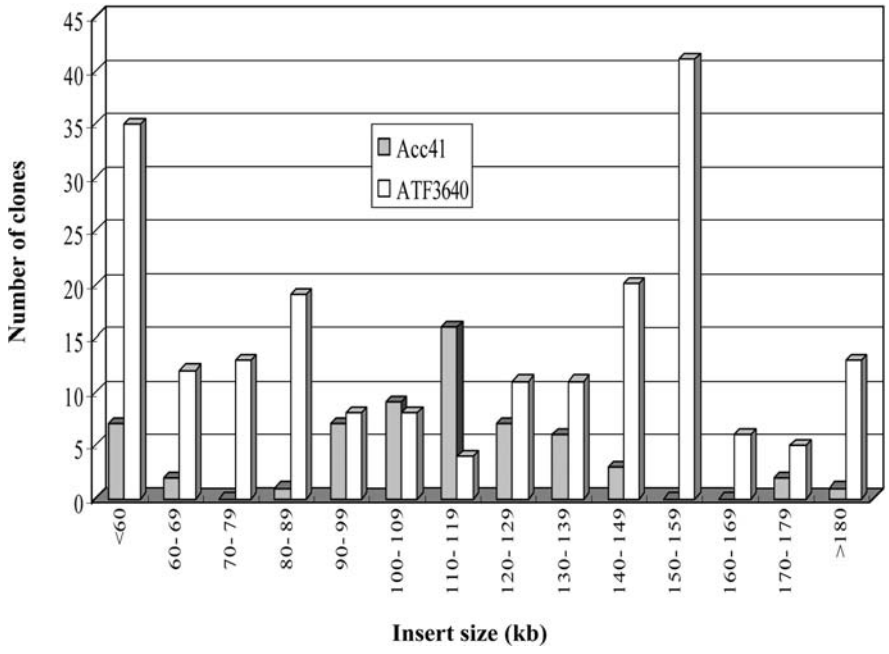

Fig. 2 Size distribution of 63 random ACC41 clones and 205 random ATF3640 clones

Discussion

Construction of mungbean BAC libraries

The mungbean BAC libraries constructed in this study were derived from two separate ligations using two different genotypes and two different restriction enzymes. One of the ligations was obtained using double-size fractionated ACC41 DNA and the other from single-size fractionated ATF3640 DNA. As seen by Zhang et al. (1996b), the double-size fractionation resulted in BAC clones with more uniform size distribution (45–180 kb) than those derived from the single-size fractionation (15–250 kb).

In total, 18,816 clones containing approximately 2,041 Mb of mungbean DNA were obtained from the two ligations. Considering that the genome size of mungbean is approximately 579 Mb (Arumuganathan and Earle 1991), these BAC clones represent 3.5 mungbean genome equivalents. Theoretically, the probability of identifying a particular clone corresponding to any mungbean DNA sequence is approximately 96%. Screening of these BAC clones with nine random RFLP probes identified three positive BAC clones each on average (Table 1), which is slightly lower than the theoretical figure of 3.5.The small number of RFLP probes tested would be unlikely to generate the exact genome average because restriction sites are unlikely to be positioned uniformly throughout the genome.

Unlike many other plant species, mungbean still lacks many of the basic molecular genetic resources. Linkage maps constructed so far (Young et al. 1992; Lambrides et al. 2000; Humphry et al. 2002) provide only limited genome coverage and, as yet, no locus-specific markers such as SSR or STS have been reported for this species. Although the BAC libraries obtained in this study represent only 3.5 genome equivalents, which may be inadequate for projects such as physical mapping or map-based cloning, these BAC clones could significantly enhance our capacity in gene mapping and in generating user-friendly markers for this 'orphan' crop.

SSR-containing sequences in mungbean

Sequences containing dinucleotide repeats of 20 bp or more were considered to be an SSR, a definition of the minimum length for a microsatellite widely used and generally accepted (Wang et al. 1994; Cregan et al. 1999). It has been estimated that on average there is one SSR in every 21.2 kb in dicotyledonous plants (Wang et al. 1994).

Table 2 Sequences of six sets of SSR and STS primers that produced PCR products in mungbean

Primer	Forward primer (5′–3′)	Reverse primer (5′–3′)	T_m(°C)
SSRbr1	ATGGGTAGCGTGATGCTG	TGTCAAAATGTGGTTGGCG	53
STSbr1	CAGAAAACAAATCACAAGGC	GTAAGCATTGAAAAAGGGTG	56
STSbr2	CCACCCTATTCAATGCTTAC	ACACTTCAATGGCGGACG	55
STSbr3	CAAAAGTCCAACGCTGTTCCCTG	CCATCTGTGTAGAATCTCTCGGTG	65
STSbr4	GGTAAGGGTAGGGGTTTCCATTAG	GAGACAAAAAGAGGACCAAAGCC	68
STSbr5	TCAGTCTTCCGTTTACG	TTGAGTGCTCAGGGGA	50

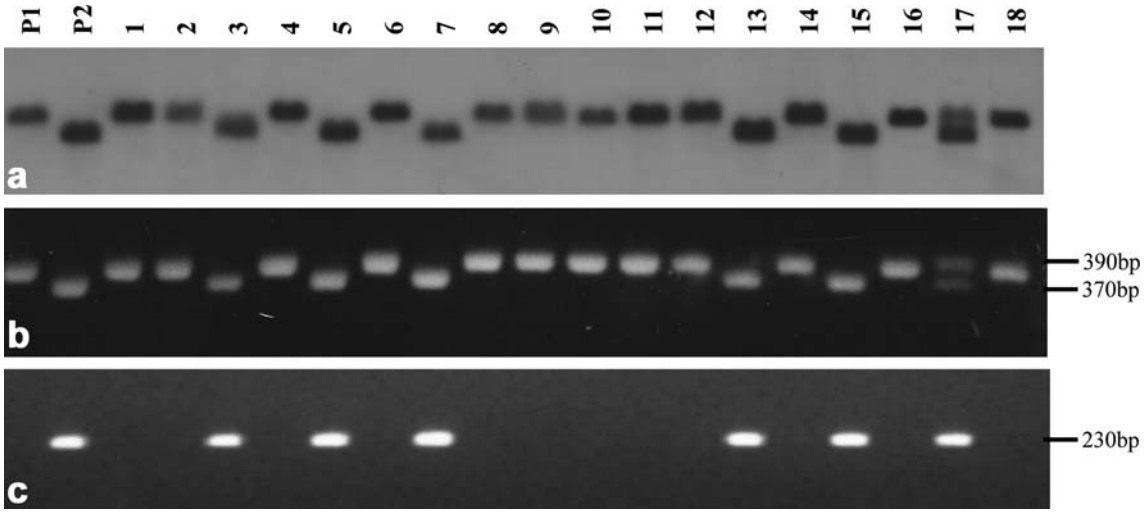

Fig. 3 Segregation patterns of the RFLP marker Mgm213 (**a**) and two of the PCR markers derived from it—STSbr1 (**b**) and STSbr2 (**c**)—in a recombinant inbred line population (*1–18*) between Berken (*P1*) and ACC41 (*P2*). Estimated sizes of the amplified products are shown on the *right*

Several subclones with strong signals were detected using $(AT)_{15}$, $(CT)_{15}$ and $(CTT)_{10}$ as probes. Of the 20 random sequences obtained, only one contained a repeat long enough to be considered an SSR. Considering that one of the three positive BAC clones used for isolating SSR-containing sequences was 135 kb (Table 1), several SSR-containing sequences were expected. The low number of SSR-containing sequences recovered could be due to the fact that sequence data were obtained from one end only for each of the subclones and that the SSRs could reside in the regions that were not sequenced; i.e. the distribution of SSR sequences along the mungbean genome might not be even. This possibility was supported by results from similar research for a major locus conditioning resistance to powdery mildew (Humphry et al. 2003). Several SSR-containing sequences were successfully isolated for this locus after screening similar numbers of subclones (M. Humphry et al., unpublished).

The two PCR-based, locus-specific markers isolated in this study co-segregated with the initial RFLP marker that was very closely linked (1.3 cM) to the major locus conditioning bruchid (*C. chinesis*) resistance. These two markers are the first reported PCR-based and locus-specific markers in mungbean. They will greatly facilitate the introgression of the bruchid resistance from wild germplasm into elite mungbean breeding material and the further characterisation of this resistance locus.

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References

Antonarakis SE (2001) BACking up the promises. Nat Genet 27:230–232

Arumuganathan K, Earle E (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:208–218

Chen H, Pulido JC, Duyk GM (1995) MATS: a rapid and efficient method for the development of microsatellite markers from YACs. Genomics 25:1–8

Cregan PB, Bhagwat AA, Akkaya MS, Rongwen J (1994) Microsatellite fingerprinting and mapping of soybean. Methods Mol Cell Biol 5:49–61

Cregan PB, Mudge J, Fickus EW, Marek LF, Danesh D, Denny R, Shoemaker RC, Matthews BF, Jarvik T, Young ND (1999) Targeted isolation of simple sequence repeat markers through the use of bacterial artificial chromosomes. Theor Appl Genet 98:919–928

Fuji K, Ishimoto M, Kitamura K (1989) Patterns of resistance to bean weevils (Bruchidae) in *Vigna radiata-sublohata* complex inform the breeding of new resistant varieties. Appl Entomol Zool 24:126–132

Humphry ME, Konduri V, Lambridges CJ, Magner T, McIntyre CL, Aitken EAB, Liu CJ (2002) Development of a mungbean (*Vigna radiata*) RFLP linkage map and its comparison with lablab (*Lablab purpureus*) reveals a high level of synteny between the two genomes. Theor Appl Genet 105:160–166

Humphry ME, McIntyre CL, Aitken EAB, Liu CJ (2003) Localization of a major QTL controlling powdery mildrew in mungbean (*Vigna radiata*). Genome 46:738–744

Kaga A, Ishimoto M (1998) Genetic localization of a bruchid resistance gene and its relationship to insecticidal cyclopeptide alkaloids, the vignatic acids, in mungbean (*Vigna radiata* L. Wilczek). Mol Gen Genet 258:378–384

Lambrides CJ, Lawn RJ, Godwin ID, Manners J, Imrie BC (2000) Two genetic linkage maps of mungbean using RFLP and RAPD markers. Aust J Agric Res 51:415–425

Lawn RJ, Ahn CS (1985) Mung bean (*Vigna radiata* (L.) Wilczek/*Vigna mungo* (L.) Hepper). In: Summerfield RJ, Roberts EH (eds) Grain legume crops. William Collins Sons, London, pp 584–623

Lawn RJ, Imrie BC (1994) The Australian mungbean industry in perspective. In: Imrie BC, Lawn RJ (eds) Mungbean: the Australian experience. In: CSIRO (ed) Proc 1st Aust Mungbean

Workshop. CSIRO Division of Tropical Crops and Pastures, Brisbane, pp 3–13

Ma Z, Weining S, Sharp PJ, Liu CJ (2000) Non-gridded library: a new approach for bacterial artificial chromosome (BAC) exploitation in hexaploid wheat (*Triticum aestivum*). Nucleic Acids Res 28:e106

Talekar NS (1988) Biology, damage and control of bruchid pests of mungbean. In: Mungbean: Proc 2nd Int Symp. Asian Vegetable Research and Development Center, Shanhua, Taiwan, pp 329–342

Tao Q, Chang Y-L, Wang J, Chen H, Nurul Islam-Faridi M, Scheuring C, Wang B, Stelly DM, Zhang HB (2001) Bacterial artificial chromosome-based physical map of the rice genome constructed by restriction fingerprint analysis. Genetics 158:1711–1724

Wang Z, Weber JL, Zhang G, Tanksley SD (1994) Survey of plant short tandem DNA repeats. Theor Appl Genet 88:1–6

Young ND, Kumar L, Menancio-Hautea D, Danesh D, Talekar NS, Shanmugasundarum S, Kim DH (1992) RFLP mapping of a major bruchid resistance gene in mungbean (*Vigna radiata*, L. Wilczek). Theor Appl Genet 84:839–844

Zhang HB, Wing RA (1997) Physical mapping of the rice genome with BACs. Plant Mol Biol 35:115–127

Zhang HB, Woo SS, Wing RA (1996a) BAC, YAC and cosmid library construction. In: Foster GD, Twell D (eds) Plant gene isolation: principles and practice. Wiley, New York, pp 75–99

Zhang HB, Choi S, Woo SS, Li Z, Wing RA (1996b) Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. Mol Breed 2:11–24