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Sugar beet BAC library construction and assembly of a contig spanning *Rf1*, a restorer-of-fertility gene for Owen cytoplasmic male sterility

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Abstract *Rf1* is a nuclear gene that controls fertility restoration in cases of cytoplasmic male sterility caused by the Owen cytoplasm in sugar beet. In order to isolate the gene by positional cloning, a BAC library was constructed from a restorer line, NK198, with the genotype *Rf1Rf1*. The library contained 32,180 clones with an average insert size of 97.8 kb, providing 3.4 genome equivalents. Five AFLP markers closely linked to *Rf1* were used to screen the library. As a result, we identified eight different BAC clones that were clustered into two contigs. The gap between the two contigs was filled by chromosome walking. To map the *Rf1* region in more detail, we developed five cleaved amplified polymorphic sequence (CAPS) markers from the BAC DNAs identified, and carried out genotyping of 509 plants in the mapping population with the *Rf1*-flanking AFLP and CAPS markers. Thirteen plants in which recombination events had occurred in the vicinity of the *Rf1* locus were identified and used to map the molecular markers relative to each other and to *Rf1*. In this way, we were able to restrict the possible location of the *Rf1* gene to a minimum of six BAC clones spanning an interval of approximately 250 kb.

Keywords BAC library · Cytoplasmic male sterility · Fertility restorer gene · Sugar beet

Introduction

Cytoplasmic male sterility (CMS) in higher plants represents one of the few systems in which interactions between mitochondrial and nuclear genomes, and their role in developmental processes, can be investigated (Hanson and Bentolila 2004). CMS is a maternally inherited defect in pollen production that is thought to result from the expression of unusual or aberrant mitochondrial genes (Schnable and Wise 1998). The mitochondrial defect can be compensated for by specific nuclear genes, termed *Restorer of fertility (Rf)* genes (Hanson and Bentolila 2004). In several of the cases that have been analyzed in detail, these *Rf* genes appear to inhibit the expression of the CMS-associated mitochondrial gene at a post-transcriptional level (Hanson and Bentolila 2004).

The occurrence of CMS in sugar beet was first documented by Owen (1945). He reported that the CMS (Owen CMS) is controlled by interaction between at least two restorer genes (designated *X* and *Z*) and the sterilizing cytoplasm. Owen CMS has been used to produce nearly all commercial beet hybrids (Bosemark 1993), but the molecular mechanism of this CMS remains to be fully defined. We recently found, using in organello translation experiments, that a 35-kDa mitochondrial protein is associated with Owen CMS (Yamamoto et al. 2005). Interestingly, this protein proved to be antigenically related to the 387-codon mitochondrial ORF (designated *preSatp6*) which is fused in frame with the downstream *atp6* ORF (Yamamoto et al. 2005). However, in contrast to the situation in other CMS systems, the presence of *Rf* genes has no detectable effect on the expression of *preSatp6* (Yamamoto et al. 2005). In order to understand the mechanism of fertility restoration in Owen CMS, we therefore decided to clone the *Rf* genes involved using a positional cloning approach. This approach has allowed the isolation of *Rf* genes in petunia (Bentolila et al. 2002), radish (Koizuka et al. 2003; Brown et al.

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2003; Desloire et al. 2003), and rice (Kazama and Toriyama 2003; Komori et al. 2004; Akagi et al. 2004). All of these *Rf* genes were reported to encode a pentatricopeptide repeat protein that may prevent the accumulation of the mitochondrial protein responsible for male sterility.

As sugar beet possesses a relatively small genome of 758 Mb (Arumuganathan and Earle 1991), positional cloning is technically feasible if the molecular resources are available (Cai et al. 1997). We identified AFLP markers tightly linked to an *Rf* gene (named *Rf1*, probably the same as *X*) for Owen CMS by employing bulked segregant analysis (Hagihara et al. 2005). These markers provide one tool required for map-based cloning. This report describes the construction of a sugar beet BAC library, which is another resource necessary for the cloning strategy (Gindullis et al. 2001; Hohmann et al. 2003; Fang et al. 2004; McGrath et al. 2004). We also describe the use of the BAC library in the assembly of a 250 kb contig spanning *Rf1*.

Materials and methods

Plant materials

TK76-MS and TK81-MS are male-sterile Owen cytoplasm lines, while NK198 is a restorer line with the genotype *Rf1Rf1*. Pollen from NK198 was used to pollinate TK76-MS. A male-fertile F1 plant was then crossed with TK81-MS instead of TK76-MS because of failure to synchronize the flowering between the F1 plant and TK76-MS. The resultant (three-way cross) population consisted of 509 plants and segregated in a 1:1 ratio for the presence/absence of *Rf1*. Male fertility was determined by visual observation of anther color and pollen dehiscence, and by microscopic observation of the percentage of cotton-blue stained pollen (Hagihara et al. 2005).

Isolation of high molecular weight DNA

About 10 g of fresh green leaves was collected from NK198 and used to isolate nuclei according to Liu and Whittier (1994) with minor modifications: our nuclear isolation buffer consisted of 10 mM Tris-HCl (pH 9.5), 20 mM EDTA (pH 8.0), 80 mM KCl, 0.5 M sucrose, 4 mM spermidine, 1 mM spermine and 0.1% mercaptoethanol; the leaf homogenate was filtered through miracloth (Calbiochem, La Jolla, CA, USA) instead of nylon mesh; the isolation buffer added to the filtrate was not supplemented with Triton X-100. The nuclei were pelleted at 600 g for 10 min. Plugs of high molecular weight DNA were prepared after adding 1.5% low-melting agarose (45°C). Plugs were equilibrated in lysis buffer (1% Sarkosyl, 0.2 M EDTA pH 8.0, 0.1 mg/ml proteinase K) at 50°C for 48 h with

one change of lysis buffer, followed by dialysis with TE50 buffer (10 mM Tris-HCl pH 8.0, 50 mM EDTA) at 4°C for more than 2 h. The proteinase K was inactivated by a 2 h incubation at 50°C in TE50 buffer supplemented with phenylmethylsulfonyl fluoride (PMSF) (final concentration 2 mM). Plugs were then washed in TE50 buffer and stored at 4°C until use. Plugs were equilibrated in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and then kept in buffer containing 10 mM TRIS-HCl pH 7.5, 1 mM DTT, 50 mM NaCl, 0.01% BSA and 15 U/ml *Hind*III (Takara Bio, Otsu, Japan) at 4°C for 5 h. After incubation in the presence of 10 mM MgCl₂ at 4°C for a further hour, plugs were kept at 37°C for 10 min. Restriction digestion was terminated by adding 0.5 M EDTA (pH 8.0) and chilling the reaction. The partially digested DNA was fractionated by pulsed-field gel electrophoresis (PFGE) using a CHEF-DRII apparatus (Bio-Rad, Hercules, CA, USA): electrophoresis was done at 14°C in a 1.0% pulsed-field certified agarose gel (Bio-Rad) at 6 V/cm with a switch time of 90 s for 8 h, followed by a switch time of 6 s for 16 h. Gel pieces containing DNA fragments ranging in size from 200 to 500 kb were excised, and the DNA was electroeluted as described by Strong et al. (1997).

Construction of the BAC library

The vector pBeloBAC11 (Kim et al. 1994) was prepared as described by Woo et al. (1994). The size-fractionated, *Hind*III-digested sugar beet DNA was ligated to pBeloBAC11 at a molar ratio of 1 (beet DNA) to 10 (vector DNA) and the ligation mixture was dialyzed according to Woo et al. (1994). The ligated DNA was utilized to transform *E. coli* Electro Max DH10B cells (Invitrogen, Carlsbad, CA, USA). The BAC clones were picked manually with sterile toothpicks and addressed in 96- or 384-well microtiter plates. BAC DNA was prepared from 2 ml cultures in LB medium by an alkaline lysis method (Woo et al. 1994), and electrophoresed in a 1% gel using the CHEF-DRII apparatus (6 V/cm, 5–15 s switch time, 14 h run-time, 14°C).

Screening of the BAC library based on AFLP analysis

The BAC library was screened with the AFLPs using plate pools, and then row and column sub-pools of BAC DNAs. The library stored in 384-well plates was replicated onto LB-plates using a 384-pin replicator, and colonies were grown at 37°C for 16 h. The plates were washed with liquid LB medium to collect clones and the BAC DNAs were isolated. The resultant plate pools of BAC DNA were subjected to AFLP analysis (Vos et al. 1995; Habu et al. 1997) using AFLP Analysis System I (Invitrogen) with ³²P-labeled primers. Pooled DNA was digested with *Eco*RI and *Mse*I. The adaptor-ligated DNA was pre-amplified using primers without selective

nucleotides. The selective-amplification was performed using primers with two selective nucleotides. Plate pools containing clone(s) of interest were further subdivided in 16 rows and 24 columns to give 40 sub-pools, which were subsequently used for AFLP-based screening to identify individual positive clones.

Screening of the BAC library based on colony hybridization

Using a 384-pin replicator, BAC clones were spotted onto Hybond N membranes (Amersham Biosciences, Piscataway, N J, USA) placed on LB agar plates. Colonies were grown at 37°C for 12 h. Colony filters were processed and hybridized using standard protocols (Sambrook et al. 1989) according to the manufacturer's instruction manual. The hybridization probe was labeled with ³²P using the Megaprime DNA labeling system (Amersham Biosciences).

Sub-cloning of BAC insert ends

About 50 ng aliquots of BAC DNA were digested with *Bam*HI or *Sph*I, and then self-ligated. BAC insert ends were generated by vectorette-PCR (Riley et al. 1990), using BR3 (5'-TGACCATGATTACGCCAAGC-3') and BL3 (5'-ACCTGCAGGCATGCAAGCTT-3') primers. TAIL-PCR (Liu and Whittier 1995) was also used to isolate BAC ends. The specific primers used to amplify the right ends were BR1 (5'-CACTTTA-TGCTTCCGGCTCG-3'), BR2 (5'-GTTGTGTGGAA-TTGTGAGCG-3') and BR3, while the left ends were amplified with BL1 (5'-GGGATGTGCTGCAAGGC-GAT-3'), BL2 (5'-GGTTTTCCAGTCACGACGT-3') and BL3. The resultant PCR products were cloned into pBluescript vector (Stratagene, La Jolla, CA, USA).

Cloning of AFLP fragments

AFLP products were electrophoresed on a standard sequencing gel. After exposure, the autoradiogram was aligned with the dried gel to isolate the amplified frag-

ment of interest from the gel. The gel piece was then boiled in water for 15 min as described by Habu et al. (1997). After a brief centrifugation, the supernatant was subjected to PCR amplification using the same sets of AFLP primers. The amplification products were recovered from the agarose gel and cloned into pBluescript vector.

Cleaved amplified polymorphic sequence (CAPS) analysis

Primer sequences and restriction enzymes used for CAPS analysis are listed in Table 1. About 20 ng of total cellular DNA was subjected to PCR amplification using the following reaction parameters: 35 cycles of 30 s at 94°C, 30 s at 54°C and 90 s at 70°C. The amplification products were digested with appropriate restriction endonucleases and resolved by electrophoresis in 1.5% agarose gels.

Other molecular analyses

Cellular DNA extraction, DNA and RNA gel blot analyses and DNA sequencing followed standard techniques (Sambrook et al. 1989; Kubo et al. 2000; Hagi-hara et al. 2005).

Results and discussion

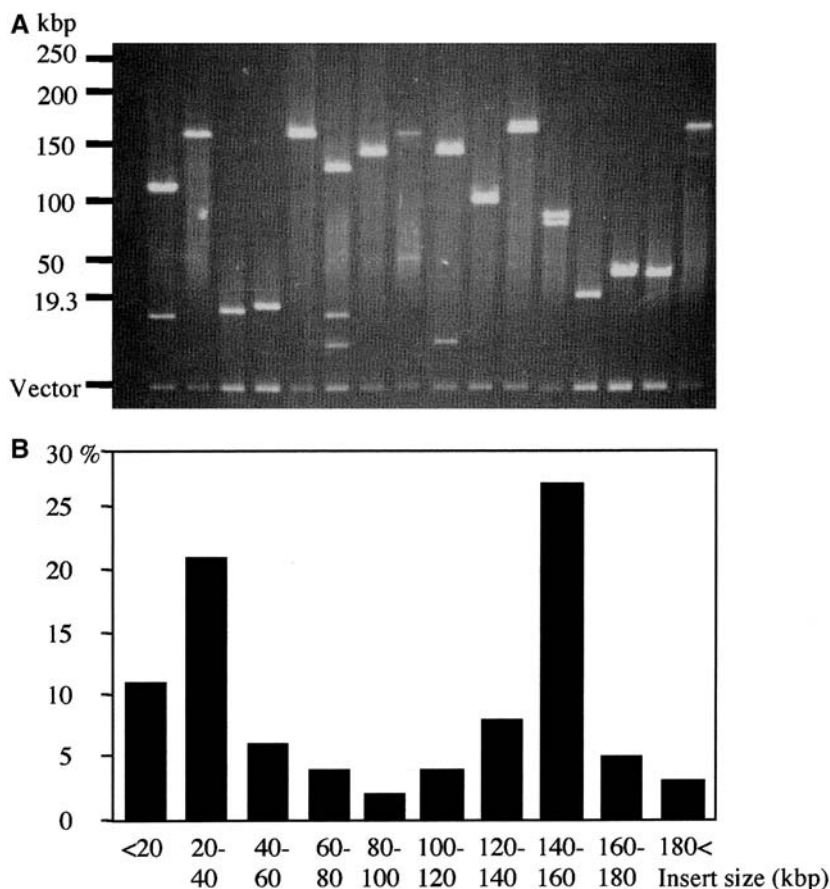
Construction of the NK198 BAC library

A restorer line, NK198, was chosen as the source of genomic DNA for the construction of the BAC library. Using three different ligation mixes, a total of 32,180 BAC clones was collected and constitutes the library. The insert-size distribution of the library was evaluated by analyzing 100 randomly selected individual clones. Figure 1a shows an ethidium bromide-stained pulsed-field gel of 16 of these clones digested with *Not*I. The average size of the inserts in the 100 clones was 97.8 kb, and the range was 20–180 kb. Approximately 58% of the clones carried inserts larger than 80 kb, though a

Table 1 Primers and restriction enzymes used for CAPS analysis

Marker	Primers	Restriction enzyme
mCP-K2	GATCAGCTAGCATTACCCATCATC CGAGCTTTGCCGAGTTCATAACAG	<i>Hae</i> III
mCP-L45	GCTTCACTATTATCACGATGTG AACATATCTTCCCAGCTCATCC	<i>Hae</i> III
mCP-L6	GGAATTAACCTCCGCTCGTCCTTC CAAAGAGGAATCTGGGCGTTTCTC	<i>Hap</i> II
mCP-A54	AATGCCATGGAGGAAAAGTGTG ATATCCTGCACTGAGCAATTCTG	<i>Dra</i> II
MP-A16	CTGATTTTGGACGGAGCTTGTTCG TGCATTGTAGAAACACCCGCGTAG	PCR products differ in size

Fig. 1 Analysis of selected clones from the NK198 BAC library. **a** Representative gel profiles of 16 BAC DNA samples digested with *NotI*. The positions of the size markers are indicated on the left. **b** Distribution of insert lengths based on 100 randomly chosen BAC clones



significant number of clones (30%) had inserts smaller than 40 kb (Fig. 1b).

To examine the stability of sugar beet DNA fragments maintained as BACs in *E. coli*, four BAC clones with inserts larger than 100 kb were selected and grown for about 100 generations (3 days). We analyzed the *HindIII* restriction digestion patterns of the four BAC clones after 16 and 100 generations. No obvious changes were observed (data not shown), proving that the BAC cloning vector could stably maintain large DNA fragments in *E. coli*.

The NK198 BAC library was further investigated to determine the extent of contamination with clones originating from chloroplast DNA. For this purpose, 1,536 randomly chosen clones were hybridized with three sugar beet chloroplast DNA probes, *ndhG* (located within the small single-copy region), *psbB* (within the large single-copy region) and *ycf2* (within the large inverted repeat regions), simultaneously. These three probes are approximately equidistant from each other in the chloroplast genome, so that any BAC insert larger than 60 kb should hybridize with one of the probes.

Some 19% of the BACs hybridized with this probe set (data not shown), indicating that these positive clones contained chloroplast sequences. Based on a haploid genome size of 758 Mb for sugar beet (Arum-

uganathan and Earle 1991), the NK198 BAC library represents 3.4-fold genome equivalents, if the chloroplast fraction is subtracted out. Assuming that the BAC inserts are randomly distributed in the genome, the probability of detecting any given sequence can be calculated (Clarke and Carbon 1976) as 96.7%.

A BAC contig containing the *Rfl* locus

We previously identified five AFLP-markers (mAFEM972, mAFEM984, mAFEM004, mAFEM976 and mAFEM985) that show close linkage to the *Rfl* locus (Hagihara et al. 2005). The AFLP marker fragments were recovered from the gel and cloned, and the resultant clones were used as probes for hybridization with sugar beet genomic DNA. Two markers, mAFEM976 and mAFEM985, showed single-copy fragment patterns (Fig. 2) and therefore these markers were deemed suitable for use in colony-hybridization-based screening of the NK198 library. The remaining three markers were less suitable as probes for the same screening approach because they were shown to represent repetitive DNA (Fig. 2). We therefore attempted to screen the library with AFLP primer combinations using pools of BAC DNA as templates. Our pooling strategy utilized three

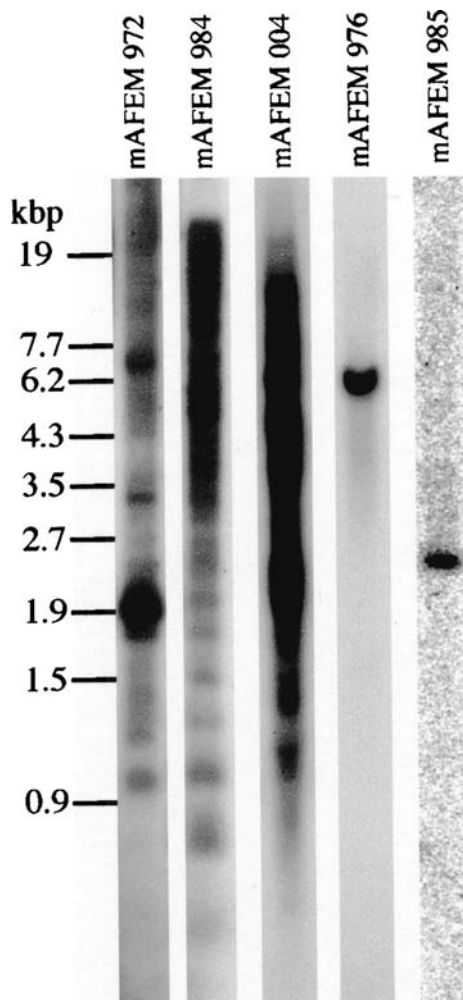


Fig. 2 Southern hybridization analysis of *Hind*III-digested genomic DNA from NK198. Five AFLP marker fragments that displayed close linkage to the *Rfl* locus (Hagihara et al. 2005) were used as probes. The positions of the size markers are indicated on the left

different pool types: plate pools of 384 single BAC clones, rows of sub-pools comprising 16 single clones each, and columns of sub-pools comprising 24 single clones each. A total of 324 pools (84 plate pools, and 240

sub-pools derived from rows and columns) were screened with the three AFLP markers. As summarized in Table 2, eight different BAC clones were identified after both hybridization- and AFLP-based screens.

In the next step, the ends of these BAC clones were sub-cloned. The BAC end clones obtained were then used for hybridization to Southern blots of *Hind*III-digested BAC DNAs. The results allowed us to assemble the eight BAC clones into two contigs (5A3/9C23/37O9/33E19/9O3/51L1 and 1M13/36I20). In order to join the two contigs, we further screened the library with the outermost ends of the contigs. This led to the identification of six additional, overlapping BACs, one (41H8) of which was found to be a bridging clone connecting the two contigs (Fig. 3). As shown in Fig. 3, a physical map of the *Rfl* region could be constructed, encompassing 14 BACs anchored by the five AFLP loci mentioned above. The genomic region of interest is flanked by the AFLP-markers mAFEM984 and mAFEM004, which are located 3.5 cM apart.

BAC-derived markers

In order to more precisely locate the *Rfl* gene in the BAC contig, we attempted to derive additional molecular markers from the BAC clones spanning the *Rfl* region. The *Hind*III sub-fragments of BAC DNAs were utilized as probes for genomic Southern hybridization. In total, 23 sub-fragments were regarded as single-copy and some of these sub-fragments were end-sequenced to design the primers for CAPS analysis. The CAPS primer pairs were used to amplify DNAs from the Owen CMS lines (TK81-MS and TK76-MS, *rfl/rfl*) and the restorer line NK198 (*Rfl/Rfl*) involved in the three-way cross (see “Materials and methods”). The amplification products were further digested with four-base-cutters to detect polymorphisms, resulting in the development of five CAPS markers, mCP-A54, mP-A16, mCP-K2, mCP-L6 and mCP-L45 (Fig. 3). Using a subset of the three-way cross population [TK81-MS × (TK76-MS × NK198)], we confirmed that all the CAPS markers segregated according to the expected ratio of 1:1 and co-segregated with *Rfl* (data not shown).

Table 2 Results of screening the BAC library with AFLP markers tightly linked to the *Rfl* gene

Marker	Identified BAC clones (insert size) ^a
Hybridization-based screen	
mAFEM976	33E19 (210), 37O9 (220), 9C23 (155)
mAFEM985	33E19 (210), 37O9 (220)
AFLP-based screen	
mAFEM004	33E19 (210), 9O3 (170), 51L1 (150)
mAFEM972	5A3 (165)
mAFEM984	1M13 (90), 36I20 (170)

^a The insert sizes of identified BAC clones are given in parentheses

Candidate *Rfl*-containing clones

We next wished to identify members of the three-way cross population that had undergone recombination close to the *Rfl* locus. We therefore determined the genotypes of DNA samples from 509 plants in the three-way cross population using eight *Rfl*-flanking markers. The screen enabled us to identify 13 plants in which recombination had occurred in the vicinity of *Rfl*, and these plants were then used to map the eight markers relative to each other and to *Rfl*. As shown in Table 3 and Fig. 3, *Rfl* could be restricted to the 250-kb region

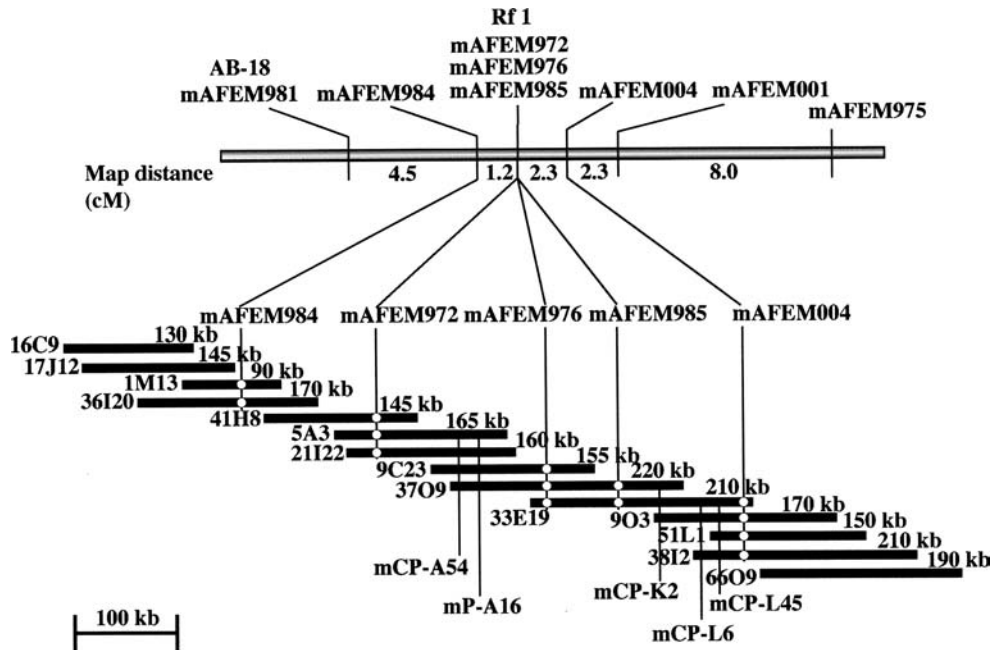


Fig. 3 Genetic and physical maps of the *Rfl* genomic region. *Upper panel* Genetic map (after Hagihara et al. 2005). The locations of the *Rfl* gene, one RAPD marker (AB-18) and eight AFLP markers are shown, together with the genetic distances between them (in cM). *Lower panel* A BAC contig containing the *Rfl* gene consists of 14 BAC clones. The five AFLP markers and five CAPS markers used to screen the BAC library are indicated. The *thick horizontal bars* represent the extents of individual BAC inserts, and are labeled with the names of the BAC clones and their insert sizes

that is delimited by the markers mP-A16 and mCP-L6. Two BACs, 37O9 and 33E19, with insert sizes of 220 and 210 kb, respectively, are candidates for cloning the *Rfl* gene.

As mentioned above, a number of *Hind*III sub-fragments from the BAC DNAs covering the *Rfl* region were labeled for Southern hybridization. These

fragments were also used to probe Northern blots of RNA prepared from flower buds, leaves and roots of the *RflRfl* (NK198) and *rf1rf1* (TK81-MS) plants in an attempt to identify regions which were transcribed in the *RflRfl* plants but not in the *rf1rf1* plants. Interestingly, a transcript difference was detected when a 7.0 kb *Hind*III fragment derived from the BAC 37O9 was utilized as a probe against flower bud RNA. This probe hybridized to a 1.7-kb transcript in NK198, but no transcripts were observed in TK81-MS (Fig. 4). It should also be noted that the 1.7-kb transcript was not detectable in leaves or roots of *RflRfl* plants (Fig. 4). To pinpoint the location of the *Rfl* gene, sequencing of BAC DNAs and identification of candidate sequences will be required. Such investigations are presently underway in this laboratory.

Table 3 Genotypes of 13 plants carrying recombination events in the vicinity of *Rfl* locus

Recombinant	Phenotype ^a	Molecular markers ^b							
		mAFEM984	mCP-A54	mP-A16	mAFEM976	mAFEM985	mCP-K2	mCP-L6	mCP-L45
H39	S	T	T	T	T	T	T	N	N
K2	F	T	T	N	N	N	N	N	N
I32	F	T	T	N	N	N	N	N	N
H22	F	T	T	N	N	N	N	N	N
H15	F	T	N	N	N	N	N	N	N
C30	F	T	N	N	N	N	N	N	N
L17	F	N	N	N	N	N	N	T	T
I24	F	N	N	N	N	N	N	T	T
I20	F	N	N	N	N	N	N	T	T
I9	F	N	N	N	N	N	N	T	T
O16	S	N	N	N	T	T	T	T	T
K15	S	N	N	N	T	T	T	T	T
F12	S	N	N	N	T	T	T	T	T

^a F, male fertile; S, male sterile

^b T, TK76-MS/TK81-MS heterozygote; N, NK198/TK81-MS heterozygote

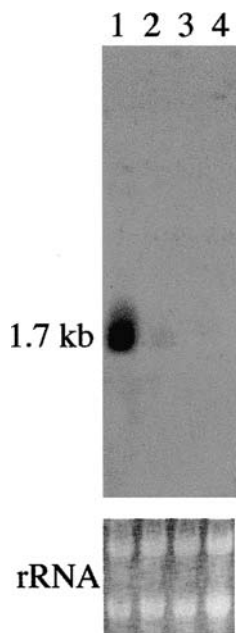


Fig. 4 Northern hybridization analysis of NK198 and TK81-MS plants. RNA from three *RfRf* (NK198) and three *r1rf1* (TK81-MS) plants was examined. Twenty micrograms of total cellular RNA was prepared from the indicated tissues (*lane 1*, flower buds of NK198; *lane 2*, leaves of NK198; *lane 3*, roots of NK198; *lane 4*, flower buds of TK81-MS) and subjected to Northern analysis using a 7-kb fragment derived from the BAC clone 3709 as the probe

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