Identification of a BIBAC clone that co-segregates with the petunia Restorer of fertility (Rf) gene

Abstract Molecular markers closely linked to the Restorer of fertility (Rf) locus in petunia were sought by conducting a bulk segregant analysis. The co-segregation of markers and Rf was tested on a large BC1 population produced from two different parental lines carrying Rf. The recombination frequency between OP704 and ECCA/MACT, the two most distal markers utilized in the fine-scale mapping, was significantly different in populations derived from parents that carry different nuclear backgrounds. The fine mapping identified an amplified fragment length polymorphism (AFLP) marker that co-segregates with Rf. A petunia BIBAC library (four genome equivalents), with an average insert size of 70 kb, was constructed and screened with the linked marker. A contiguous map was constructed from three different BIBAC clones that hybridized to the marker. As a result, we have identified a 37.5-kb BIBAC clone that co-segregates with Rf.

Keywords Petunia · Fertility restorer · Pollen development · Positional cloning · Binary BACs (BIBACs)

Introduction

Petunia plants containing mitochondria encoding cytoplasmic male sterility (CMS) are fertile if they carry a single dominant nuclear restorer allele termed Rf. The presence of Rf affects the expression of a mitochondrial gene (pcf) that is responsible for the disruption in pollen development. The pcf gene, consisting of the 5′-portion of the atp9 gene, parts of the first and second exons of the coxII gene, and an unidentified sequence (urf S), co-segregates with male sterility in a population of sterile and fertile somatic hybrids containing recombinant mitochondrial genomes (Booshore et al. 1985; Young and Hanson 1987). In the presence of Rf, the accumulation of pcf-derived transcripts and protein products is altered (Nivison and Hanson 1989; Pruitt and Hanson 1991).

In order to understand the molecular mechanism of restoration, we decided to clone Rf using a positional cloning strategy. We previously reported the identification of Randomly Amplified Polymorphic DNA (RAPD) and AFLP markers linked to Rf by applying a bulk segregant analysis, using a small mapping population (Michelmore et al. 1991; Bentolila et al. 1998). A promising result of this previous study was the relatively low ratio of physical to genetic distance in the area surrounding Rf. With an estimated ratio of 400 kb/cM, we felt it should be feasible to select enough recombinants to identify unequivocally a bacterial artificial chromosome (BAC) clone containing Rf.

Below we describe the identification of additional AFLP markers linked to Rf in tests employing more primer combinations on our segregating bulks. Having found three additional AFLP markers linked to Rf, we then evaluated the genetic distances between Rf and the linked molecular markers in a large segregating population comprised of 954 individual plants. A binary BAC (BIBAC) library was constructed from a petunia Rf/Rf line and screened with the most closely linked marker, resulting in the identification of three different BIBAC clones. These clones were used to develop a fine-scale map around the Rf locus and will be a valuable resource in future attempts to identify the Rf and rf alleles.

Materials and methods

Plant material

The original mapping population is a BC2F2 population, and has been described elsewhere (Bentolila et al. 1998). Briefly, the
original cross was intraspecific and involved two *P. hybrida* lines, 2423 CMS (*rf*) and 7037 (*Rf*). After two backcrosses to 2423, a restored (*Rf/rf*) BC2 progeny was selfed to produce a segregating population. A subset of this population comprised of 62 sterile (*rf/rf*) plants constituted our original mapping population. The populations used for fine-scale were two BC1 populations obtained by first crossing 2423 with either line 7037 (*Rf/rf*) or 7984 (*Rf/Rf*) and then backcrossing each of the restored F1s to 2423. The two BC1 populations are designated BC1 7037 or BC1 7984 according to the line used in the initial cross, and comprise 475 and 479 plants, respectively. These 954 BC1 plants were genotyped in order to find recombinants between OP704 and ECCA/MACT, the two closest markers bracketing *Rf* (Bentolila et al. 1998). The 39 BC1 recombinants obtained were further crossed in order to produce homozygous recombinants. In the case of restored recombinants (*Rf*/(*Rf*)), the plants were sired and homozygous recombinant progenies (*Rf*/*Rf*) were selected using molecular markers linked to *Rf* (*R* refers to the recombinant chromosome). The sterile (*rf*/(*rf*)) recombinants were first pollinated by a BC1 (*Rf/rf*) plant. Molecular markers linked to *Rf* were used to select *Rf*/*Rf* plants among the progenies. These restored plants were then selfed; the progenies were expected to include 25% homozygous recombinants (*rf*/(*rf*)). In this way, the recombinant plants were vector progeny. The remaining plants, either *Rf*/(*Rf*) or *rf*/(*rf*) were immortalized. Lines 2423, 7037, and 7984 were originally provided by Shumay Izhar (Volcani Center, Bet Dagan, Israel).

**Genotyping of the 954 BC1 plants**

To facilitate genotyping of 954 plants, a PCR assay was developed to identify the alleles present at two loci bracketing *Rf*, OP704 and OP413. For each of these markers, primers were designed which allowed the amplification of only the allele coming from the *Rf* parental line, not the allele coming from the other parent. Since the plants are BC1, they could either be homozygous for the allele coming from the *rf*/*rf* parent (no amplification), or heterozygous (amplification). The recombinants between OP704 and OP413 were identified by electrophoresing both PCRs in the same lane of a 2% agarose gel. The ampliﬁed bands differ in length and hence migrate to different locations. DNA from recombinant plants exhibit one band, whereas lanes loaded with DNA from non-recombinant plants carry either no bands or two bands.

OP413 was used instead of ECCA/MACT in this ﬁrst screen because the short length of the latter marker made it impossible to design primers that would amplify only the marker linked to the *Rf* allele in the original genotype. DNA was extracted from one cotyledon, adapting the procedure of Fulton et al. (1995). The ﬁrst genotypic selection identiﬁed 62 recombinants between OP704 and OP413. These plants were allowed to grow larger, and further characterization of this smaller number of plants by RFLP genotyping sorted out 39 recombinants between OP704 and ECCA/MACT.

**Construction of the bulks**

*Rf/Rf* and *rf/rf* bulks were used in order to ﬁnd markers linked in *cis* to both *Rf* and *rf* alleles. The *Rf/Rf* bulk used in this study is the same as the one previously described (Bentolila et al. 1998). The 10 plants used for the *Rf/Rf* bulk were ﬁrst selected by progeny testing, and conﬁrmed to be *Rf/Rf* by using the markers bracketing *Rf*, namely OP704 and ECCA/MACT. The *rf/rf* bulk used in this study differs from the previous one. The *rf/rf* bulk is composed of 10 plants selected from the 62 sterile BC2F2 plants that constituted the original mapping population. The plants selected for the bulk are among the closest recombinants to *Rf* identiﬁed in this population in order to ensure that any new polymorphic markers would be closer to *Rf* than the markers previously reported. They include two recombinants between *Rf* and the closest markers, OP704 and ECCA/MACT.

**AFLP analyses**

AFLP analyses were performed using the AFLP Analysis System I Kit (GibcoBRL, Life Technologies) with 32P-labeled oligonucleotides, according to the supplier’s instructions and as described by Vos et al. (1995). All primer combinations (PC), 64 in total, were used to look for any polymorphism between the rfrf and Rfrf bulks. DNA of each plant was extracted as described in Bentolila et al. (1998), and special care was taken to pool equal quantities of each plant DNA into the bulks. Polymorphic bands were named after the PC they originated from, and were further conﬁrmed to be linked to *Rf* in a two-step process. First, their segregation was tested by amplifying them from the individual DNAs composing the bulks. If the band proved to be truly linked to *Rf*, then its segregation was analyzed by using as AFLP templates the DNAs of the 39 homozygous recombinants obtained from the ﬁne mapping populations. AFLP reactions were performed with an MJ Research PTC100 Programmable Thermal Controller.

**BIBAC library**

The construction of the petunia BIBAC library followed very closely the procedure reported for preparation of the tomato BIBAC libraries (Hamilton et al. 1999), with a few minor changes concerning mainly the preparation of megabase DNA. Nuclei from leaves of 7984, an *Rf* parental line, were embedded in agarose plugs (Liam and Whittier 1994). Prior to digestion with restriction endonuclease, the plugs were submitted to a pre-run pulsed-ﬁeld gel electrophoresis using a CHEF-Mapper or DR11 apparatus (both from Bio-Rad); the conditions used in this step were 3.7V/cm, 10 s pulse (constant), 120° angle, 14°C, 1% agarose, 0.5×TBE for 16 h. This removes the starch and degraded DNA, while the megabase DNA remains in the plugs. The BamHI digestion and size selection of petunia genomic DNA were performed as in Hamilton et al. (1999). The collection of *E. coli* DH10B cells, selection and analysis of the clones. Different size classes of digested genomic DNA, from 150 kb to 300 kb, were ligated to a BamHI-cleaved and dephosphorylated BIBAC2 vector. Different molar ratios of insert to vector, ranging from 1:10 to 1:30 were tested. The different ligation mixes were introduced into competent *E. coli* DH10B cells by electroporation. Ligation mixes were evaluated by estimating in the population of clones they generated the average insert size and the frequency of clones with no insert or a rearranged pattern characterized by the absence of the vector band. As a result of this analysis four different ligation mixes were selected. The BIBAC clones were picked manually with sterile toothpicks and addressed in 384-well plates (Genetix, New Milton, Hants., UK). A replicate of the library was sent to the Clemson University Genomics Institute (CU/GI). There a robot arrayed the library on high-density filters; each filter containing 18432 clones spotted twice. The entire library is represented by ﬁve high-density filters. Filter hybridizations were done according to Hamilton et al. (1999). Clones and filters are available upon request from CUGI (http://www.genome.clemson.edu/).

**Characterization of the ends of the BIBAC clones**

The ends of selected BIBAC clones were sequenced at the Cornell BioResource Center, essentially following the protocol published by Kelley et al. (1999). The primer amount was increased to 50 pmol per sequencing reaction. Digestion of the BIBAC clones with XhoI prior to sequencing seemed to improve the quality of the sequencing. Currently an average of 450 bp can be read before the occurrence of the first N. Before implementing this technique, the PCR walking strategy described by Devic et al. (1997) was used to isolate the ends of the BIBAC clones, using a BIBAC clone as template instead of genomic DNA. Approximately 10 ng of the BIBAC plasmid was digested to completion before being ligated to the adaptor. The pair of nested primers used to amplify the left end