Verification of positive BAC clones near the *Rf1* gene restoring pollen fertility in the presence of the PET1 cytoplasm in sunflower (*Helianthus annuus* L.) and direct isolation of BAC ends

Sonia Hamrit[1], Barbara Kusterer[2], Wolfgang Friedt[2], Renate Horn[1]
[1]Institute of Biological Sciences, Department of Plant Genetics, University of Rostock, Albert-Einstein-Str. 3, 18051 Rostock, Germany, E-mail: sonia.hamrit@uni-rostock.de; renate.horn@uni-rostock.de
[2]Institute of Agronomy and Plant Breeding, Department of Plant Breeding, University of Giessen, Heinrich-Buff-Ring 26-32, 35392, Giessen, Germany,
E-mail: Kusterer@hybro.de, wolfgang.friedt@uni-giessen.de

ABSTRACT

Cytoplasmic male sterility (CMS) plays an important role in the production of hybrid seeds. In sunflower, commercial hybrid breeding is based on a single CMS-inducing cytoplasm, PET1. In this particular male sterility, which was derived from an interspecific cross between *Helianthus petiolaris* Nutt and *Helianthus annuus* L., CMS is caused by the expression of an aberrant mitochondrial gene that prevents the development of viable pollen. The restoration of pollen fertility in the presence of the PET1-cytoplasm has been reported to be controlled by two dominant nuclear genes (*Rf1* and *Rf*2). In a previous work, a linkage map has been constructed around the *Rf1* gene that consisted of 35 AFLP (amplified fragment length polymorphism) markers, 7 RAPD (random amplified polymorphic DNA) markers, and 1 SSR (simple sequence repeat) marker. These markers represent a good opportunity and an excellent basis for a map-based cloning approach. Markers tightly linked to the restorer locus *Rf1* have been used as overgo probes to be hybridized against sunflower BAC libraries. Positive BAC clones were identified and a putative closed contig around *Rf1* was constructed by fingerprinting. Here we report on: (i) the verification of the identified BAC clones near the *Rf1* gene and (ii) the inability of the DNA fingerprinting methodology alone for identifying overlapping DNA fragments that can be assembled into contigs, showed by using different restriction enzymes, and (iii) the description of a fast and efficient method to clone BAC ends into a high copy number vector based on double antibiotics selection.

Key words: BAC end – CMS – contig – fertility restoration – *Helianthus annuus* L.

INTRODUCTION

Cytoplasmic male sterility (CMS) is often associated with mitochondrial DNA rearrangements, resulting in the expression of chimeric genes believed to interfere with normal pollen development (Horn, 2006). In sunflower, commercial hybrid breeding is based on a single source of cytoplasmic male sterility, PET1, obtained from an interspecific cross between *Helianthus petiolaris* Nutt and *Helianthus annuus* L. (Leclercq, 1969). Detectable alterations in the mitochondrial genome of CMS and fertile lines are limited to a 17-kb-region and consist of two mutations: a 12-kb-inversion and a 5-kb-insertion/deletion, which lead to an altered transcript pattern of the *atp*A gene (Siculella and Palmer, 1988). CMS is associated with the expression of a novel open reading frame, *orf*H522 (Köhler et al., 1991), which encodes a 16-kDa polypeptide (Horn et al., 1991; Laver et al., 1991). Male fertility can be restored by the introduction of nuclear *Rf* (restorer of fertility) genes that compensate for this deficiency (Schnable and Wise, 1998). The isolation of *Rf* genes in sunflower may help to clarify the mechanism behind the expression of the CMS-associated mitochondrial gene.

The *Rf1* gene, responsible for fertility restoration in the presence of the PET1-cytoplasm, was mapped based on AFLP, RADP and SSR markers (Kusterer et al., 2002; Horn et al., 2003). A linkage map surrounding the restorer gene *Rf1*, which consists of 43 markers (35 AFLPs, 7 RAPDs, and 1 SSR) and covering 250.3 cM, has been constructed (Kusterer et al., 2005). These markers were used in a map-based cloning strategy as probes against the sunflower BAC library RHA325 (Özdemir et al., 2002, 2004) to identify positive BAC-clones near the restorer gene *Rf1*. This allowed the development of a preliminary putative contig around the restorer gene by fingerprinting (Kusterer et al., 2004). This contig could have provided a starting point for cloning the *Rf1* gene restoring pollen fertility in the presence of the sunflower PET1 cytoplasm.

Our objectives were: (i) To establish the resources and protocols necessary to verify the previously identified putative closed contig around the restorer gene using different restriction enzymes. We show

here that DNA fingerprinting alone is not a sufficient criterion for identifying overlapping DNA fragments that can be assembled into a contig. (ii) To present a method for isolating BAC ends from positive BAC clones. The use of BAC-end sequences (sequences adjacent to the insert sites) has been proposed as a means for selecting minimally overlapping clones for sequencing large genomic regions (Venter et al., 1996). Our aim was to develop a fast and efficient tool to obtain cloned BAC ends by ligating restriction fragments of BAC clones digested with *BamH*I into a universal cloning vector, followed by double antibiotics selection.

MATERIALS AND METHODS

The first BAC library reported here has been constructed from the restorer line RHA325 (an American public restorer line carrying the PET1 cytoplasm) using *Hind*III fragments and the pBeloBAC11 vector. The library has a 1.9 fold genome coverage and an average insert size of 60 kb (Özdemir et al., 2004). The second BAC library, constructed from the maintainer line HA383, was developed for Steven Knapp and is distributed by Clemson University Genomic Institute (CUGI, http://www.genome.clemson.edu/capabilities/bacCenter.shtml).

For cloning BAC ends, we used a modified plasmid end rescue method (Kelley et al., 1999). Ten µl of DNA from the positive BAC clones were digested with the restriction enzyme *BamH*I for 3 hours at 37°C. Four µl of the digested DNA was ligated into pUC18 in 20 µl final solution of 10 x ligase buffers, sterile H_2O, and T4 DNA ligase for 16 hours at 4°C. Prior to ligation, the vector pUC18 had been digested with the restriction enzyme *BamH*I and dephosphorylated with calf intestinal alkaline phosphatase. In the mean time, *E. coli* (DH5α) bacteria were treated with calcium chloride to make them competent. For the transformation of *E. coli*, 4 µl ligation was added to 100 µl of competent *E. coli*, incubated on ice for 20 min, and heat shocked at 42°C for exactly 60 sec. The tubes were immediately returned to ice for a minimum of 5 minutes. Transformed cells were incubated in 250 µl SOC media for 1 hour at 37°C, plated on LB agar medium containing X-Gal, IPTG, and two antibiotics for selection: chloramphenicol (12 µg/ml) for the pBeloBAC11 vector and ampicillin (50 µg/ml) for the pUC18 vector. The cultures were incubated at 37°C overnight to allow the colonies to grow. Only colonies containing pBeloBAC11 and pUC18 could grow on both antibiotics. To estimate the insert size of the cloned BAC end, DNA from the obtained clone was isolated by the alkaline lysis method, resuspended in 100 µl H_2O (for sequencing purpose), completely digested with *BamH*I and separated on a 0.8% agarose gel. For cycle sequencing, we used the SP6 universal primer for the promoter that flanks one cloning site of the pBeloBAC11 vector.

RESULTS AND DISCUSSION

In previous studies, a linkage map for the *Rf1* gene was constructed using a segregating F_2 population of the cross between RHA325 (restorer line carrying the PET1 cytoplasm) and HA342 (maintainer line). Forty-three markers (7 RAPD-markers, 35 AFLP-markers and 1 SSR-marker) were identified that were localized on both sides of the restorer gene (Fig. 1). This corresponds to an average marker distance of 5.8 cM for this linkage group and a marker density of 1/0.56 cM in an area of 3.9 cM around the restorer gene *Rf*1. This area consists of two RAPD and five AFLP markers.

The closest markers from both sides of the restorer gene *Rf1*, OP-K13_454 and E33M61_136 were used as probes against the BAC library RHA325 to identify positive BAC clones (Kusterer et al., 2004). Five positive BAC clones were identified. BAC fingerprinting using *Hind*III as restriction enzyme was performed. The BAC clones 67I5 and 67N4 showed an identical banding pattern, which overlapped with the smaller BAC clone 59J13 from one side. From the other side, the BAC clones 22408 and 22407 also shared *Hind*III fragments. The banding pattern was confirmed by Southern hybridization using the *Hind*III digested BAC clone 67N4 as probe. A 3.7-kb-fragment shared between the clones 224O8, 67N4 and 67I5 was identified. This procedure allowed the development of a preliminary putative contig at the restorer gene *Rf*1. This could also be an indication that the contig around the restorer gene *Rf*1 might be closed (Fig. 2)

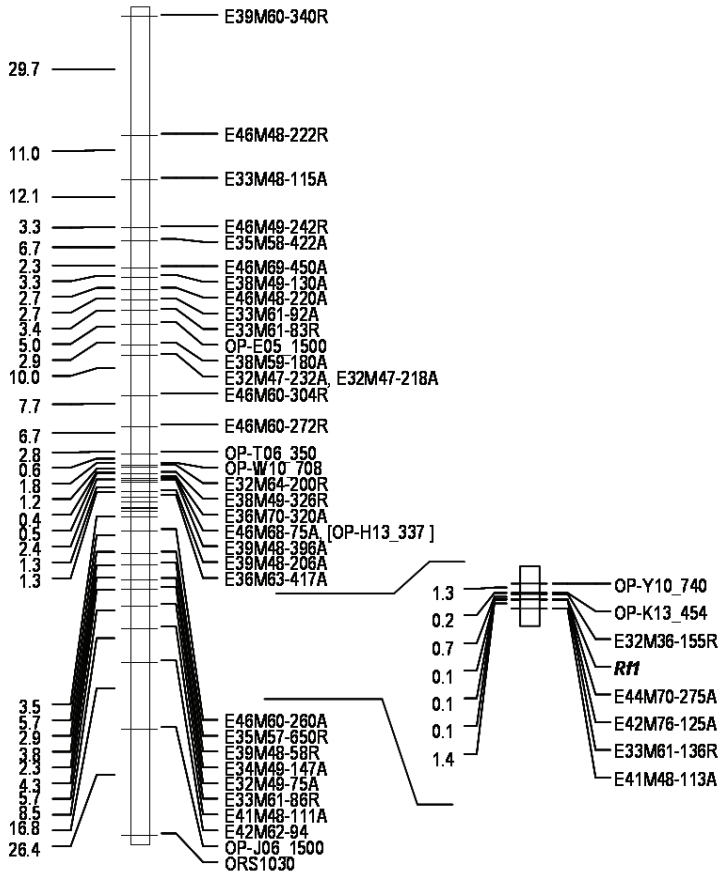

Fig. 1. Marker-saturated map of the linkage group surrounding the restorer gene *Rf1* of sunflower. A region of 3.9 cM around the *Rf1* gene is shown enlarged (Kusterer et al., 2005).

The putative closed contig around the *Rf1* gene of the PET1 cytoplasm needed to be verified. The 3.7 kb-fragments from the BAC clones 67N4 and 22408 were cloned. Four restriction enzymes *Hind*III, *Pst*I, *Eco*RI and *Kpn*I were used to verify the identity of the putative overlapping 3.7-kb-fragment (Fig. 3). Surprisingly, the restriction patterns obtained for these clones with *Pst*I and *Eco*RI were not identical. These digests proved that we had cloned two different fragments and have no closed contig around the *Rf1* gene, so far (Fig. 2).

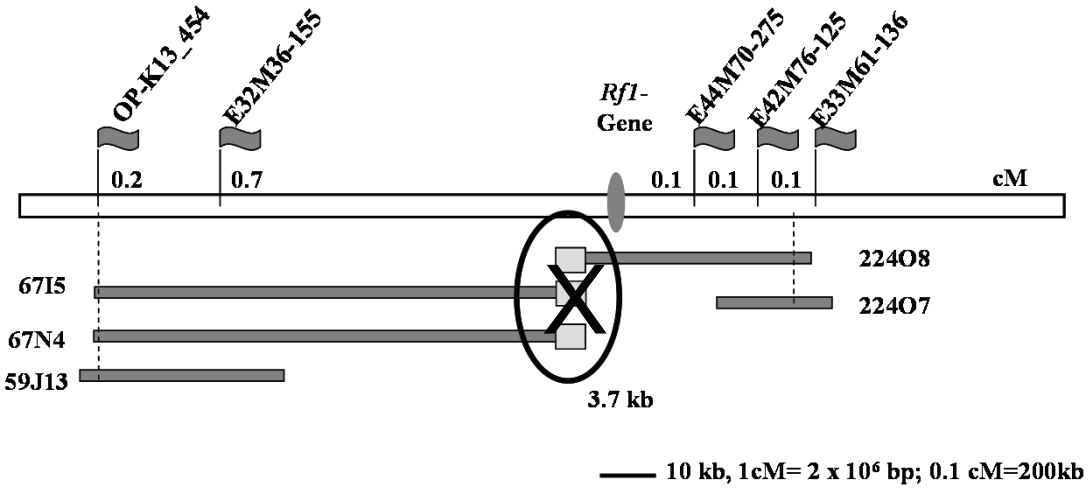

Fig. 2. Preliminary putative contig around the restorer gene *Rf1*.

In addition, we sequenced the 3.7-kb-fragment from the 67N4 BAC clone. The analysis showed homology to a retrotransposon, which might explain the observed cross hybridization between the 3.7-kb-fragments of the two BAC-clones 67N4 and 22408. It has been reported that overlaps can be detected simply by hybridization but it is not a satisfactory criterion because dispersed repeats can generate false-positive (Hong et al., 1997). We believe that to obtain reliable fingerprints, digestion with several restriction enzymes should be part of the whole process.

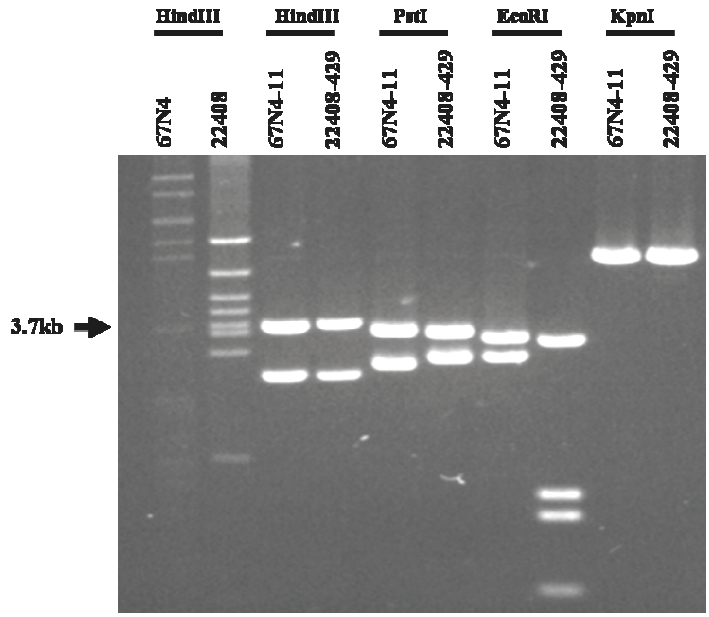

Fig. 3. Investigation of the putative closed contig. The cloned 3.7-kb-fragments from 67N4 and 22408 BAC clones were digested with four restriction enzymes. The intensity of the sub-cloned fragments is due to the high copy number of the vector pUC18.

The RAPD marker OP-K13_454 was also used for hybridization against the BAC library of the line HA383 (maintainer line), developed for Steven Knapp and distributed by Clemson University Genomic Institute (CUGI, http://www.genome.clemson.edu/capabilities/bacCenter.shtml). Four positive BAC-clones 216F17, 307N02, 225D09 and 401E15 were identified that, according to the obtained *Hind*III-BAC-fingerprinting data, belonged to one contig.

The first objective of this study was to identify positive BAC clones near the *Rf1* gene restoring male fertility in sunflower in order to conduct a map-based cloning strategy for the isolation of the gene. This involves screening of BAC libraries with cloned markers and the assembly of contigs. Since chromosome walking requires the isolation of the BAC ends to be used as probes for further steps, we developed a method for an efficient and reliable isolation of BAC ends.

Fig. 4 shows a scheme to isolate BAC ends by cloning restriction fragments of BAC clones. In our case, we used *BamH*I that cuts several times within the insert and once within the BAC vector, followed by ligation into the pUC18 vector, transformation of *E.coli*, and plating on double antibiotics selection media. When using two antibiotics: ampicillin resistance for the pUC18 vector and chloramphenicol resistance for the pBeloBAC11 vector, we could be sure that the resulting plasmid in pUC18 contained the BAC vector pBeloBAC11 and a *BamH*I-BAC-end as insert.

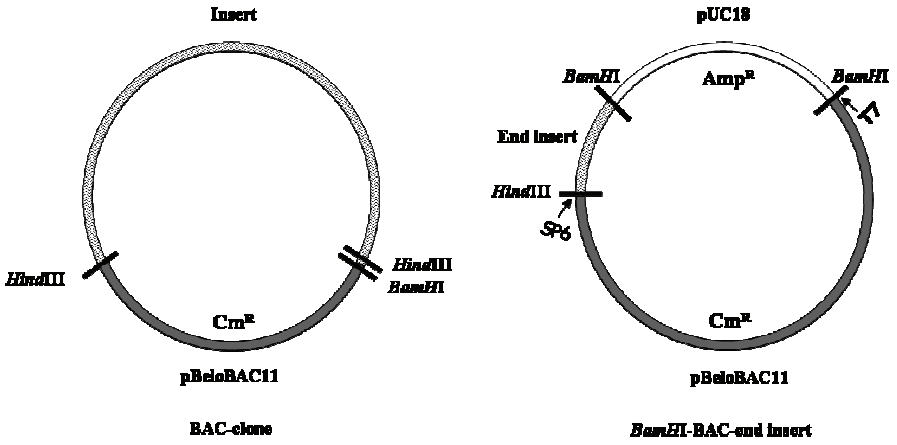

Fig. 4. Schematic diagram of the generation of end-specific probes from BAC clones. Digestion of the BAC clone with the restriction enzyme *BamH*I cuts the insert of the BAC clone into several fragments, one containing the *BamH*I-BAC-end together with the pBeloBAC11 vector as *BamH*I has only one restriction site within pBeloBAC11. Ligation of the *BamH*I-BAC-end into pUC18 combined with a double antibiotics selection results in clones with a high copy number vector construct inside.

During chromosome walking at the restorer locus, using this strategy for BAC-end cloning we were able to isolate 17 BAC-ends out of 21 positive BAC-clones, which had been identified by various overgo probes.

The cloned *BamH*I-BAC-ends were released from the pUC18 vector by complete digestion with *BamH*I (Fig. 5A). For the failure of cloning the four remaining BAC ends, possible explanations could be that the restriction fragments generated by *BamH*I digestion were either too large for an efficient ligation or too small to be detected. To check the reliability of our method, we performed additional hybridizations using pBeloBAC11 as probe against the cloned BAC ends. The results confirmed that all obtained sub-clones contained the pBeloBAC11 vector and a BAC end (Fig. 5B) as all obtained fragments gave a hybridization signal and had a size larger than the size of the pBeloBAC11 vector (7.5 kb). Knowing the sequence of the pBeloBAC11, the BAC-end sequence could be easily separated from the vector sequence.

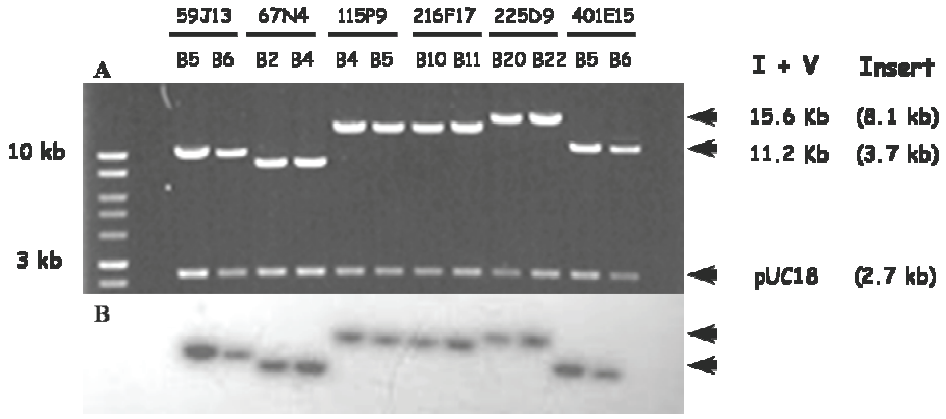

Fig. 5. *BamH*I digest of six cloned BAC ends. **(A)** For higher reliability, at least two sub-clones for each BAC end were analysed. Two fragments were obtained in each digest: one for the pUC18 vector (2.7 kb) and a second fragment with a size larger than the vector pBeloBAC11 (7.5 kb). **(B)** Hybridization of the BAC ends 59J13, 67N4, 115P9, 216F17, 225D9, and 401E15 digested with *BamH*I against pBeloBAC11 vector as probe. The larger *BamH*I-fragments harbouring the cloned BAC ends hybridized to the pBeloBAC11 vector probe, which means that the pBeloBAC11 vector is definitely part of these inserts. I= *BamH*I-BAC-end, V= pBeloBAC11

We believe that this method to clone BAC ends, despite having just one end, is simple, fast, very reliable, and not costly. First, there is no risk of cloning non-target fragments because of the double antibiotics selection. Second, due to cloning into pUC18, the cloned BAC end is present in a high copy vector, which makes minipreparations using the simple alkaline lysis method much more efficient.

The sequences of the BAC ends are now used to develop markers that allow the back mapping of these BAC clones and contigs to the restorer gene *Rf1*. Markers developed from the BAC end of 216F17 proved that the contig of the BAC clones 216F17, 307N02, 225D09 and 401E15 is not localized on the linkage group of the *Rf1* gene.

Our study proved that there are a lot of pitfalls in map-based cloning. However, more steps of chromosome walking will be performed by verification of the new obtained BAC clones. Furthermore, new AFLP markers will be identified and mapped to the restorer gene to saturate the region of the gene with more markers, especially with markers cosegregating with the *Rf1* gene.

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