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Molecular mapping of a fertility restorer gene for Owen cytoplasmic male sterility in sugar beet

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Abstract We report here the molecular mapping of a fertility restorer gene (named *Rf1*) for Owen cytoplasmic male sterility in sugar beet. Eight AFLP and two RAPD markers, tightly linked to the *Rf1* locus, were identified using bulked segregant analysis. Three AFLP markers, mAFEM972, mAFEM976 and mAFEM985, were found to co-segregate with the *Rf1* allele in our mapping populations. With the help of RFLP markers, previously mapped on the sugar beet genome, we showed that *Rf1* is positioned in the terminal region of linkage group Kiel III/Koeln IV. This map location agrees well with that found for the restorer gene *X*, which suggests that the *Rf1* locus corresponds to the *X* locus. The availability of the molecular markers will facilitate the selection of maintainer–pollinator lines in breeding program and provide the foundation for map-based cloning of the *Rf1* gene.

Introduction

Cytoplasmic male sterility (CMS) is a mainstay for the hybrid seed production in sugar beet because large-scale emasculation is not feasible, owing to the small perfect flowers and indeterminate flowering habit of beet plants (Bosemark 1993). The production of all hybrid beet cultivars relies upon a single source of CMS, which was discovered by Owen (1945). Owen (1945) reported that the male sterility resulted from the interaction of two recessive nuclear genes [named *x* and *z*, restorer-of-fertility (*Rf*) genes] with the sterilizing cytoplasm. A plant having the sterilizing cytoplasm is hypothesized to be fully male-fertile only if it carries dominant alleles at both *X* and *Z* loci. CMS beet plants can be propagated by fertilization with pollen from so-called maintainer plants, which have the *xxzz* genotype and normal fertile cytoplasm.

Several attempts have been made to improve upon the original assumption of Owen (1945). This is because the results in some crossing experiments could not be fully explained on the basis of the original premise. It should also be noted that the maintainer genotype exists at low frequencies (3–5%) in most sugar beet populations, and can be identified only through test-crossing to CMS plants and scoring of the resulting F_1 for male sterility/fertility (Bosemark 1993; K. Taguchi, personal communication). Hence, selection and development of inbred maintainer lines is the most laborious and expensive part of a hybrid breeding program in sugar beet (Owen 1950; Bosemark 1993). The identification of molecular markers tightly linked to *Rf* loci in sugar beet would permit the classification of new breeding lines or germplasm accessions as the maintainer, without the need for test crosses.

Research investigating the molecular mechanism of Owen CMS has largely focused on the mitochondrial genome (Kubo et al. 1999; Ducos et al. 2001; Satoh et al. 2004; M. P. Yamamoto, T. Kubo, and T. Mikami, submitted). The restorer locus *X* has been mapped onto the terminal region of linkage group Kiel III/Koeln IV corresponding to chromosome III and the *Z* locus onto chromosome IV (Pillen et al. 1993; Schondelmaier and Jung 1997). However, nothing further is known about these two loci. In this paper, we attempted to find

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RAPD and AFLP markers tightly linked to the *Rf* gene(s) for the Owen CMS using a bulked segregant analysis approach.

Materials and methods

Sugar beet lines and phenotypic classification of male fertility

Three male sterile lines [TK76-MS, TK81-MS, and I-12 CMS(R)] carrying the Owen cytoplasm and a restorer line NK198 were used in this study. TK81-MS is self-incompatible, whereas the others contain genes for self-fertility. The S2 plants of NK198 were crossed with I-12 CMS(R), and subsequent F_1 and BC_1 progenies were evaluated for pollen fertility. A BC_1 plant with the highest fertility was allowed to self-pollinate and set seed (BC_1F_2). Pollen from NK198 was also used to pollinate TK76-MS. A male-fertile F_1 plant was crossed with TK81-MS instead of TK76-MS because of the failure to synchronize the flowering between the F_1 plant and TK76-MS. The same F_1 plant was also allowed to self-pollinate for F_2 seed.

A sample of pollen grains or anthers was collected at anthesis from each plant. Fertility was checked visually and microscopically by staining pollen grains with cotton-blue solution (Yui et al. 2003). Plants classified as "fertile" produced more than 20% stainable pollen and shed pollen abundantly from most yellow anthers. "Partial fertile" plants bore light-yellow or orange anthers with less than 20 % stainable pollen. Such anthers scarcely dehisce. "Sterile" plants had only shriveled anthers that were either white or brown. In these plants, nearly all microspores aborted soon after being released from tetrads.

RAPD and AFLP analyses

Total cellular DNA was extracted from fresh green leaves according to the procedure described by Doyle and Doyle (1990). Bulked segregant analysis was performed with plants obtained from the three-way cross, TK81-MS × (TK76-MS × NK198). Bulks were composed of five individuals sharing the same phenotype, i.e., fully fertile or fully sterile. In RAPD analysis, 20 ng of DNA was subjected to PCR amplification in a reaction mixture containing 0.5 μ*M* of a single 10-mer oligonucleotide (Qiagen, Hilden, Germany). Cycling parameter was 35 cycles of 94°C for 1 min, 43°C for 2 min, and 72°C for 2 min. RAPD marker fragments were cloned into pBluescript vector (Stratagene, La Jolla, Calif., USA) and sequenced. Two RAPD markers, AB-18 and AD-11, were then converted to sequence-tagged site (STS) markers. Nucleotide sequences of the STS primers are AB-18Fw, 5′-TTGGCAATGCAAGAGAAAGGATACG-3′; AB-18Rv, 5′-GTATCGTCAAATTCCCCTGCTCAAC-3′; AD-11Fw, 5′-CAATCGGGTCTCGCAGGTATTCTGG-3′; and AD-11Rv, 5′-CAATCGGGTCCATGGGAATCCACGC-3′. AFLP analyses were carried out using the AFLP Analysis System I (Invitrogen, Carlsbad, Calif., USA) with ^{32}P-labeled primers, as described by Vos et al. (1995) and Habu et al. (1997). Total cellular DNA was digested with *Eco*RI and *Mse*I. The adaptor-ligated DNA was pre-amplified using primers with a single selective nucleotide. For selective-amplification, *Eco*RI-NNN and *Mse*I-NNN primers were employed. The three additional, selective nucleotides used for amplification of AFLP markers are as follows: mAFEM972 (*Eco*RI-AAT/ *Mse*I-GAC), mAFEM975 (*Eco*RI-AGA/ *Mse*I-TAA), mAFEM976 (*Eco*RI-AGT/ *Mse*I-GAT), mAFEM981 (*Eco*RI-CAG/ *Mse*I-TAC), mAFEM984 (*Eco*RI-CTA/ *Mse*I-GCA), mAFEM985 (*Eco*RI-CTG/ *Mse*I-GAT), mAFEM001 (*Eco*RI-GAA/ *Mse*I-GAG), and mAFEM004 (*Eco*RI-GTA/ *Mse*I-CTT).

DNA gel blot analysis

Three micrograms of total DNA were digested with restriction endonucleases, electrophoresed in 1% agarose gel, and capillary-transferred to Hybond-N$^+$ membrane (Amersham Biosciences, Piscataway, N.J., USA). The DNA fragment of interest was labeled using the Alkphos Direct System (Amersham Biosciences), and hybridization was done according to the instruction manual. The RFLP probes used in this study are described in Pillen et al. (1993) and Schumacher et al. (1997).

Linkage analysis

Linkage map was constructed using Map Manager (version 2.6.5) and MAPL98 (Ukai et al. 1991; http://linkage.rockefeller.edu/soft/list.html). Map distances in centiMorgans (cM) were calculated from recombination frequencies using the Kosambi function (Kosambi 1944).

Results

Segregation of male fertility

All F_1 plants of the cross I-12 CMS(R) × NK198 were fertile, having 70% or more stainable pollen (data not shown). The BC_1 generation yielded 66 partial-fertile or fertile and 71 completely male-sterile plants, which fitted the expected 1:1 ratio for monogenic inheritance (Table 1). In the BC_1F_2 generation, approximately 25% of the plants produced no pollen, which showed good fits to a 3 male-fertile:1 male-sterile ratio. As shown in Table 1, the segregation in the F_2 generation of the cross, TK76-MS × NK198 as well as in the three-way

cross, TK81-MS × (TK76-MS × NK198), also gave a better fit to the monogenic hypothesis than to the complementary two gene model (see "Discussion"). We tentatively designate this single gene *Rf1*.

Molecular markers linked to the *Rf1* gene

The three-way cross [TK81-MS × (TK76-MS × NK198)] population that consisted of 88 plants was used for bulked segregant analysis (Michelmore et al. 1991) to isolate RAPD markers linked to fertility restoration. The contrasting bulked DNA samples were formed separately from the DNA of five fully fertile and five sterile plants. Approximately 6,900 RAPD fragments were amplified with 814 primers, and two primers, AB-18 and AD-11, were confirmed to give reproducible polymorphic amplifications that co-segregated with pollen fertility when they were tested in a number of individual fully fertile and sterile plants (Fig. 1; data not shown). These two RAPD markers were further converted into two dominant STS markers. Co-segregation analysis was performed with the whole mapping population to confirm linkage between the STS markers and the *Rf1* locus. Our results indicated that the *Rf1* locus is flanked by AB-18 and AD-11 at a genetic distance of 5.7 cM and 24.5 cM, respectively (data not shown; see below).

Using an AFLP analysis, we obtained additional markers that are closer to *Rf1* than AB-18. To eliminate the false-positive fragments, three different bulks were created for both phenotypic classes (fully fertile vs sterile) of plants, each containing DNA samples of five individuals, and were used for three-round screening. In total, 2,721 primer combinations were used to compare the fully fertile and the sterile bulks. Approximately 180,000 fragments were scored, giving an average of 66 fragments per primer combination. We finally identified eight polymorphic fragments, all of which were always present in the fertile bulks and absent from the sterile bulks (Fig. 2; data not shown). Analysis of all members of the three-way cross population indicated that the eight AFLP markers were located on the same linkage group as the *Rf1* locus and the two STS markers. Figure 3 shows the map position of *Rf1* relative to the linked molecular markers. It should also be mentioned that mAFEM972, mAFEM976 and mAFEM985 completely co-segregated with the *Rf1* allele in 88 plants assayed so far [95% CI (0-4.19)].

We further used a separate mapping population [the BC_1 generation of the cross I-12 CMS(R) × NK198] to verify this linkage relationship. Out of the eight AFLP markers, four failed to reveal polymorphism between the parents of the BC_1 population. The remaining four markers were found to co-segregate with the *Rf1* allele in 137 plants examined (Fig. 3). This BC_1 population was subsequently used to determine the position of the *Rf1* locus in the genetic map of sugar beet.

The *Rf1* gene probably corresponds to the *X* gene

The *X* locus first described by Owen (1945) is located on the terminal region of the linkage group Kiel III/Koeln IV, which corresponds to chromosome III (Pillen et al. 1993). Three RFLP markers (pKP1194, pKP1168, and 28; see Pillen et al. 1993; Schumacher et al. 1997) that are linked to the *X* locus were used to analyze a subset of the BC_1 population: 17 male-fertile and 19 male-sterile plants. All the RFLP markers detected polymorphism and were shown to be linked to the *Rf1* gene and to the four AFLP markers (Fig. 3). This makes it likely that the *Rf1* gene corresponds to the *X* gene, though the definite proof remains to be seen.

Discussion

In spite of numerous studies, genetic basis for the restoration of Owen CMS remains to be fully determined. Some authors reported that a single dominant gene controlled fertility restoration (Theurer 1971; Pillen et al. 1993), whereas others indicated the combined action (complementary or epistatic interaction) of at least two major genes (Bliss and Gabelman 1965; Owen 1945; Theurer and Ryser 1969). A recent QTL mapping revealed three loci to be responsible for restoration (Hjerdin-Panagopoulos et al. 2002). Our data presented here are consistent with a single-gene model: *Rf1* derived from a breeding line NK198 exhibited monogenic inheritance with the Owen CMS tester lines. These observations do not necessarily contradict each other,

Table 1 Inheritance of fertility restoration on the Owen cytoplasmic male-sterility (*CMS*) cytoplasm, using NK198 as restorer, in four segregation populations

Cross/generation	Segregation				χ^2 (ratio[a])	*P*-value
	Fertile	Partial-fertile	Sterile	Total		
I-12 CMS(R) × (I-12 CMS(R) × NK198)/BC_1F_2	14	32	17	63	0.12 (3:1)	$0.7 < P < 0.8$
I-12 CMS(R) × (I-12 CMS(R) × NK198)/BC_1	2	64	71	137	0.18 (1:1)	$0.5 < P < 0.7$
TK76-MS × NK198/F_2	23	12	12	47	0.005 (3:1)	$0.9 < P < 0.99$
TK81-MS × (TK76-MS × NK198)/three-way cross	19	25	44	88	0 (1:1)	$0.99 < P$

[a]For genetic modeling, fertile and partial-fertile classes were combined into a single class

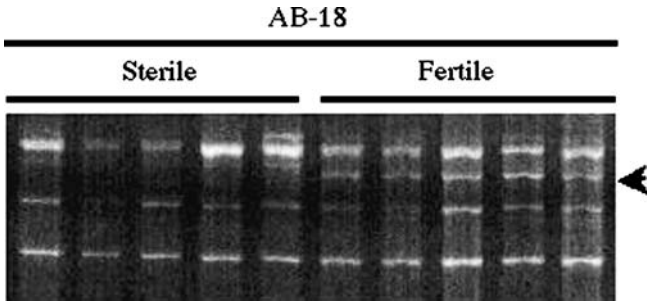

Fig. 1 Image of RAPD marker AB-18. Five fully fertile and five sterile individuals from TK81-MS × (TK76-MS × NK198) population were used in these panels. A PCR fragment linked to the *Rf1* gene is shown by an *arrow*

but suggest that the inheritance of fertility restoration in hybrids with the Owen cytoplasm varied depending on the nuclear backgrounds of the parental lines involved.

We note that there was large variation in pollen fertility of the fertile and partial-fertile segregants that were grouped into a single phenotypic class for genetic modeling. For example, in the F_2 generation of the cross TK76-MS × NK198, 9 out of 35 pollen-producing plants had over 70% stainable pollen. Twelve of the 35 plants, however, produced 20% or less stainable pollen (see Table 1, details not shown). The question remains to be answered whether or not the fully fertile phenotype reflect the *Rf1Rf1* homozygous genotype. The *Rf1*-linked co-dominant markers (e.g., CAPS marker) could allow the accurate genotyping (*Rf1Rf1* or *Rf1rf1*) of the fully fertile segregants. This approach is currently in progress. It is also possible that more than one restorer locus was segregating in the population examined. One simple model would assume that (1) the dominant allele of a second *Rf* gene (e.g., *Z*) enhances the pollen-producing ability of the plants having *Rf1*, leading to complete fertility restoration, and (2) the recessive *rf1* gene is independent of and epistatic to the second gene. Such a gene action would give a ratio of 3 fertile to 1 partial-fertile in the F_2 and a 1:1 ratio in the three-way cross. The data from the crosses involving TK76-MS appear to approximate these ratios, whereas segregation of the I-12CMS(R) × NK198 cross did not give ratios that fitted the same model (see Table 1). More-refined experiments will be necessary to further delineate the inheritance and interactions of *Rf* genes in Owen CMS.

The present study also revealed that the combined strategy using bulked segregant analysis and AFLP markers is an efficient means to identify the markers linked to the *Rf1* gene, as has recently been shown for mapping of the *RfG1* gene in CMS *G* of wild beet (Touzet et al. 2004). We constructed a regional map encompassing the *Rf1* locus, with eight AFLP markers being closely linked to *Rf1*. In addition, our data provided a clear indication of the map location of the *Rf1* locus on the terminal region of linkage group Kiel III/Koeln IV, which makes it likely that *Rf1* corresponds to the *X* gene mapped previously on the same region (Pillen et al. 1993). It should also be noted that three markers, mAFEM972, mAFEM976, and mAFEM985, co-segregated with the *Rf1* allele. These markers are not only available for marker-assisted selection of the maintainer genotype, but also useful for the map-based cloning of *Rf1*.

Until now, five restorer loci have been cloned and functionally characterized: *Rf2*, conditioning male fertility restoration in T-cytoplasm maize (Cui et al. 1996), *Rf* in petunia (Bentolila et al. 2002), *Rfk1*/*Rfo* in *Brassica* (Koizuka et al. 2003; Brown et al. 2003), and *Rf-1* in rice (Kazama and Toriyama 2003; Komori et al. 2004; Akagi et al. 2004). The functional *Rf2* allele in maize was shown to encode a mitochondrial aldehyde dehydrogenase, which in some way compensates for the expression of the mitochondrial URF13 protein that causes male sterility (Schnable and Wise 1997). Other four *Rf* genes encode a mitochondrial-targeted, pentatricopeptide repeat protein that may disrupt accumulation of the mitochondrial protein responsible for male sterility. The final aim of our study is to clone the *Rf1* gene. To this end, we have recently constructed a bacterial artificial chromosome library from the restorer line NK198 (E. Hagihara, H. Matsuhira, T. Mikami and T. Kubo, manuscript in preparation). At present, we are

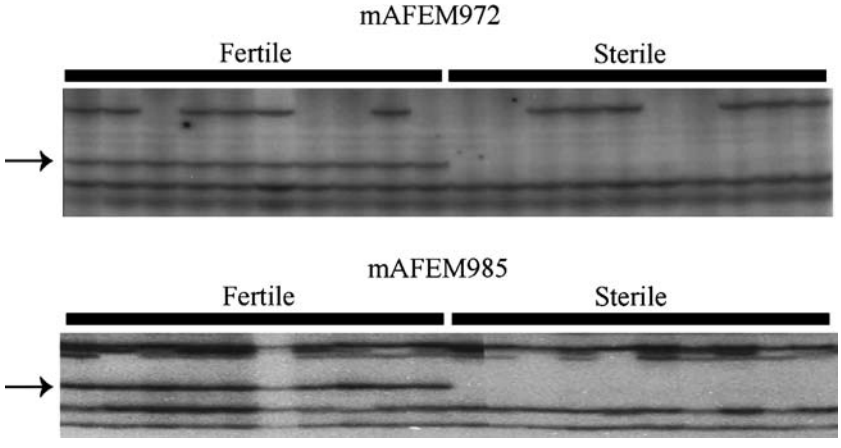

Fig. 2 Images of two AFLP markers, mAFEM972 (*upper panel*) and mAFEM985 (*lower panel*). Ten fertile and ten sterile individuals from TK81-MS × (TK76-MS × NK198) population were used in these panels. The PCR fragments linked to the *Rf1* gene are shown by *arrows*

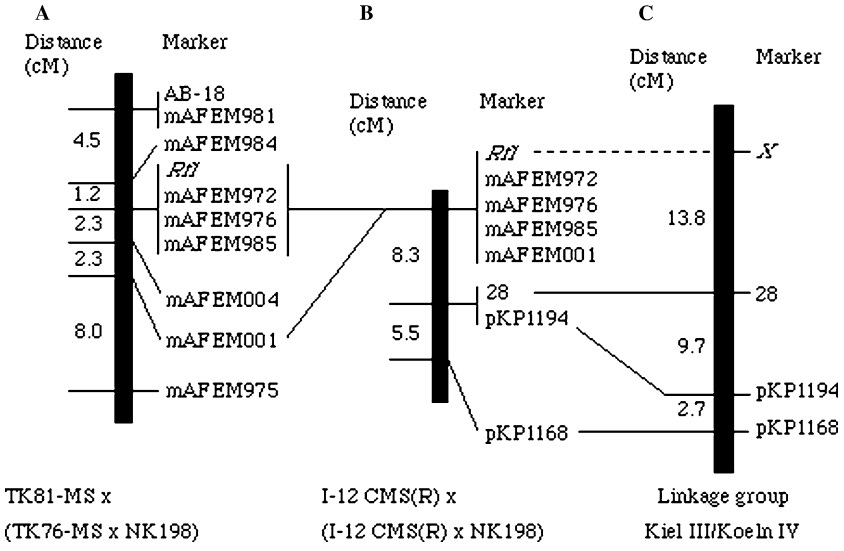

Fig. 3 Linkage map of the chromosomal region around *Rf1* locus and comparison with the maps of linkage group Kiel III/Koeln IV. **a** Map is based on the segregating progeny from three-way cross TK81-MS × (TK76-MS × NK198). **b** Map is based on the BC_1 population of the cross I-12 CMS(R) × NK198. **c** Map of linkage group Kiel III/Koeln IV. The *Rf1* locus, an RAPD marker, eight AFLP markers, and three RFLP markers are located

attempting to establish a contig of BAC clones that span the *Rf1* locus. This information would provide a necessary starting point for identifying candidate gene(s).

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