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Construction of a Bacterial Artificial Chromosome Library Containing Large BamHI Genomic Fragments from *Medicago truncatula* and Identification of Clones Linked to Hypernodulating Genes

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Abstract In the model legume *Medicago truncatula*, two mutants, *sickle* and *sunn*, exhibit morphologically and genetically distinct hypernodulation phenotypes. However, efforts to isolate the single recessive and single semidominant genes for *sickle* and *sunn*, respectively, by map-based cloning have so far been unsuccessful, partly due to the absence of clones that enable walks from linked marker positions. To help resolve these difficulties, a new bacterial artificial chromosome (BAC) library was constructed using BamHI-digested genomic fragments. A total of 23,808 clones were collected from ligation mixtures prepared with double-size-selected high-molecular-weight DNA. The average insert size was 116 kb based on an analysis of 88 randomly selected clones using NotI digestion and pulsed-field gel electrophoresis. About 18.5% of the library clones lacked inserts. The frequency of the BAC clones carrying chloroplast or mitochondrial DNA was 0.98% and 0.03%, respectively. The library represented approximately 4.9 haploid *M. truncatula* genomes. Hybridization of the BAC clone filters with a C_0t-1 DNA probe revealed that approximately 37% of the clones likely carried repetitive sequence-enriched DNA. An ordered array of pooled BAC DNA was screened by polymerase chain reactions using 13 sequence-characterized molecular markers that belonged to the eight linkage groups. Except for two markers, one to five positive BAC clones were obtained per marker. Accordingly, the *sickle*- and *sunn*-linked BAC clones identified herein will be useful for the isolation of these biotechnologically important genes. The new library will also provide clones that fill the gaps between preexisting BAC contigs, facilitating the physical mapping and genome sequencing of *M. truncatula*.

Key words: *Medicago truncatula*, BAC library, PCR screening, molecular markers, map-based cloning, hypernodulating genes

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The symbiotic associations between plants and microorganisms, unlike the pathogenic associations [3, 18], are mutually beneficial interactions that provide essential nutrients to both partners. For example, legumes are the primary plant hosts for nitrogen-fixing rhizobia and phosphate-supplying arbuscular endomycorrhizal fungi [1]. The nitrogen fixation by rhizobia, *per se*, takes place in legume root nodules and is initiated based on the perception of Nod factors from the infecting rhizobia, and subsequently regulated by multiple factors involving several intracellular and hormonal signaling pathways [21]. Numerous genetic studies have identified plant mutants displaying abnormal nodulation phenotypes, and among these, hypernodulating mutants have attracted much attention owing to their potential biotechnological applications. Nonetheless, despite the recent isolation of a *CLAVATA1*-like receptor kinase gene from supernodulating soybeans [29], the regulatory mechanisms controlling the nodule number remain to be elucidated.

Over the past decade, two legume species, *Medicago truncatula* [4] and *Lotus japonicus* [15], have been extensively used as the primary model legumes for molecular genetic analyses [35]. *M. truncatula* is taxonomically related to the forage crop alfalfa (*M. sativa*), yet has attributes, such as a small diploid genome (2n=2x=16; 450 Mbp/1C) [5], short life cycle, and the ability to be regenerated and transformed [34], that are suitable for genomics research programs. Furthermore, *M. truncatula* symbiotically associates with *Sinorhizobium meliloti*, one of the best characterized nitrogen-fixing symbionts [21]. An extensive genetic map of *M. truncatula* has been developed with hundreds of sequence-characterized molecular markers [8], a number of which have been identified as conserved orthologous sequences in related species. A comparison of the genetic maps of several crop legumes with these consensus markers has revealed an octagonal macrosyntenic relationship among the genomes [9]. The eight *M. truncatula* chromosomes

are currently being sequenced, and more than 25% of the entire *M. truncatula* genome has already been sequenced (http://www.medicago.org).

The genes that are associated with the initial signaling stages of nodulation have recently been isolated by map-based cloning. The *DMI1* [2], *DMI2* [13], and *DMI3* [19] genes of *M. truncatula* [6], as well as other cloned genes from *L. japonicus* [27, 31] and pea [20], have provided an emerging picture of the molecular events that occur during nodulation from the early stages of Nod factor perception to cortical cell division [11]. However, despite these interesting findings, the two hypernodulating genes of *M. truncatula*, *sickle* [24] and *sunn* [25, 28], still remain to be isolated. The two mutants exhibit morphologically and genetically distinct phenotypes, where the nodules of the *sickle* mutants are deformed and irregularly positioned, whereas those of the *sunn* mutants are fully differentiated and functional [25]. In addition, *sickle* fails to display an ethylene triple response, suggesting its involvement in the ethylene signaling pathway [24]. The two genes have already been placed in the genetic map, linked to specific molecular markers [8, 28]. Thus, in principle, chromosomal walks toward each gene can be implemented from any large insert clone that contains particular marker sequences. However, recent investigations have suggested that the currently available large insert library clones from *M. truncatula* may not be sufficient to support the completion of such walks (R. V. Penmetsa and D. R. Cook, personal communication).

Bacterial artificial chromosome (BAC) libraries serve as an essential resource for the map-based cloning of important plant genes and structural and comparative genome analyses. BAC libraries are capable of carrying and stably maintaining inserts of 100–500 kb in size [30], and have comparative advantages over yeast artificial chromosome (YAC) libraries [14] as regards a low rate of chimera formation [33, 36] and easily manipulated individual clones [39]. Since the early 1990s, a number of BAC libraries have been constructed from major plant species [7, 10, 22, 23, 33, 36, 37]. For *M. truncatula*, the first BAC library was constructed with HindIII genomic fragments [22], and contained 30,720 clones that covered approximately five haploid genome equivalents. Subsequently, the HindIII library was expanded to a size equivalent to 25 haploid genomes (D. R. Cook, unpublished). Another BAC library was separately constructed with EcoRI genomic fragments (F. Debelle, unpublished). Yet, despite the extensive usefulness anticipated from these two preexisting BAC libraries, recent investigations have revealed that the contiguous HindIII or EcoRI BAC segments are often separated by gaps that are present in a number of genomic locations (D. R. Cook and F. Debelle, personal communication), raising the issue that a third novel restriction fragment BAC library may be required to fill the gaps.

Accordingly, this study reports on the construction of a new BAC library using BamHI genomic fragments from *M. truncatula*. The preparation of a multiplex DNA pool system followed by screening with sequence-characterized molecular markers is described. As a result, the BAC clones identified herein as linked to two hypernodulating loci provide opportunities for cloning these biotechnologically important genes.

MATERIALS AND METHODS

Plant Material and HMW DNA Preparation

The *Medicago truncatula* cultivar "Jemalong" (ecotype A17) was used to isolate high-molecular-weight (HMW) DNA. Seeds were released from pods, treated with mild sulfuric acid, and subsequently germinated, as described previously [22]. The seedlings were grown in flats in a growth chamber with a daily cycle of 14 h of light and 10 h of dark at 25°C for three weeks. Before isolating the nuclei, the plants were kept in the dark for 48 h to minimize polysaccharide accumulation. The nuclei isolation, embedding in low-melting-point agarose plugs, and subsequent lysis of the nuclear envelopes were all carried out as described in Ref. [38].

Library Construction

The HMW DNA was partially digested with BamHI, as described in Ref. [23], with minor modifications. In each plug-containing digestion tube, 2.0, 4.0, or 6.0 U of BamHI was added and subsequently incubated at 37°C for 7 min. The resulting DNA fragments were separated by pulsed-field gel electrophoresis (PFGE), as described in Ref. [23]. The gel sections containing the 100–250 and 250–400 kb zones, respectively, were excised and electroeluted. The resulting DNA preparations were then subjected to a second round of PFGE, as described in Ref. [23]. Double-size-selected DNA fragments, over 90 kb in size, were recovered in a volume of less than 100 μl. The ligation was carried out using the pECBAC1 vector prepared as described in Ref. [23] at a molar ratio of 5–10 (vector) to 1 (insert). Thereafter, the electroporation of the ligation mixtures, colony selection, and library arraying were all carried out as described in Ref. [23].

Pooling of Library for PCR Screening

The preparation of the BAC DNA pools for the efficient multiplex PCR screening [16, 17] was carried out essentially as described in Ref. [23] with minor modifications. Since the *M. truncatula* BamHI library consisted of 62 384-well microtiter plates, the BAC clones were arrayed in two consecutive plates per set. All the clones were combined entirely from two fresh BAC plates, resulting in 31 set pools. For the column and row pools, the two plates comprising a given set were arranged vertically, and 24 column and 32 (2×16) row pools prepared by combining

all the clones belonging to the same column (1 to 24) or row (A to P). Each primary pool was then subjected to plasmid DNA isolation using the alkaline method [26]. The isolated DNA preparations were dissolved in 100 μl (set pools) or 25 μl (row and column pools) of 1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The resulting column and row DNA pools were then pooled two-dimensionally, as described in Ref. [23], except for the horizontal superpools that were given lower numbers. As such, R1–R4 and C1–C4 represented the horizontally combined row and column superpools, respectively, whereas R5–R12 and C5–C10 represented the vertically combined superpools. The DNA in each superpool was estimated to be approximately 20 ng/μl per tube.

Molecular Markers and Screening of BAC DNA Pools

Thirteen sequence-characterized molecular markers located in the eight linkage groups (LG) of the *M. truncatula* genetic map [8] were used to characterize the library. These markers included MDH2 (LG-1), ACCO (LG-2), PRTS (LG-3), DNABP (LG-4), NUM1 (LG-4), FAL (LG-5), PTSB (LG-5), CrS (LG-6), CAK (LG-7), ENOL (LG-7), VBP1 (LG-7), CDC16 (LG-8), and SDP1 (LG-8). The primer nucleotide sequences for these markers and their expected fragment sizes are described in Ref. [8]. Each marker was used to screen the BAC DNA pool. Typically, 1 μl of the intact (set pools) or 1/5-diluted (row or column superpools) DNA template was added to a tube containing the reaction mixture (2.5 mM $MgCl_2$, 50 μM dNTPs, 0.25 pmol primers, and 0.1 unit *Taq* polymerase). The polymerase chain reaction (PCR) amplification conditions were 94°C, 20 s; 54°C, 30 s; 72°C, 1 min for 40 cycles. After the PCR, the products were analyzed on a 1.5% agarose gel, as described in Refs. [22, 23].

Probes, Filter Hybridization, and BAC Clone Analysis

The genomic DNA was isolated from *M. truncatula* leaves and subsequently used to prepare C_0t-1 DNA [40], as described in Ref. [22]. Two sets of double-spotted high-density BAC clone filters in a 4×4 format were prepared using a Microgrid II robot (Biorobotics) (FnP, Suwon, Korea). Two barley (*Hordeum vulgare*) chloroplast cDNAs (*rbcL* and *psbA*) and four pepper (*Capsicum annuum*) mitochondrial cDNAs (*cox*II, *cox*III, *atp*6, and *atp*9) were used as the extranuclear DNA probes for the BAC filter hybridization, as described in Ref. [23]. The probe labeling and filter hybridization were also carried out as described in Ref. [23]. The plasmid DNA was isolated from the BAC clones using an alkaline mini-preparation [26], then analyzed by NotI digestion and PFGE, as described in Ref. [23]. Thereafter, the gel-separated BAC DNA fragments were transferred to a Hybond N (+) nylon membrane (Amersham Pharmacia), and the blot hybridized, as described in Ref. [23].

RESULTS

Construction of BAC Library

As a measure of constructing a large insert-carrying library, agarose plugs were prepared to immobilize the *M. truncatula* nuclei. The HMW DNA resulting from the nuclear lysis was partially digested with BamHI at optimal concentrations for producing the maximum number of DNA fragments of 100–400 kb. Double-size selection of the digested HMW DNA typically gave two DNA preparations from 100–250 kb (MtB1) and 250–400 kb (MtB2), and four DNA preparations from two independent experiments (Mt10 and Mt12 from Experiments 10 and 12, respectively) contained sufficiently abundant DNA (~1 ng/μl) for successful ligation. The subsequent electroporation of each of these four ligation mixtures then produced sufficient numbers of white colonies for the library construction. A PFGE analysis of randomly selected clones showed that almost every clone carried inserts (Fig. 1A). In total, 18,432 and 5,376 clones were collected from the Mt10 (Mt10B1 and

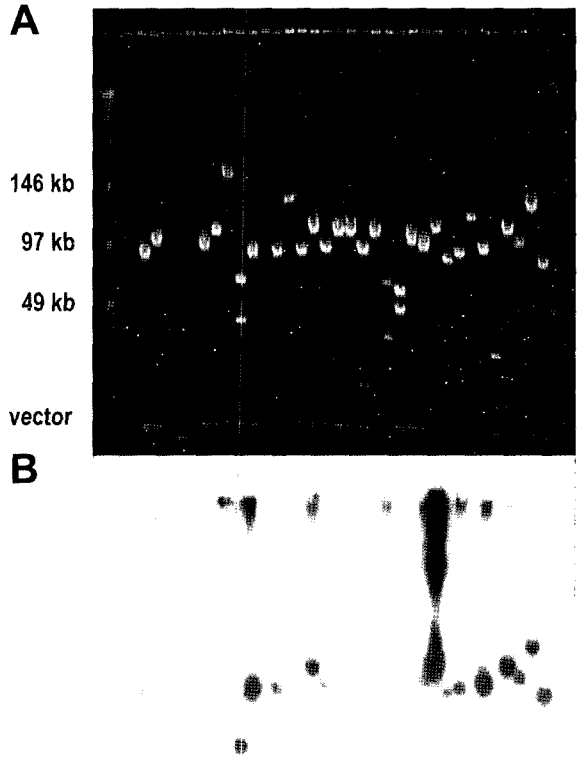

Fig. 1. PFGE analysis of *M. truncatula* BamHI BAC clones.
A. Thirty BAC clones were randomly selected and their plasmids analyzed by NotI digestion and PFGE. The lambda concatemer markers are shown on the left. **B.** The gel-separated DNA fragments in panel A were transferred to a nylon filter and hybridized with the *M. truncatula* C_0t-1 DNA as a probe.

Mt10B2) and Mt12 (Mt12B1 and Mt12B2) ligation mixtures, respectively, resulting in 23,808 clones arrayed in 62 384-well microtiter plates.

Characterization of Library

The percentage of no insert-carrying clones in the library and the average size of clone inserts were estimated. For this, plasmids were isolated from 108 randomly selected BAC clones and analyzed by NotI digestion and PFGE. The results showed that 88 clones carried inserts and 20 clones had no inserts, giving a blank ratio of approximately 18.5%. The majority of the insert-carrying clones were shown to have 90–130 kb inserts (Fig. 1A). The calculated average insert size was 116 kb (Fig. 2).

The content of repetitive DNA in the library was also estimated, where the BAC DNA separated by PFGE (Fig. 1A) was transferred to a nylon filter, and the blot hybridized with *M. truncatula* C_0t-1 DNA as a probe. Since C_0t-1 DNA is prepared by excluding the fraction of genomic DNA that fails to reanneal in a calculated time period after denaturation [40], it is enriched with highly and moderately repetitive DNA sequences. Therefore, the hybridization of randomly selected BAC inserts with the C_0t-1 DNA was expected to provide a rough estimate of the repetitive DNA content in the library. Of the 30 BAC DNAs analyzed, eleven hybridized strongly and ten hybridized weakly with the C_0t-1 DNA (Fig. 1B), indicating that approximately 70% of the library clones apparently contained repetitive DNA.

To further characterize the library, high-density colony filters were prepared using robotic instruments. For example, filter hybridization with specific probes was used to identify particular clones that carried inserts derived from extranuclear origins. Thus, when a ^{32}P-labeled mixture of chloroplast *rbcL* and *psbA* cDNAs was used as the probe, 156 clones were identified (data not shown). Based on the assumption that at least two-thirds of chloroplast DNA-carrying BAC inserts can be identified with these two genes [36], approximately 0.98% of the library clones were estimated to carry DNA derived from the chloroplast genome. Similarly, the use of a radiolabeled probe containing four mitochondrial cDNAs identified eight positive clones (data not shown). Thus, approximately 0.03% of the library clones were estimated to carry DNA derived from the mitochondrial genome.

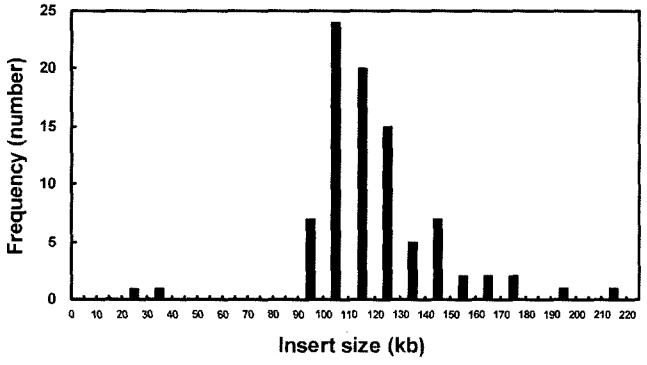

Fig. 2. Distribution of insert sizes of *M. truncatula* BamHI BAC library clones.
The BACs isolated from 88 randomly selected and insert-carrying clones from the library were analyzed by NotI digestion and PFGE. The estimated insert sizes are plotted versus the frequencies.

Preparation of BAC DNA Pools and Screening with Molecular Markers

To facilitate library screening with PCR-based molecular markers, a multiplex DNA pool system was developed. In contrast to the strategy used for the previously constructed HindIII library, where two-dimensional pooling was applied uniformly to the entire library plates [22], the rows and columns of the present BamHI library were only pooled two-dimensionally within a given set of two consecutive plates (see Materials and Methods). As a result, 31 set pools and 10 row and 12 column superpools per set were prepared. Three rounds of PCR screening were required to identify the individual BAC clones when using this pool system: the initial round of 31 reactions on the set pools was followed by a second round that identified the specific column and row intersects, then the final round identified the specific positive BAC clones from the original library.

The DNA pool system was screened with thirteen selected *M. truncatula* sequence-characterized molecular markers [8], corresponding to genes that encode various metabolic (e.g., ENOL for enolase), regulatory (e.g., DNABP for an SAR DNA-binding protein; VBP1 for a TGA-type basic leucine zipper protein), or structural proteins (e.g., NUM1 for a homolog of mammalian nucleolin), and belong to the eight linkage groups of *M. truncatula* (Table 1). The three rounds of PCR screening with each marker resulted in 11 markers identifying 27 positive BAC clones, where the number of positive clones per marker varied from one to five (Table 1), giving an average of 2.4 clones per marker. However, the CAK and SDP1 markers belonging to Linkage Groups 7 and 8, respectively, were unable to identify any BAC clones.

Identification of BAC Clones Linked to Hypernodulating Genes

The two markers, ENOL and VBP1, surround the *sickle* locus in a 3.6-cM genetic interval in Linkage Group 7 [8]. Two and one positive BAC clones were identified with ENOL and VBP1, respectively (Table 1). The procedure used to screen the BAC DNA pools with the ENOL marker is illustrated in Fig. 3. As such, these ENOL- and VBP1-linked BAC clones most likely delimited the location of *sickle* within the corresponding physical region. Similarly, the two *sunn*-linked markers, DNABP and NUM1, identified two positive BAC clones, one for each marker (Table 1),

Table 1. Results from screening BAC DNA pools using polymerase chain reaction with sequence-characterized molecular markers

Primer	Linkage group	No. of positives	BAC address (insert size in kb)[a]
MDH2	1	2	07H21 (130), 15L10 (135)
ACCO	2	5	06M21 (115), 13G16 (160), 25J04 (145), 48I16 (105), 60I19 (125)
PRTS	3	4	09F01 (105), 09M14 (95), 09N13 (115), 46P18 (155)
DNABP	4	1	14C21 (115)
NUM1	4	1	08E16 (105)
PTSB	5	5	07A16 (100), 10G13 (110), 22B19 (115), 41B07 (100), 52D23 (90)
FAL	5	1	07I17 (110)
CrS	6	4	14D14 (125), 20J23 (125), 28F22 (85), 55K13 (120)
CAK	7	0	
ENOL	7	2	22H02 (125), 57C05 (5)
VBP1	7	1	37E04 (120)
CDC16	8	1	45L13 (50)
SDP1	8	0	

[a]Positive BAC DNA was isolated and analyzed by NotI digestion and PFGE.

that also likely determined the physical boundaries of the *sunn* locus. An insert size estimation revealed that, except for the ENOL-linked 57C05, the BAC clones all carried inserts that were sufficiently large to serve as cores for physical mapping around the targeted regions (Table 1).

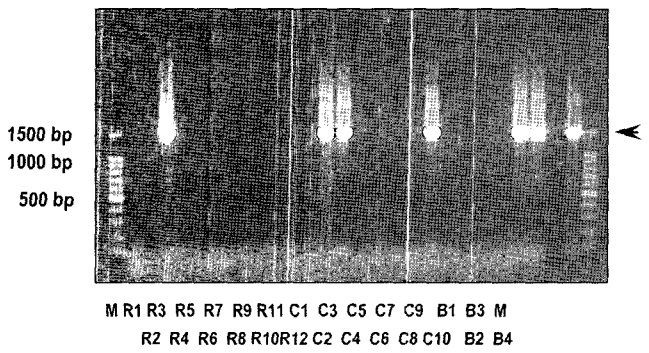

Fig. 3. PCR screening of BAC DNA pools with primers specific to the ENOL marker.
An initial set pool screening was carried out, and two positive sets, #11 and #29, identified (data not shown). The 12 row (R1–R12) and 10 column (C1–C10) superpools from set #11 were analyzed by PCR amplification, as shown. The positive signals from the row (R3 and R12) and column (C1 and C6) superpools were then used to deduce the coordinates (2H and 02) of the original BAC clone. Consequently, the address of the positive BAC clone was identified as 22H02 (22H from row 2H in set #11, and 02 from column 02). M: 100-bp DNA size standards; B1: 22H02; B2: 57C05; B3: negative control without template DNA; B4: positive control with genomic DNA. The arrowhead indicates the expected size (~1,500 bp) of the PCR products.

DISCUSSION

To facilitate map-based cloning of the hypernodulating genes of the model legume *M. truncatula*, a new BAC library was constructed using large BamHI-digested genomic DNA fragments. The library included a total of 2,223 Mbp of nuclear DNA, equivalent to approximately 4.9 haploid genomes of *M. truncatula*. The probability of any particular sequence being found at least once in the library was estimated to be greater than 95% [39]. Thus, the apparent capacity for genome representation by this new library is equivalent to that of the previously constructed HindIII library [22].

Like many recently constructed plant BAC libraries [7, 23, 33, 37], the present BamHI library was constructed using HMW DNA embedded in agarose plugs. When compared with agarose microbeads [10, 22], the plugs were expected to increase the yield of double-size-selected DNA fragments and provide higher quantities of restriction enzyme-accessible DNA per unit volume of digestion mixture [23, 33]. These characteristics typically enable the effective removal of fragments smaller than ~90 kb in size. Indeed, the insert size distribution of the randomly selected BAC clones revealed that the percentage of small (<90 kb) DNA fragments was as low as 2.3% (Fig. 2). Presumably, the relative absence of small interfering DNA fragments elevated the probability for larger inserts to ligate with the vector. Consequently, these factors contributed to an increase in the average insert size (116 kb) compared with that (100 kb) for the previous HindIII library [22]. However, despite the substantial increase in the insert size, the percentage of no insert-carrying clones was still relatively high (18.5%), probably due to overdigestion or overdephosphorylation of the cloning vector.

When the randomly selected BAC clones were analyzed by PFGE and gel-blot hybridization, only four out of 30 clones were shown to contain more than two NotI-digested inserts (Fig. 1A). This relative absence of NotI sites in the *M. truncatula* genome is a common characteristic of dicotyledonous species [7, 10, 22, 23, 37]. As estimated by C_0t-1 DNA hybridization, the repetitive DNA content in the library was approximately 70% (Fig. 1B). However, only 37% of the clones hybridized relatively strongly, indicating the presence of moderately or highly repetitive sequences in this proportion of the hybridized clones (Fig. 1B). These and previous estimates from the HindIII library [22] suggest that the *M. truncatula* genome is composed of a significant amount of repetitive DNA.

In the mapping population derived from *M. truncatula* ecotypes A17 and A20, most molecular markers have been primarily developed using PCR technology, such as CAPs or dCAPs [8]. To efficiently screen for such sequence-specific markers, a multiplexed DNA pool system was

constructed for rapid identification of the BAC clones. When compared with the previously constructed DNA pool system from the HindIII library [22], the BamHI BAC DNA pools were expected to increase the efficiency and economy of the library screening by avoiding the use of all the pools for every marker based on limiting the range of plates to be screened to the few that turned out to be positive from the initial set screening. Some of the sequence-characterized markers used in this study were linked to important nodulation genes in specific linkage groups. For example, PTSB is linked to *sym2* [13] in Linkage Group 5, whereas CDC16 is linked to *dmi3* [19] in Linkage Group 8 [8]. Nonetheless, despite the effectiveness of the DNA pool screening, as demonstrated by the results, two markers failed to identify positive clones (Table 1). Moreover, the average number of BACs identified (2.4) was substantially smaller than that expected from the calculated genome coverage (4.9), apparently reflecting an underrepresentation of particular genomic sequences in the library. Yet, the amplification of specific sequences from the initial set pools may also have been inefficient, since when compared with the moderately high clone complexities in the row and column superpools (1/192–1/96), the clone complexity in the set pools was fairly low (1/768), thereby decreasing the probability for the amplification of rare DNA fragments. Partly supportive of this view, BAC filter hybridization with a single-copy gene *ENOD8* [12] as a probe identified ten positive BACs, of which eight hybridized strongly and two weakly (data not shown).

The *sickle* and *sunn* loci are two distinct hypernodulating genes of *M. truncatula*. Thus, identification of the linked BAC clones is the first step toward positional cloning of these biotechnologically important genes. For example, the *sickle*-linked BACs can be analyzed to develop additional genetic markers between ENOL and VBP1 in Linkage Group 7, enabling more effective physical mapping within a narrower chromosomal range. Furthermore, it would be extremely useful to identify an ethylene-insensitive gene from the designated genomic region. Since *sickle* seedlings exhibit typical ethylene-insensitive phenotypes [24], a candidate gene for *sickle* could then be easily isolated by functional complementation. Although this targeted approach was unsuccessful with the EIN3 gene [22], continued searches using the currently available genetic map [8] may still prove worthwhile.

The two molecular markers, DNABP and NUM1, are tightly linked to the *sunn* locus [8]. In fact, the DNABP marker is so tightly linked to *sunn* that no recombinant has been found among the 93 individuals in the F_2 mapping population [28]. Therefore, the BAC clone positively identified herein (14C21) provides a convenient landmark for chromosomal landing [32]. Contig assembly and sequencing of the overlapping BAC clones around this BAC clone will likely provide crucial information for the isolation of *sunn*, the most spectacular supernodulating gene of *M. truncatula* [25, 28].

The BamHI library constructed herein can easily complement the preexisting BAC libraries of *M. truncatula*. Despite the seemingly extensive genome coverage predicted by the sizes of the (×25) HindIII and EcoRI libraries, genome-wide contig assembly still occasionally faces an unexpected lack of clones that can connect isolated segments of contiguous BAC clones (D. R. Cook, personal communication). Accordingly, because different restriction enzymes often exhibit varying accessibilities to a particular genomic region [37], and alternative DNA fragments generated with multiple restriction enzymes can significantly increase the capacity of genome coverage [7, 33], the new BamHI library is expected to facilitate connection between such isolated BAC contigs. Indeed, such a possibility is currently being explored, as the new BamHI library is now being used for the construction of a complete physical map of the 3rd chromosome of *M. truncatula* and determination of its nucleotide sequences (F. Debelle, INRA-CNRS, France, personal communication). Consequently, these concerted efforts will eventually promote the feasibility of isolating, characterizing, and utilizing biotechnologically important genes in legume-microbe interactions.

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