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A Gene-enriched BAC Library for Cloning Large Allele-specific Fragments from Maize: Isolation of a 240-kb Contig of the *bronze* Region

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A generic bacterial artificial chromosome (BAC) library from a complex plant genome like maize may not be suitable for some types of genomic analysis, for example, for establishing correlations between the genetic and the physical organization of a given chromosome region. Previously, we carried out extensive genetic analysis of the *bronze* (*Bz*) region in *Zea mays* using a W22 inbred line carrying the *Bz–McC* allele; however, BAC libraries of that line are neither available nor under construction. Here, we report the isolation of large, adjacent BAC clones of this region from a partial BAC library of W22. We developed a BAC vector suitable for cloning *Not*I fragments and used it to clone size-fractionated genomic DNA that had been cut to completion with the methylation-sensitive, rare-cutting enzyme *Not*I. This strategy resulted in a very significant enrichment of large genic DNA. From a library of about 20,000 BACs, containing just two-thirds of a maize genome, we isolated 16 BAC clones of the 110-kb distal *Bz* fragment and 10 BAC clones of the 130-kb proximal *Bz* fragment. This recovery means that our strategy resulted in a 15- to 24-fold enrichment of specific sequences. The order of the BAC clones in the 240-kb contig, predetermined from an internal *Not*I site in the *Bz–McC* allele was confirmed by hybridization with sequences from sites previously mapped proximal and distal to *Bz* and by sequencing. To show the general utility of our approach and the value of our partial BAC library, we also isolated BAC clones of other sequences, such as *tub4* and the complex *R–r* allele, contained in the same size fraction of DNA. This is the first report of the use of a BAC vector to clone allele-specific large DNA fragments from a plant with a large genome, circumventing the need to construct a complete BAC library.

Genetic background is a critical, yet often overlooked, experimental parameter. In extreme instances, erroneous conclusions about the phenotypic effect of a mutation may be reached from ill-advised comparisons of wild-type and mutant phenotypes in different genetic backgrounds. A more recent trap facing geneticists is to assume that genomic organization is a constant in different genetic backgrounds. Because the type of resource required for genomic analysis —a library of large DNA fragments—is difficult to generate, the temptation to ignore the genetic source of the library is great and increases with genome size.

Bacterial artificial chromosomes (BACs) (Shizuya et al. 1992) have become the vectors of choice for cloning large (>100 kb) DNA fragments from plants. To date, BAC libraries have been constructed for several plants: *Arabidopsis* (Mozo et al. 1998), rice (Wang et al. 1995; Xu et al. 1998), sorghum (Woo et al. 1994), tomato (Hamilton 1997), sugarcane (Tomkins et al. 1999b), and soybean (Tomkins et al. 1999a). Because of the effort required to construct a complete BAC library of organisms with large genomes, such libraries are being developed either by commercial concerns or specialized genome centers. Maize has a large genome (2.5×10^9 bp; Arumuganathan and Earle 1991) and is highly polymorphic (Walbot and Messing 1988). Assuming an average insert size of 100 kb, almost 300,000 colonies would be required for an 11-fold representation of the haploid maize genome, the level of redundancy of the Nipponbare BAC library being used in the rice genome project (Budiman et al. 1999). The only maize libraries currently available are based on the inbred line B73 and are considerably shallower than the existing rice or *Arabidopsis* libraries, although deeper libraries based on the inbred lines B73 and LH132 will soon be publicly available (R. Wing, pers. comm. and http://www.genome.clemson.edu/libframe.html).

Our laboratory has a long-standing interest in the relationship between genetic and physical distance in the *Bz* region of the short arm of chromosome 9 (*9S*). We have shown that the *Bz* gene is at least 100 times more recombinogenic than the average segment of the maize genome (Dooner 1986; Dooner and Martinez-Ferez 1997) and are interested in studying recombination immediately outside of *Bz*. To achieve this goal, we need to isolate and analyze large DNA fragments corresponding to defined genetic intervals on the proximal and distal side of the *Bz* locus. A possible source of these fragments is the existing BAC library from the inbred line B73. However, all our previous work on intergenic recombination in the region has

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been carried out with a different genetic line, namely a version of the W22 inbred line carrying an introgressed *Bz-McC* allele (Dooner and Belachew 1989). Because of the extensive DNA polymorphisms in maize, we were concerned that the *Bz* region in B73 might differ from that in the *Bz-McC* version of W22 and, as it turned out, our initial concern proved justified. We analyzed a *Bz* BAC clone from a commercially available B73 BAC library (kindly provided by Dr. V. Llaca) and found that the makeup of the DNA distal to *Bz* in that clone differed substantially from that in a previously isolated λ clone of the *Bz-McC* allele (Ralston et al. 1988; H. Fu and H.K. Dooner, unpubl.). Thus, to make a proper comparison, it became necessary for us to isolate the *Bz* region from the line used in our genetic experiments.

We were neither interested in nor in a position to construct a complete BAC library of our W22 maize line, so we decided to try to isolate large fragments of the *Bz* region from a partial library highly enriched in the fragments of interest. That is, we set out to use BAC vectors in much the same way that λ phage vectors have been used to construct partial libraries of size-fractionated DNA from total genomic digests. To that end, we modified pBeloBAC 11 (Fig.1; Kim et al. 1996a) to make it suitable for cloning *Not*I fragments. We chose the restriction enzyme *Not*I because it has an 8-bp recognition sequence and is sensitive to cytosine methylation. Consequently, it cuts maize DNA infrequently. Furthermore, *Not*I cuts once within *Bz-McC* (Ralston et al. 1988), producing a *Bz*-proximal and a *Bz*-distal fragment, which can be aligned and oriented

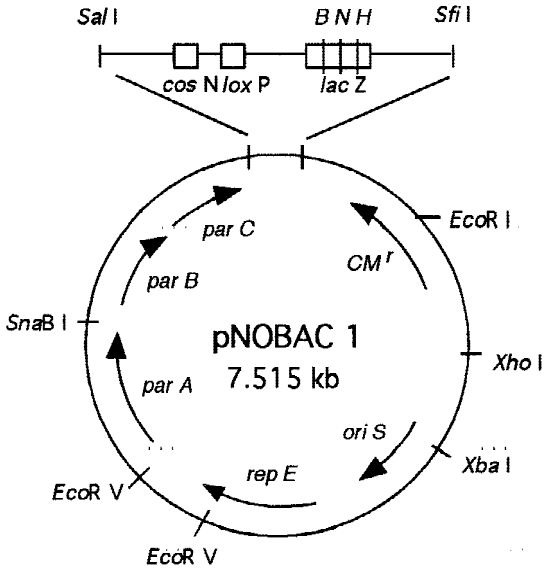

Figure 1 Schematic representation of the pNOBAC 1 vector. pNOBAC 1 is a *Not*I cloning BAC vector derived from pBeloBAC 11 (Kim et al. 1996b). It lacks the two *Not*I sites located on either side of the *lacZ* gene in pBeloBAC 11, has a new *Not*I cloning site between *Bam*HI and *Hin*dIII in the polylinker, and retains the blue-white selection feature of its progenitor.

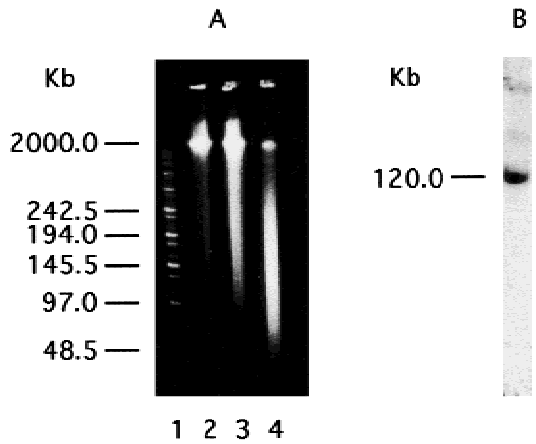

Figure 2 CHEF gel electrophoresis of maize genomic DNA. (*A*) Ethidium bromide-stained gel of maize genomic DNA that was not digested (lane *2*) or was digested with either *Not*I (lane *3*) or *Sfi* I (lane *4*). (Lane *1*) DNA size markers. (*B*) Autoradiogram of a different gel containing *Not*I-digested maize DNA. The membrane was hybridized with the probe *Bz*-528 (see Fig. 3).

along the known *Bz-McC* sequence. Here, we report the successful implementation of our strategy in the cloning of adjacent *Bz* fragments, which together comprise a 240-kb contig of the *Bz* region in 9S. We also isolated a 140-kb *tub4* and a 180-kb *R* clone from the same partial BAC library, thus demonstrating the general utility of our approach. This is the first report of the use of a BAC vector to clone allele-specific large DNA fragments from a plant with a large genome, thus bypassing the daunting task of having to construct a complete BAC library when such a library is not needed.

RESULTS

A BAC Vector Suitable for Cloning *Not*I DNA Fragments

A large fraction of the maize genome consists of methylated, repetitive DNA (Hake and Walbot 1980; Bennetzen et al. 1994) that is not cut by methylation-sensitive enzymes. In contrast, most genes exist in hypomethylated CpG islands (Antequera and Bird 1988). The enzyme *Not*I cuts maize DNA infrequently because it recognizes an 8-bp sequence, GCGGCCGC, and is sensitive to cytosine methylation. As seen in Figure 2A lane 3, a complete *Not*I digest of maize DNA produces very large fragments, most of which are larger than 2 Mb and cannot be resolved in a conventional CHEF gel. In contrast, *Sfi* I, another octanucleotide-recognizing enzyme which is less sensitive to cytosine methylation, produces smaller DNA fragments with an average size around 130 kb (Fig. 2A, lane 4).

Probing a *Not*I digest of DNA from the W22 *Bz-McC* maize line with a *Bz* probe that contains an internal *Not*I site (*Bz*-528 in Fig. 3) produces a thick band (Fig. 2B) that can be resolved into two bands of very

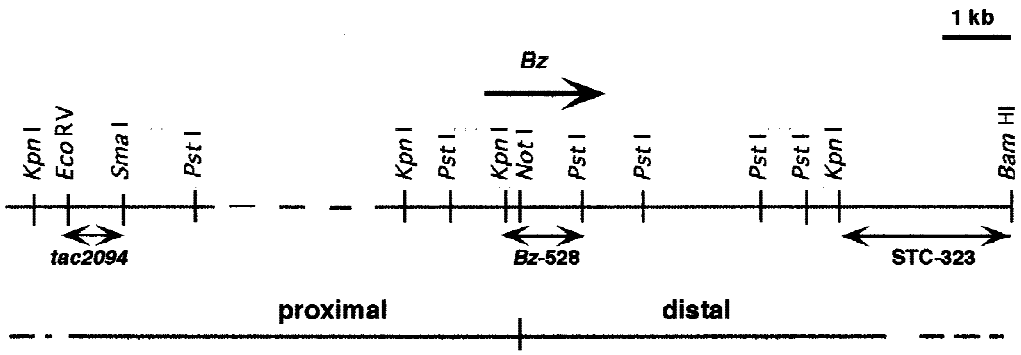

Figure 3 Partial restriction map of the *Bz-McC* and *Ac2094* λ genomic clones (Ralston et al. 1988, 1989). (Large arrow) Location and orientation of *Bz* transcript; (double-headed arrows) locations and extents of the three probes used in this work, *Bz*-528, STC-323, and tac2094. The *Not*I site in the *Bz*-528 fragment divides the *Bz-McC* gene into a proximal and a distal fragment, as shown (Dooner et al. 1985).

similar size (~110 kb) if the gel is run longer under different electrophoresis conditions (not shown). Thus, digestion of genomic DNA with *Not*I followed by CHEF gel fractionation of fragments in the 100- to 180-kb range should provide a very significant enrichment of both *Bz-McC* fragments and allow their BAC cloning without having to construct an entire BAC library.

To be able to clone *Not*I fragments into a BAC vector while retaining the bacterial colony color discrimination feature of existing BAC vectors, we modified pBeloBAC 11 (Kim et al. 1996a) as described in Methods. The resulting vector, pNOBAC 1 (Fig. 1), lacks the two *Not*I sites located on either side of the *lacZ* gene in pBeloBAC 11 and has a new *Not*I cloning site between *BamH*I and *Hin*dIII in the polylinker.

Isolation of Proximal and Distal Bz Clones from a Partial BAC Library

A partial BAC library of about 20,000 colonies was constructed from *Bz-McC* DNA that had been digested completely with *Not*I and enriched for fragments in the 100- to 180-kb range. The bacterial colonies were transferred to a nylon membrane and hybridized with a labeled 1.2-kb *Kpn*I–*Pst*I fragment from *Bz-McC* (*Bz*-528 in Fig. 3: Ralston et al. 1988), which contains an internal *Not*I site 180 bp downstream of the *Kpn*I site. Seventeen clones giving the strongest signals in the colony lift were isolated. BAC DNA from these positive clones was extracted, digested with *Not*I, and analyzed on pulsed-field gels. Figure 4A shows the ethidium bromide stain of a CHEF gel containing DNA from five positive clones (lanes 2–6), one negative control clone (lane 7), and high-molecular-weight markers (lane 1). Two patterns can be distinguished among the positive BAC clones: one given by the BAC clone in lanes 2–5 (pattern A) and the other given by the BAC clone in lane 6 (pattern B). This gel was blotted and hybridized sequentially to a series of probes.

Probe *Bz*-528 from *Bz-McC* hybridized to a 90-kb band—the largest *Not*I fragment—in lanes 2–5 and to a 10-kb band in lane 6 (Fig. 4B). The common band seen at 8 kb in all BAC lanes is due to nonspecific hybridization of the *Bz*-528 probe to the BAC vector. Sixteen of the seventeen positive clones gave pattern A upon digestion with *Not* I. We suspected that these corresponded to the distal *Bz-McC* fragment because most of the *Bz* probe used in the screen hybridizes to the distal *Not*I fragment as a consequence of the asymmetric location of its internal *Not*I site. This suspicion was confirmed when the membrane was hybridized to probe STC-323, corresponding to a sesquiterpene cyclase (B. Shen, Z. Zheng, and H.K. Dooner, unpubl.), located 5 kb distal to the *Not*I site in *Bz-McC* (Fig. 3). As seen in Figure 4C, probe STC-323 detects the same 90-kb band as the *Bz* probe in lanes 2–5, but does not hybridize to any *Not*I fragments in lane 6. To confirm that the BAC in lane 6 contains the proximal *Not*I fragment, the membrane was hybridized with a probe from *tac2094*, a locus that maps 0.05 cM proximal to *Bz* (Dooner and Belachew 1989). *tac2094* corresponds to the site of insertion of *Ac2094*, a transposed *Ac* element from the *bz-m2(Ac)* allele. The *tac2094* site has been cloned and sequenced and shown to correspond to unique DNA (Ralston et al. 1989). Because *tac2094* hybridized to the same band as a *Bz* probe in *Not*I genomic digests (Fig. 2B and data not shown), we expected that it would also hybridize to the proximal BAC clone, and it did. As seen in Figure 4D, *tac2094* detects a band of about 63 kb in lane 6, but does not hybridize to any *Not*I fragments in the other lanes. In an attempt to isolate additional clones of the proximal *Bz-McC* fragment, the library was rescreened with the proximal *tac2094* probe. Nine additional clones were obtained from this screen, and all gave the same B pattern of fragments upon digestion with *Not*I. Thus, several clones of both *Bz-McC Not*I fragments were recovered from the partial BAC library.

Both types of BACs contained other *Not*I fragments besides the ones hybridizing to the *Bz* probe, indicating that the cloned *Not*I genomic fragments

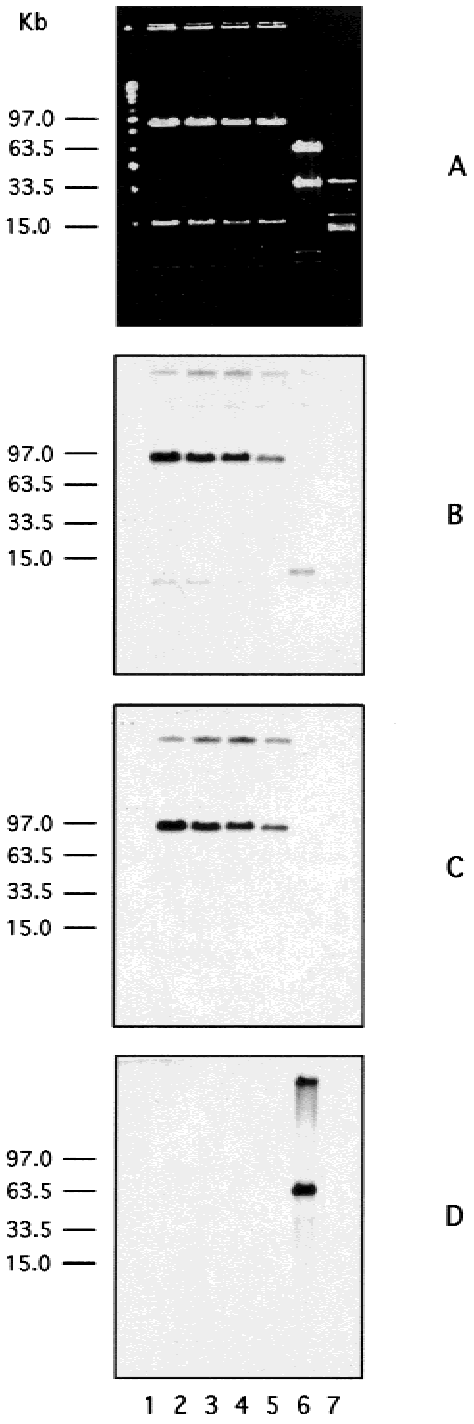

Figure 4 Analysis of BAC clones that hybridized to the probe *Bz*-528. BAC DNA from positive clones was extracted, digested with *Not*I, and separated by CHEF gel electrophoresis. (*A*) Ethidium bromide-stained gel containing DNA from five positive clones (lanes *2–6*), one negative clone (lane *7*), and high-molecular-weight markers (lane *1*). The gel was transferred to a nylon membrane and hybridized successively with three different probes, as shown in *B–D*. (*B*) Hybridization with probe *Bz*-528; (*C*) hybridization with probe STC-323, distal to *Bz*; (*D*) hybridization with a *tac2094* probe, proximal to *Bz*.

have internal *Not*I sites that are not cleaved by the enzyme because they are probably methylated. The BACs producing the A pattern of *Not*I fragments have an 18-kb *Not*I fragment, in addition to the 90-kb fragment, that hybridized to both the *Bz* and STC-323 probes. Thus, the overall size of the insert in the BAC containing the distal *Not*I fragment from *Bz-McC* is 108 kb. Because *Bz* defines the proximal end of the insert, the 90-kb *Not*I fragment must lie proximal to the 18-kb fragment in the chromosome. The BACs producing the B pattern have *Not*I fragments of 40, 5, and 3 kb, in addition to the 10-kb, *Bz*-hybridizing fragment and the 63-kb, *tac2094*-hybridizing fragment. Thus, the overall size of the insert in the BAC containing the proximal *Not*I fragment from *Bz-McC* is 121 kb. Except for the *Bz*-hybridizing fragment, which must lie at the distal end of the BAC clone, we do not know at this point the relative order of the *Not*I fragments in the proximal BAC, but we are currently in the process of characterizing the physical organization of both BACs vis-á-vis the genetic organization of the *Bz* region.

Further Characterization of the Partial BAC Library

The methylation-sensitive *Not*I enzyme should cut maize DNA in and close to genes. Thus, our *Not*I partial BAC library can be expected to be enriched for genic DNA. To determine whether the library could be used to isolate large fragments from other well-characterized genes, we screened the library with five different pobes: *wx* (Varagona et al. 1992), an *enod93* homolog (W. Park and H.K. Dooner, unpubl.), *tub4* (Villemur et al. 1994), *R* (Ludwig et al. 1989), and *sh2* (Bhave et al. 1990).

Southern blot data indicated that the *Not*I fragments containing the *tub4* and *R* genes should be present in the 100- to 180-kb size fraction used to construct our BAC library, but that the *Not*I fragments containing the *wx*, *enod93*, and *sh2* genes are either too small or too large to be present in the library (Fig. 5). In agreement with this expectation, we succeeded in isolating six 140-kb *tub4* and two 180-kb *R* clones from the partial BAC library (Fig. 6). These BAC clones are present in ninefold and threefold excess, respectively, confirming that the *Not* I partial BAC library has a disproportionate representation of chromosome fragments containing genes and is, thus, a valuable source of high-molecular-weight genic DNA from maize. It should be possible to recover large *Not*I BAC clones of *wx* and *enod93* from a similar size fractionation of a *Not*I partial digest and a *sh2* clone from a larger size fraction of a complete *Not*I digest, although the *sh2* *Not*I fragment (300 kb) is close to the upper limit of the size normally cloned into BAC vectors.

To assess the composition of our partial BAC library, 70 random clones were analyzed. Of the 70 clones, 67 (>95%) had inserts. The clone insert size

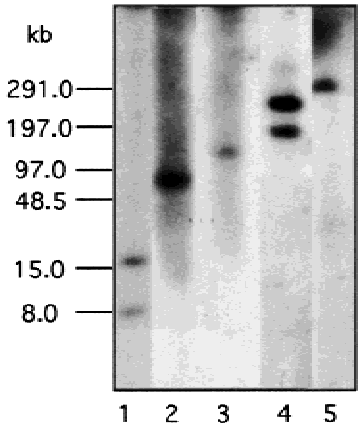

Figure 5 Southern blot analysis of high-molecular-weight maize genomic DNA. High-molecular-weight DNA was digested with *Not*I, separated by CHEF gel electrophoresis, and hybridized to different cDNA probes. (Lane *1*) *wx*; (lane *2*) *enod93*; (lane *3*) *tub4*; (lane *4*) *R*; (lane *5*) *sh2*.

averaged 106 kb and ranged from 60 to 180 kb. The identical makeup of the 16 distal and 10 proximal *Bz-McC* clones, of the six *tub4* clones, and of the two *R* clones suggests that there is little or no chimerism in the library.

DISCUSSION

Here, we report the BAC cloning of large, allele-specific genomic fragments from a partial BAC library of the maize inbred line W22. The library was constructed for the specific cloning of two adjacent, >100-kb *Not*I fragments from the *Bz-McC* allele present in that line. Several clones of both fragments were recovered, as well as two clones of the *R-r* allele carried in that line (Dooner and Kermicle 1971) and six clones of the *tub4* gene

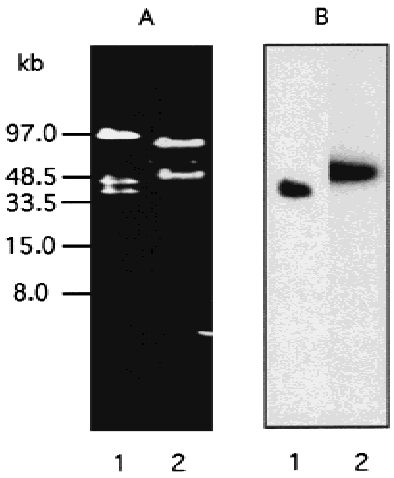

Figure 6. Analysis of *R* and *tub4* BAC clones. BAC DNA was extracted, digested with *Not*I, and separated by CHEF gel electrophoresis. (*A*) Ethidium bromide-stained gel. (Lane *1*) *R* clone; (lane *2*) *tub4* clone. (*B*) Autoradiogram of the gel blot in *A* hybridized to *R* cDNA (lane *1*) and *tub4* cDNA (lane *2*).

(Villemur et al. 1994), which also produce large fragments upon *Not*I digestion. This study constitutes the first example of targeted BAC cloning of allele-specific genomic fragments from a plant with a large genome and illustrates the feasibility of this approach when the construction of a complete BAC library is not warranted. This approach should be particularly applicable to the cloning of loci consisting of clustered gene families in which the members of the family either exhibit high line-to-line polymorphism or contain uniquely interesting variants. Examples of the former are the *Rp1* locus for resistance to races of the maize rust fungus (Hulbert and Bennetzen 1991; Richter et al. 1995) and the *R* and *P* loci that encode transcriptional regulators of anthocyanin biosynthesis in maize (Robbins et al. 1991; Eggleston et al. 1995; Chopra et al. 1998); an example of the latter is the α-zein cluster of the maize inbred line BSSS53, which contains a *dzr* variant that conditions high methionine accumulation in the endosperm (Chaudhuri and Messing 1995; Llaca and Messing 1998).

In this work, we have shown that it is possible, without a huge commitment of resources, to clone specific large fragments of DNA from a particular inbred line of maize, a plant with well-known limitations for genomic analysis because of its large genome. So far, only two maize lines (B73 and LH132) have been used to construct BAC libraries. These are complete BAC libraries constructed by researchers in specialized centers in both the public and the private sectors (R. Wing, pers. comm. and http://www.genome.clemson.edu/lib_frame.html; Genome Systems, Inc.). As geneticists with a long-term interest in the relationship between genetic and physical distance inside and outside of the *Bz* gene, we wanted to isolate large DNA fragments of the *Bz* region from the specific line where we had conducted all our previous studies on intergenic recombination. That line is a color-converted version of the inbred line W22 that carries an introgressed *Bz-McC* allele (Dooner and Belachew 1989). Early on, we learned that the region immediately distal to the *Bz* locus differed in B73 and in our line (H. Fu and H.K. Dooner, unpubl.), so it became essential for us to isolate BAC clones of our own line. We developed a strategy and a vector to accomplish this task and, in less than four months, succeeded in isolating multiple clones of the desired region from a partial BAC library containing less than one genome's worth of maize DNA. Our present success should encourage others who may want to isolate large allele-specific genomic fragments to pursue a similar strategy.

Southern blot analysis of *Bz-McC* DNA digested with the methylation-sensitive enzyme *Not*I and separated by CHEF gel electrophoresis revealed two *Bz*-hybridizing fragments of about 110 kb each. Maize genes exist in regions of hypomethylated DNA (Ante-

quera and Bird 1988; Bennetzen et al. 1994), so it is not surprising that the internal *Not*I site of *Bz-McC* (Ralston et al. 1988) is cleaved by *Not*I. Because *Not*I recognizes an 8-bp restriction site and is sensitive to cytosine methylation, restriction of genomic DNA with *Not*I, followed by size fractionation of the digested DNA should result in a very significant enrichment of large DNA fragments containing genes. To clone these *Not*I fragments, we converted pBeloBAC 11 into a *Not*I cloning vector (pNOBAC 1) and ligated *Not*I fragments in the 100- to 180-kb range to the new vector. From a library of about 20,000 BACs, containing a total amount of DNA equivalent to approximately two-thirds of a maize genome, we isolated 16 BAC clones of the 110-kb distal and 10 BAC clones of the 130-kb proximal *Bz* fragments. This recovery means that our strategy resulted in a 15- to 24-fold enrichment of specific sequences.

To show the general utility of our approach and the value of our partial BAC library, we also set out to isolate BAC clones of *tub4* and *R*, two genes that should be present in the library based on the size of their respective *Not*I fragments. The complex *R-r* allele in our line contains three copies of the *R* coding sequence, designated *P*, *S1*, and *S2* (Robbins et al. 1991), within a stretch of about 190 kb (Walker et al. 1995). We succeeded in isolating two identical 180-kb BAC clones of *R-r* and six identical 140-kb *tub4* clones. The lower recovery of *R-r* and *tub4* clones relative to *Bz-McC* clones can be explained by their larger size. Not only are larger fragments harder to clone, but the 180-kb *R* insert is at the high end of the size fractionation range used in the construction of the library. The insert size in the resulting BAC library ranged from 60 to 180 kb and averaged 106 kb.

The large 240-kb BAC contig of the *Bz-McC* allele was assembled from DNA completely digested with *Not*I. We took advantage of an internal *Not*I site in the previously cloned *Bz-McC* allele (Ralston et al. 1988) to define the contig junction, instead of basing the assembly on the sequences of overlapping BAC clones obtained from incompletely digested genomic DNA. Thus, the occurrence of a cleavable internal *Not*I site allows a very efficient use of a partial library in assembling a contig. All four classes of BAC clones—the proximal and distal *Bz-McC* clones, the *tub4* clones, and the *R-r* clone—contained internal *Not*I sites, confirming that many *Not*I sites in maize genomic DNA are not cleaved because they are probably methylated. The presence of internal *Not*I sites in the BAC clones allows a quick assessment of clone identity. All 16 distal *Bz-McC* clones had an identical *Not*I fragment makeup, as did all 10 proximal *Bz-McC* clones, the six *tub4* clones, and both *R-r* clones, suggesting that there is little or no chimerism in our *Not*I BAC library.

METHODS

Plant Material

The maize stock used in this study carried the *Bz-McC* allele introgressed into the genetic background of the inbred W22. This is the normal progenitor allele of the *bz-m2(Ac)* mutation (McClintock 1955), which served as the donor locus of many transposed *Ac* elements (*trAc*s) that have been used as markers in recombination experiments (Dooner and Belachew 1989).

Preparation of High-Molecular-Weight DNA from Maize

High-molecular-weight DNA was extracted from the shoots and leaves of 4-week-old greenhouse-grown maize plants, as described (Wang et al. 1995; Yang et al. 1997) with some modifications. About 50 grams of tissue was used for nuclear isolation. The tissue was washed with tap water and ground manually to a fine powder in liquid nitrogen. The powder was suspended in 200 ml of ice-cold 1× GH buffer (1 mM spermidine, 1 mM spermine, 10 mM Na_2-EDTA, 10 mM Tris, 80 mM KCl, 0.5% Triton-X 100, 0.15% β-mercaptoethanol, and 0.5 M sucrose at pH 9.4–9.5). After incubation in ice for 20 min, the suspension was filtered by squeezing with gloved hands through four layers of cheesecloth into a pre-chilled centrifuge bottle. The filtrate was centrifuged at 1800*g* (Beckman model JA14, maximum speed, 3500 rpm) for 20 min at 4°C. Then, the supernatant was discarded, and the nuclei pellet was resuspended in 30 ml of 1× GH buffer. The resuspended nuclei were filtered by gravity through two layers of Miracloth (Calbiochem) and spun down at 1800*g* for 15 min at 4°C. The resulting nuclei pellet was washed three times with 1× GH buffer, resuspended in 1.5 ml 1× GH buffer without β-mercaptoethanol and embedded in an equal volume of 2% low-melting-point agarose. Plugs containing about 5–8 μg DNA in a volume of 80 μl were lysed in 30 ml of lysis buffer (0.5 M EDTA, 1% sodium lauryl sarcosine, 2 mg/ml proteinase K, at pH 9.3–9.4) for 48 hr at 50°C, with one change of buffer after 24 hr.

Digestion of High-Molecular-Weight DNA and Size Fractionation by Pulsed-Field Gel Electrophoresis

After lysis, the agarose plugs were washed once in 0.5 M EDTA (pH 9.3) for 1 hr at 50°C, dialyzed four times against TE buffer (10 mM Tris, 1 mM EDTA, at pH 8.0) containing 1 mM phenylmethyl sulfonylfluoride (PMSF) for 1 hr at 4°C with gentle shaking, and equilibrated twice with *Not*I buffer for 1 hr at 4°C. Sixty units of *Not*I were added per plug and the enzyme was allowed to diffuse into the plug for 1 hr on ice. Complete digestion was achieved by incubating at 37°C for 10 hr. The digested DNA in four plugs was loaded into a 1% gel made with pulsed-field certified agarose (Bio-Rad) and fractionated by pulsed-field gel electrophoresis (CHEF-DR II system, Bio-Rad). The digested DNA was resolved in three steps as described previously (Osoegawa et al. 1998), with some modifications. The first step allowed the DNA to migrate from the wells toward the nearest gel edge (about 1 cm away from the well). Small DNA fragments were electrophoresed out of the gel by running it at 14°C and 6 V/cm for 3 hr with a 15 sec pulse time. In the second step, the direction of the gel was changed back to normal, and the gel was run under the same conditions as in step 1 to bring all the fragments remaining in the gel back to the well. Finally, the fragments were resolved at 6 V/cm for 16 hr with a 0.1- to 40-sec pulse time. The section of the agarose gel containing DNA fragments between

100 and 180 kb was cut out, and the fragments were recovered by electroelution.

Vector Modification

The polylinker of pBeloBAC 11 has *Hin*dIII and *BamH*I cloning sites (Kim et al. 1996a). To add *Not*I cloning capability to the vector, the following modifications were performed. A *Not*I site was added to the polylinker of pBeloBAC 11 by site-directed mutagenesis with the 40-bp oligonucleotide CCTCTAGAGTCGACCTGCGGCCGCGCAAGCTTGAGTATTC and the two *Not*I sites flanking the polylinker were eliminated by cutting the vector with *Not*I and filling in with the Klenow fragment of DNA polymerase. The modified vector, pNOBAC 1, has one *Not*I site between *BamH*I and *Hin*dIII in the polylinker and retains all the functions of pBeloBAC 11 (Fig. 1), including the blue-white selection feature on X-gal. It is available from the authors upon request.

Preparation of Vector, Ligation, and Transformation

Vector DNA was isolated as described previously (Wang et al. 1995) with some modifications. Briefly, a single colony of pNOBAC 1 was cultured overnight in 30 ml of LB containing 30 µg/ml of chloramphenicol at 37°C with shaking at 250 rpm. A 30-ml aliquot of the overnight culture was diluted in 5 liters of LB containing 30 µg/ml of chloramphenicol and incubated at 37°C for 12 hr. The plasmid DNA was isolated by alkali lysis and purified by CsCl–ethidium bromide differential centrifugation (Sambrook et al. 1989). Purified pNOBAC 1 DNA was digested with *Not*I, dephosphorylated with CIP (New England Biolabs), and used for ligation.

Size-fractionated and electroeluted genomic DNA was ligated to 50 ng of *Not*I-digested and dephosphorylated pNOBAC 1 DNA at an ~1:5 to 1:10 molar ratio of insert:vector in a 50-µl total volume with 1 unit of T4 DNA ligase (Promega) at 16°C for 8–10 hr. The reaction was placed on a 25-mm, 0.025-µm pore size microdialysis filter (Millipore) and allowed to dialyze passively, first against sterile deionized water on ice for 2–3 hr and then against 0.5× TE containing 30% PEG 8000 (Sigma) on ice for 0.5–1 hr to reduce the volume to about 20 µl before transformation. Two microliters of ligation reaction was used to transform 40 µl ElectroMAX DH 10B competent cells (Life Technologies) by electroporation with a Gene Pulser II (Bio-Rad). The electroporation conditions were 2.5 KV, 25 µF, 100 Ω, and a 0.1-cm cuvette. After the electroporation, the cells were immediately added to 1 ml of SOC medium (Sambrook et al. 1989) and incubated at 37°C for 1 hr by shaking at 250 rpm to express the antibiotic resistance gene. The cells were then plated on LB plates (100 × 15 mm) containing 20 µg/ml chloramphenicol, 5 µg of X-gal and 100 µg/ml of IPTG, and incubated at 37°C for 24 hr to a colony diameter of 1–2 mm.These plates were used in the primary screen.

Screening and Analysis of the BAC Partial Library

Bacterial colonies were transferred to Hybond-N+ nylon membranes (Amersham), hybridized with a *Bz* radioactive probe, and washed at high stringency following the manufacturer's instructions. Probes were labeled by use of random primer extension. The washed membranes were exposed to X-ray film overnight to reveal positive clones. Seventeen colonies giving the strongest signals were picked, and all turned out to be positive upon subsequent testing.

The bacterial colonies from the plates used in the primary screen (~20,000) were transferred individually to a series of 384-well microtiter plates for long-term storage. The colonies were replica-plated onto Hybond-N+ nylon membranes, and the membranes were hybridized with a *tac2094* probe (Ralston et al. 1989) to isolate additional BAC clones of the *Not*I fragment on the proximal side of *Bz-McC* and with several maize cDNA probes to isolate BAC clones of other maize genes (see Results).

Restriction enzyme digestion and genomic blotting were carried out as described (Dooner et al. 1985).

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