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ORIGINAL PAPER

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Construction of a binary BAC library for an apomictic monosomic addition line of *Beta corolliflora* in sugar beet and identification of the clones derived from the alien chromosome

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Abstract A plant-transformation-competent binary BAC library was constructed from the genomic DNA of the chromosome 9 monosomic addition line of *Beta corolliflora* Zoss. in sugar beet (*B. vulgaris*. L). This monosomic addition line (designated M14) is characterized by diplosporic reproduction caused by the alien chromosome carrying the gene(s) responsible for diplospory. The library consists of 49,920 clones with an average insert size of 127 kb, representing approximately 7.5 haploid genome equivalents and providing a greater than 99% probability of isolating a single-copy DNA sequence from the library. To develop the scaffold of a physical map for the alien chromosome, *B. corolliflora* genome-specific dispersed repetitive DNA sequences were used as probes to isolate BAC clones derived from the alien chromosome in the library. A total of 2,365 positive clones were obtained and arrayed into a sublibrary specific for *B. corolliflora* chromosome 9 (designated *bc*BAC-IX). The *bc*BAC-IX sublibrary was further screened with a subtractive cDNA pool generated from the ovules of M14 and the floral buds of *B. vulgaris* by the suppression subtractive hybridization method. One hundred and three positive binary BACs were obtained, which potentially contain the genes of the alien chromosome specifically expressed during the ovule and embryo development of M14, and may be associated with apomictic reproduction. Thus, these binary BAC clones will be useful for identification of the genes for apomixis by genetic transformation.

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Introduction

Apomixis is a plant reproductive mode that is of great significance for hybrid seed production and heterosis utilization in agriculture and for studies of plant reproductive biology. Therefore, isolation and characterization of the gene(s) controlling apomixis has attracted the interest of many scientists. We are working towards the isolation and characterization of the genes controlling apomixis in an apomictic monosomic addition line of *Beta corolliflora* Zoss. in sugar beet, designated M14. This monosomic addition line was isolated by a wide cross between cultivated sugar beet and *B. corolliflora*. Cytological studies showed that it contains the 18 normal chromosomes of sugar beet plus the *B. corolliflora* chromosome 9 (Guo et al. 2001), and the alien chromosome has an average transmission frequency of 96.7% through eggs (Fang et al., submitted). The reproduction mode of M14 was characterized as *Allium-odorum* type diplospory, and the gene(s) or a linkat responsible for apomixis were located on the alien chromosome (Fang et al., submitted).

Several approaches, including map-based cloning, transposon tagging and inverse genetics combined with a candidate gene approach, are being used to clone the genes controlling apomixis in some plant taxa. For transposon tagging, sample sizes much larger than those of the model plant species are needed because most apomicts are polyploid and have much larger genomes. As a result, the probability of knocking out the apomixis reproduction behavior by transposon and/or T-DNA random insertion is relatively low. Moreover, although this approach is efficient for the traits controlled by single genes, it might not be effective if apomixis is genetically complex, i.e., controlled by multiple genes, which has

been observed in several published works (Savidan et al. 2001). In spite of this, transposon tagging and T-DNA insertion mutation was carried out in *Hieracium* and two mutants with dysfunctional apomixis were isolated (Bicknell et al. 2001). This is a promising method; however, it does not appear to be suitable in our case not only because an available transposon system has not been developed in M14 or sugar beet to our knowledge, but also the structure of this plant is much bigger than that of *Hieracium* or certain other model plants. A large population of M14 theoretically saturated for transposon or T-DNA insertion would consume too much field space, and require intensive labor.

Isolation of the genes for apomixis by map-based cloning is also extremely challenging although several groups have made promising progress toward this end. One of the key steps to the success of map-based cloning is the ability to genetically position and fine map the trait of interest to its locus using molecular markers. This approach needs a well-characterized genetic background to facilitate marker development. Although the inheritance of sugar beet has been well studied, and several genetic linkage maps are available (Pillen et al. 1992; Barzen et al. 1995; Uphoff et al. 1995; Weber et al. 1999), little is known about *B. corolliflora*. Futhermore, apomictic reproduction itself overrides certain processes essential to the analysis of inheritance for determination of the position of the apomixis loci (Sherwood 2001). In the crossing of apomicts with their sexual counterparts to generate segregating populations, most apomicts can only serve as pollen donors because of the absence of meiosis during megasporogenesis and fertilization-independent embryogenesis (Sherwood 2001). This is a significant limitation for M14 that was found to be almost male sterile in reciprocal crosses with *B. vulgaris* (Fang et al., submitted). Finally, recombination repression at the apomixis loci, for both aposporic and diplosporic variants, has been reported in several well-studied apomictic taxa, including *Pennisetum squamulatum* (Ozias-Akins et al. 1998), *Paspalum simplex* (Pupilli et al. 2001), *Tripsacum dactyloides* (Grimanelli et al. 1998) and *Erigeron annuus* (Noyes and Rieseberg 2000). The existence of recombination repression would make map-based cloning difficult because markers that are closely linked genetically may be at great physical distances from the apomixis loci (Grossniklaus and van Dijk 2001). In the absence of recombination, physical mapping becomes a tedious but essential process for ordering molecular markers associated with the trait (Ozias-Akins et al. 1998).

We report here the construction of a large-insert, plant-transformation-competent binary BAC library for M14 and the identification of BAC clones covering the alien chromosome. We also developed a subtractive cDNA pool corresponding to the reproduction of M14 by forward suppression subtractive hybridization (Diatchenko et al. 1996) between the cDNAs from the ovules of M14 and the floral buds of sugar beet. This subtractive cDNA pool was used as a probe to screen the *B. corolliflora* chromosome-specific BAC clones to identify clones containing genes potentially responsible for diplosporic reproduction in M14. Large DNA fragment transformation for the identification of apomixis gene(s) will be discussed.

Materials and methods

Plant materials

Seeds of M14 were germinated in a greenhouse, and young plants were subjected to cytological analysis and dot-blot hybridization to confirm the presence of the extra chromosome from *B. corolliflora*. Cytological analysis was described by Liu et al. (1996). For the dot blot hybridizations, plant genomic DNA was extracted according to Ausubel et al. (1995) and the *B. corolliflora* genome-specific dispersed repetitive DNA sequence pBC1054, which was kindly provided by Dr. Christian Jung (Institute of Crop Science and Plant Breeding, Christian-Albrechts University, Kiel, Germany) was used as a probe. The young leaves of positive plants (almost all the plants) were used for isolation of high-molecular-weight (HMW) DNA for BAC library construction.

BAC library construction

The plant-transformation-competent binary vector pCLD04541 (Tao and Zhang 1998) was prepared as described by Zhang et al. (1996). HMW DNA preparation, size selection of partially digested HMW DNA, BAC cloning and library construction were carried out according to Zhang et al. (1996). Nuclei were extracted from about 100 g leaves collected from young positive M14 plants, resuspended in 4 ml of 1% low-melting-point (LMP) agarose, and pipetted into molds to make plugs (100 μl/plug). The solidified plugs were transferred into 10 volumes of lysis buffer (0.5 M EDTA, pH 9.0, 1% lauryl sarcosine, and 1 mg/ml proteinase K), and incubated at 50°C with gentle shaking for 24 h. The HMW DNA was purified in the plugs by successive washes in 20 volumes of 0.5 M EDTA (pH 9.0) for 1 h at 50°C and 20 volumes of 50 mM EDTA (pH 8.0) for 1 h on ice, and then stored in 50 mM EDTA (pH 8.0) at 4°C.

Before partial digestion with *Bam*HI, the plugs were washed three times (1 h each time) in 20 volumes of ice-cold TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) plus 0.1 mM phenylmethylsulfonyl fluoride, and three times (1 h each time) in 20 volumes of ice-cold TE. The plugs to be subjected to partial digestion were cut into smaller pieces approximately equal in size, equilibrated on ice for 1 h in 1×React 3 buffer (Gibco BRL, USA) plus 2 mM spermindine, 1 mM DTT, and 0.2 mg/ml BSA. The equilibrated plug slices were transferred into the fresh ice-cold reaction mixture containing 0.6 U/tube of *Bam*HI, incubated on ice for 1 h, and then incubated in a 37°C water bath for 7 min. The reactions were stopped by adding 1/10 volume of 0.5 M EDTA (pH 8.0). Partially digested DNA was fractionated by pulsed-field electrophoresis on a 1% agarose gel using the CHEF DRIII (Bio-Rad, USA) in 0.5×TBE buffer (45 mM Trizma base, 45 mM boric acid, 1.0 mM EDTA, pH 8.3) for 18 h. The CHEF apparatus was set at 6 V/cm, 12°C, 120° angle and a switch time of 90 s. After electrophoresis, DNA fragments ranging from 100 to 300 kb were excised from the gel and subjected to a second size selection on a 1% agarose gel using conditions that compress the DNA fragments larger than 100 kb into a thin band (4 V/cm, 5 s switch time, 10 h at 12°C in 0.5×TBE). The compressed DNA band was cut out of the gel and recovered from the gel slices by electroelution. The recovered DNA solution was dialyzed against 1,000 volumes of ice-cold 0.5×TE twice for 8 h at 4°C.

The dialyzed DNA was collected, quantified and then ligated into the *Bam*HI-digested and dephosphorylated pCLD04541 vector at a molar ratio of vector: insert DNA of 4:1 in the presence of 2.0 U T4 DNA ligase (Gibco BRL) per 100 μl ligation mixture. The ligation mixture was directly used to transform *E. coli* DH10B competent cells (Gibco BRL) by electroporation. The device for

electroporation (Cell Porator and Voltage Booster System, Gibco BRL) was set at 350 V, 330 μF capacitance, low ohm impedance and fast charge rate with the Voltage Booster at 4 kΩ resistance. Each 20 μl aliquot of -transformed cells was diluted with 1 ml SOC medium (Sambrook et al. 1989) and cultured at 37°C for 1 h before being plated on selective LB agar medium (Sambrook et al. 1989) containing 15 μg/ml tetracycline, 0.55 mM IPTG, and 80 μg/ml X-gal. White colonies were arrayed into 384-well microtiter plates containing 50 μl of freezing medium (Zhang et al. 1996) in each well, incubated overnight at 37°C, and stored at −80°C. From the original copy of the BAC library, two more copies were duplicated and stored in separate −80°C freezers.

Estimation of BAC insert sizes

BAC clones were grown overnight at 37°C in 5 ml LB broth (Sambrook et al. 1989) containing 15 μg/ml tetracycline with violent agitation. BAC DNA was isolated using the alkaline lysis method (Sambrook et al. 1989). To estimate the insert sizes, 2 μl of each BAC DNA miniprep was digested with 0.1 U *Not*I (Gibco BRL) in a 20 μl reaction. The digests were separated by pulsed-field gel electrophoresis on a 1% agarose gel. Electrophoresis was run in 0.5×TBE buffer with an initial pulse time of 5 s, a final pulse time of 15 s, and a voltage of 6 V/cm at 12°C for 16 h. The gel was stained with ethidium bromide and photographed.

BAC library screening and Southern analysis

High-density colony filters were prepared using the GeneTAC Robotic Workstation (Genomic Solutions, USA). Each BAC clone of the library was gridded in double spots using a 3×3 array on 8×12 cm Hybond-N+ membranes (Amersham-Pharmacia Biotech, USA), each filter thus containing 1,536 clones. The clones were grown on the filters placed on LB agar plates containing 15 μg/ml tetracycline at 37°C for 16 h, and the filters were processed according to Zhang et al. (1996).

For BAC library screening, DNA probes were prepared from the *B. corolliflora* genome-specific, dispersed repetitive DNA sequence clones pBC1054, pBC305 and pBC227 (Gao et al. 2000) by PCR using primers KS and SK, during which [^{32}P]-dCTP was incorporated. Labeled DNA was purified using PCR purification columns (Qiagen) and hybridization was carried out according to Sambrook et al. (1989). Each set of eight filters was pre-hybridized in a bottle containing 160 ml hybridization buffer (5×SSC, 5×Denhardt's solution, 100 μg/ml salmon sperm DNA, and 0.5% SDS) at 65°C for 8 h. Hybridization was performed overnight at 65°C in 25 ml fresh hybridization buffer plus the denatured radioactive probe. Filters were washed at 65°C as follows: once in 1×SSC, 0.1% SDS for 20 min, then twice in 0.1×SSC, 0.1% SDS for 20 min each time. To estimate the number of clones that originated from chloroplast DNA, fragments of the *psb*A gene (Wu et al. 1987) and the *rbc*L gene (Nishizawa and Hirai 1987) from the rice chloroplast genome were used as probes to screen four of the library filters randomly selected. The fragments were labeled with [^{32}P]-dCTP by random priming according to the manufacture's instructions (Promega, USA).

To further verify the library screening results, DNA from the BACs randomly selected from the positive clones was digested with *Bam*HI and separated by electrophoresis on a 1% conventional agarose gel. Southern blots were prepared (Sambrook et al. 1989) and hybridized with the probe pBC1054.

Development of the subtractive cDNA pool

To develop a subtractive cDNA fragment pool corresponding to the reproduction of M14, forward suppression subtractive hybridization (SSH) was carried out according to Diatchenko et al. (1996) using mRNAs (2.0 μg) from the ovules of M14 as the tester and those from the flower buds of sugar beet (2.0 μg) as the driver. The floral buds of M14 were dissected and the ovules were isolated on ice using a dissecting microscope (Olympus) and quickly transferred into liquid nitrogen. About 300 mg ovules at definite development stages were obtained from thousands of floral buds of M14. Messenger RNA was isolated from the ovules of M14 and the floral buds of sugar beet using the QuickPrep micro mRNA purification kit according to the manufacture's instructions (Amersham Pharmacia). cDNA synthesis, adaptor ligation, hybridizations and PCR amplifications were performed according to the manufacturer's instructions (Clontech, USA). To estimate the positive frequency of the SSH, the products of the secondary amplification were cloned into pGEM-T Easy vector (Promega) and transferred into the bacterial strain XL1-Blue. About 1,000 recombinant clones were randomly selected, spotted on Hybond-N+ membrane and subjected to reverse Northern blot (Zegzouti et al. 1997) with reverse transcribed mRNA from the floral buds of M14 and sugar beet as probes. To estimate the size of the cDNA fragments, DNA was prepared from randomly selected recombinant clones using the alkaline lysis method (Sambrook et al. 1989), digested with *Eco*RI, and separated on a 2% agarose gel by electrophoresis. The insert sizes of cDNA fragment clones were estimated by comparison to the 200 bp DNA ladder.

For the use of the subtracted cDNA pool as a probe to screen *B. corolliflora* genome-specific BAC colonies the first-round PCR amplification products from the subtractions were labeled with [^{32}P]-dCTP during the secondary PCR amplification, and the products of this secondary PCR amplification were purified with QIAquick PCR purification Kit (Qiagen), then denatured before use.

Results

BAC library construction and characterization

We have constructed a plant-transformation-competent binary BAC library for an apomictic monosomic addition line of *B. corolliflora* in sugar beet (M14), with the aim at developing the scaffold of a physical map for the alien chromosome and ultimately for cloning the apomixis gene(s). The library consists of 49,920 clones stored in 130 384-well microplates. Random sampling of 280 BACs from the library indicated that it has an average insert size of 127 kb with a range of 50–240 kb (Fig. 1). Approximately 0.9% of the clones were derived from chloroplast DNA. Based on the haploid genome size of 758 Mb (Arumuganathan and Earle 1991) of sugar beet

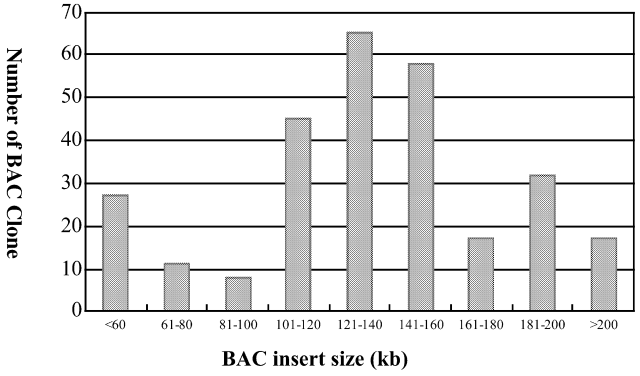

Fig. 1 Insert size distribution of clones randomly selected from the M14 binary BAC library. BAC inserts were released by digestion of BAC DNA with *Not*I, fractionated on a pulsed-field gel, and their sizes estimated by comparison with the lambda size markers

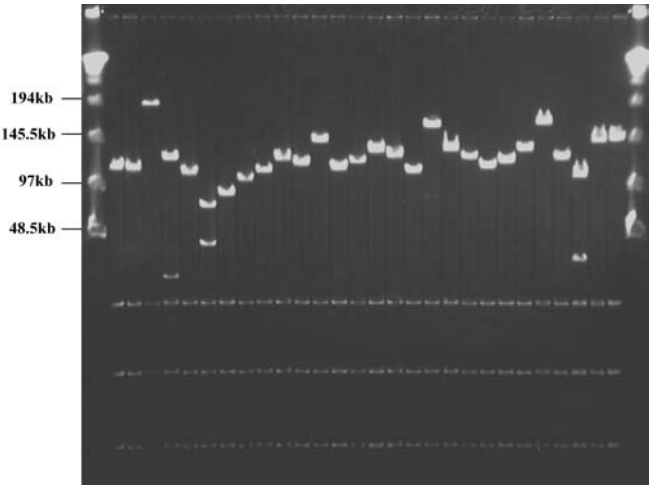

Fig. 2 Examples of randomly selected M14 binary BAC clones. The three common bands in all lanes were derived from the vector pCLD04541 whereas the remaining band(s) was from the inserts of the clones

plus the expected 80 Mb of *B. corolliflora* chromosome 9, the coverage of the library is about 7.5 equivalents of the haploid genome of the monosomic addition line, resulting in a probability greater than 99% of recovering any single-copy DNA sequence from the library. Figure 2 shows randomly selected BAC clones digested with *Not*I. As there are two other *Not*I sites on the cloning vector pCLD04541 besides the double sites flanking the multiple cloning site, the vector was digested into three fragments. Most BAC clones of M14 had one insert (Fig. 2). This is a typical pattern observed in the large-insert BACs of dicotyledonous plants that contain fewer *Not*I recognition sites than those of monocotyledon plants, such as rice, wheat and sorghum.

Library screening with *B. corolliflora* genome-specific dispersed repetitive sequences

Beta corolliflora genome-specific dispersed repetitive sequences pBC227, pBC305 and pBC1054 were kindly provided by C. Jung. These repetitive DNA sequences are dispersed over the whole of chromosome 9 of *B. corolliflora*, as revealed by chromosome fluorescent in situ hybridization and Southern hybridization of genomic DNA from *B. corolliflora* (Gao et al. 2000), and thus, are suitable for use as probes to isolate BAC clones originating from the alien chromosome in the BAC library. A total of 2,365 clones were obtained, which was consistent with the number of clones expected [80/(758× 2+80)× 49,920=2,502]. These clones were estimated to be equivalent to approximately 3.8 equivalents of the alien chromosome. To further verify the positive clones, DNAs were isolated from a set of random positive clones, digested with *Bam*HI, Southern blotted and hybridized with probe pBC1054 (Fig 3). At least one *Bam*HI fragment of each of the BACs hybridized strongly with the *B. corolliflora* genome-specific dispersed repetitive sequence probe at high stringency (0.1×SSC, 65°C), indicating that the clones originated from the *B. corolliflora* chromosome. To facilitate further analysis of these BACs, we re-arrayed the 2,365 positive BAC clones in 384-well microplates and developed them into a sublibrary specific for *B. corolliflora* chromosome 9 (*bc*BAC-IX).

Screening of the sublibrary specific for *B. corolliflora* chromosome 9 with a subtractive cDNA pool

We successfully developed a subtractive cDNA pool specific for the reproduction of M14 from the ovules of M14 and the floral buds of sugar beet by using the forward suppression subtractive hybridization (SSH) method (Diatchenko et al. 1996). The sizes of the subtractive cDNA fragments ranged from 200 to 400 bp (Fig. 4), with a positive frequency of approximately 40%,

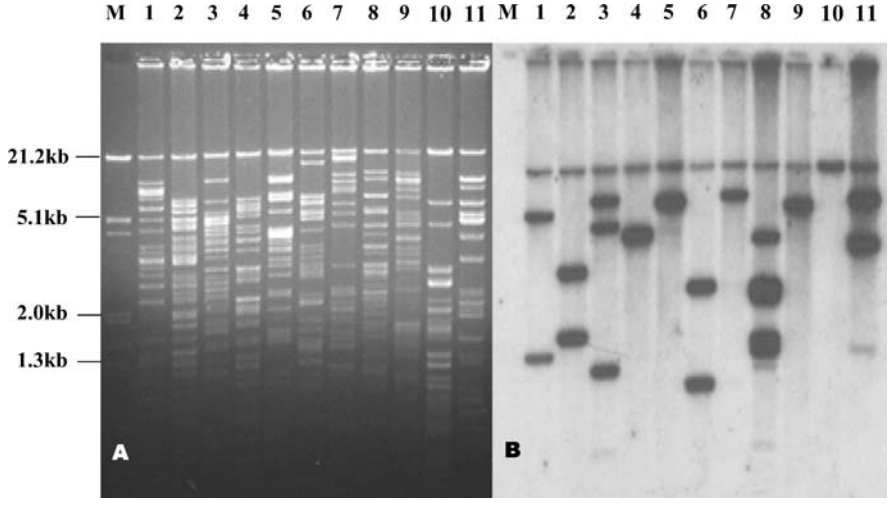

Fig. 3A, B BACs of *Beta corolliflora* chromosome 9 isolated with its genome-specific, dispersed repeated sequences. **A** Agarose gel of the BACs digested with *Bam*HI. *M* indicates lambda DNA *Eco*RI/*Hin*dIII marker and lanes 1–11 show randomly selected positive BAC clones. **B** Southern blot hybridization of the BACs with the pBC1054 probe

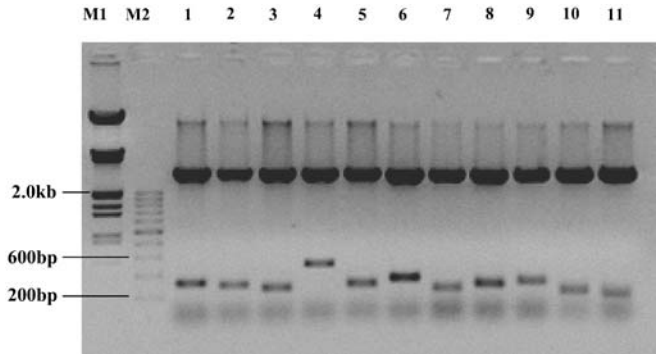

Fig. 4 An ethidium bromide-stained agarose gel of randomly selected subtractive cDNA clones. The DNA of the clones was isolated, digested with *Eco*RI to release their inserts and subjected to agarose gel electrophoresis. The insert sizes of the clones are arranged from 200 to 600 bp. *M1* indicates the lambda *Eco*RI/*Hin*dIII marker, *M2* indicates the 200 bp ladder, and the band around 3.0 kb appearing in all lanes is the linearized pGEM-T Easy vector

as revealed by reverse Northern hybridization (data not shown). This is a typical pattern of SSH products that are suitable to serve as a pooled cDNA probe. Filters of the sublibrary specific for *B. corolliflora* chromosome 9 were then screened with this subtractive cDNA pool, giving a total of 103 positive signals. These signals represented the BAC clones containing the genes specifically expressed in the ovule of M14 residing on the alien chromosome, and consequently may be correlated with the diplosporic reproduction in M14. Thus, they are candidate clones for identification of the genes controlling apomixis by large DNA fragment genetic transformation.

Discussion

We constructed a plant-transformation-competent binary BAC library for the *B. corolliflora* chromosome 9 monosomic addition line in sugar beet (M14). This library was estimated to have an average insert size of 127 kb and to be equivalent to 7.5 haploid genomes of the addition line, which contains not only the entire genome of sugar beet, but also the *B. corolliflora* chromosome 9 carrying the genes responsible for apomixis. Therefore, this library will be useful for isolation of the genes for apomixis in M14, as well as for genome research in sugar beet in general. Furthermore, because the library was cloned into an *Agrobacterium*-mediated, plant-transformation-competent binary vector pCLD04541 (Jones et al. 1992; Tao and Zhang 1998), it can be directly transformed into plants via *Agrobacterium*. Therefore, this library will streamline the identification of apomixis genes and large-scale functional analysis of sugar beet genome sequences by transformation.

We identified the BACs that originated from the *B. corolliflora* chromosome 9 and developed a sublibrary (*bc*BAC-IX) of clones specific for this chromosome. The sublibrary contains a total of 2,365 clones, providing genome coverage of approximately 3.8 equivalents of the alien chromosome (80 Mb). The genome coverage of the sublibrary is highly consistent with that which was expected. This sublibrary represents an important resource for the molecular characterization of the alien chromosome and the final isolation of the genes for apomixis. However, it is possible that this sublibrary may not completely cover the whole alien chromosome. This may be due to the use of the three dispersed repetitive DNA sequences for the M14 library screening. These repetitive sequences may not be evenly dispersed over the whole length of the chromosome such that at least one repeat is present in every 127 kb. Consequently, clones from the segments in which the repeats are not present will be absent from the sublibrary. Additionally, the BAC library was constructed from a partial digestion of genomic DNA using only one restriction enzyme (*Bam*HI), which could lead to preferential cloning given the uneven distribution of restriction sites throughout the genome. Furthermore, considering the difficulty of cloning certain sequences in bacteria it is possible that the BAC library may not completely cover the entire M14 genome. If this is the case, the problem could be easily minimized by developing more clones for the library using one or two new restriction enzymes in different vector and/or host systems. The problem of underrepresentation of repeat sequences could be overcome by using genomic DNA of *B. corolliflora* as a probe, masked with an excess of sugar beet genomic DNA, to screen the M14 BAC library at high hybridization stringency as is widely used in genomic in situ hybridization. Furthermore, we are also trying to develop additional dispersed repeat sequences and/or marker sets that are specific for the alien chromosome by using genetic analysis and comparative genome studies.

The use of *B. corolliflora* repetitive DNA sequences to screen the M14 library revealed BAC clones spanning the regions rich in repetitive sequences. This experiment may have increased the chances of isolating BACs containing the apomixis locus. One of the postulated mechanisms to explain the recombination suppression seen at the apomixis locus is that this locus could be positioned in centromeric, paracentromeric, or other heterochromatic regions rich in repetitive sequences known to be characterized by suppressed recombination (Ozias-Akins et al. 1998; Pupilli et al. 2001). Since it seems to be difficult to saturate the recombination-suppressed chromosomal regions with molecular markers (Ozias-Akins et al. 1998), which has frustrated the conventional map-based cloning, the use of suppression subtractive hybridization or mRNA differential display in conjunction with large-insert BAC libraries to obtain ESTs derived from recombination-suppressed regions has become promising. Combined with the binary BAC-based physical map of the alien chromosome, we hope to order the ESTs expressed specifically during the reproduction of M14 that were isolated in this study along the physical map and determine their relationships with each other. The ESTs of interest will be analyzed further, and at the same time,

the BAC clones that contain the promising ESTs will be subject to plant transformation for identification of the genes for apomixis.

Successful transformation of large DNA fragment via binary BAC or TAC has been reported by Hamilton et al. (1996, 1999) and Liu et al. (1999). This system would make it feasible to study the expression of plant genes or gene clusters in their native genomic context and might eliminate genomic site-dependent gene expression. Therefore, it would be applicable for the isolation of genes that encode complex quantitative traits as well as genes within complex loci located in a chromosomal region of low recombination frequency (Hamilton et al. 1996; Liu et al. 1999). Combined with the physical map and genetic delimitation of the chromosomal region in which the apomixis locus resides, the genes for apomixis could be identified by genetic transformation of successive binary BAC clones in the contigs, or selective transformation of candidate binary BAC clones containing ESTs of interest within the delimitated chromosomal region. Thus, the practically impossible long distance chromosome walking and precisely positioning of the apomixis gene(s) in the conventional positional cloning could be avoided.

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