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ORIGINAL PAPER

Allelic discrimination of the *Restorer-of-fertility* gene and its inheritance in peppers (*Capsicum annuum* L.)

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Abstract Cytoplasmic male sterility (CMS), one of the most important traits in crop breeding, is used for commercial F_1-hybrid seed production in peppers (*Capsicum annuum* L.). A nuclear gene, *Restorer-of-fertility* (*Rf*), can induce normal pollen production in CMS plants resulting in fertility. Since the first report of fertility restoration in peppers, various inheritance modes have been suggested, including the presence of a third haplotype of the locus. The pepper *Rf* gene has not been cloned, and calculated genetic distances of linked markers have varied between research groups. A more precise allelic test and additional genetic mapping are needed to accurately select recombinants for use in marker-assisted backcrossing (MAB). Therefore, the reliability and application of these markers for allelic selection of the *Rf* gene was tested. Two different F_2 populations, Buja and Tamna, were used for the construction of a linkage map. From these linkage groups, a new closely linked flanking marker of the *Rf* gene were identified. Previous allelic testing revealed the existence of a third haplotype, $Rfls^{7701}$, which can function as dominant (*Rf*) or recessive (*rf*). In a previous report, $Rfls^{7701}$ was considered to be linked to unstable male sterility (MS). However, our results suggest that unstable MS was induced by a gene residing at another locus rather than by $Rfls^{7701}$ haplotype-linked allele.

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Introduction

Cytoplasmic male sterility (CMS) refers to the inability to produce functional pollen in plants. Most of its causal factors are in the maternally inherited mitochondrial genome. CMS systems are widely used for production of commercial F_1 hybrid seeds by breeding companies. For seed crops, however, CMS systems are exploited only if a nuclear restorer gene (*Rf*) is introduced to suppress male sterility (MS) in the hybrid plants (Schnabel and Wise 1998; Budar et al. 2003; Hanson and Bentolila 2004). Therefore, reliable markers for *Rf* are needed for accurate marker-assisted backcrossing.

At present, several mitochondrial genes that can induce CMS have been identified in various crop species (Hanson and Bentolila 2004). However, restorer genes (*Rf*) from nuclear genomes have only been cloned from maize (Cui et al. 1996), petunia (Bentolila et al. 2002), radish (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003), and rice (Komori et al. 2004). Since Peterson (1958) first reported CMS in peppers, the inheritance of fertility restoration has been extensively studied by several research groups with diverging results. Peterson (1958), Yu (1990), and Gulyas et al. (2006) have reported that pepper fertility is restored by a single dominant nuclear gene. However, Wang et al. (2004) have reported that fertility restoration is controlled by one major and four minor quantitative trait

loci (QTL). A recent report links a third haplotype (*Rfls*^{7701}), which can be recessive or dominant and may be related to the instability of MS (Min et al. 2008). Despite the conflicting reports, the presence of a single major determinant gene in fertility restoration is widely accepted in the field.

An efficient method for detecting fertility restoration is essential for facilitating F_1 hybrid breeding by eliminating laborious emasculation and test crosses. Furthermore, *Rf*-linked markers are essential for efficient recombinant selection during marker-assisted backcrossing. Recently, one *Rf*-flanking marker, AFRF8CAPS, was reported (Kim et al. 2006) to be located on the same side of the *Rf* gene as the OP13 RAPD marker (Min et al. 2008). Min et al. (2008) reported that the *Rf*-linked sequence in a third haplotype (*Rfls*^{7701}) is different from the formerly known allele, *Rf*. Therefore, *Rf* or *rf* gene function of the third haplotype can only be practically divided via test crosses.

To detect recombinants of the *Rf* locus and to more accurately define map distances of *Rf*-linked markers, precise linkage groups were constructed using amplified fragment length polymorphisms (AFLP) and genome walking in two F_2 mapping populations. Three additional *Rf*-linked markers, AFRF1, AFRF3, and AFRF4, were identified in this study. We also tested allelism to examine the inheritance patterns of *Rfls*^{7701} in various breeding lines and found that *Rfls*^{7701} alone could not induce unstable MS. Therefore, at least one other locus is required to regulate the instability of the *Rf* gene.

Materials and methods

Plant materials and fertility scoring

Male-sterile (S *rf*/*rf*) and restorer (S *Rf*/*Rf*) parental lines of a commercial F_1 cultivar, Buja, were used as control plants, each containing homozygous recessive and dominant *Rf* alleles, respectively. The parental lines of the F_1 cultivar Tamna were used for homozygous recessive *rf* (*Rfls*^{7701} haplotype) and dominant *Rf* alleles. When isolating the flanking sequence of the *Rf* locus, F_2 segregating populations, produced by self-pollination of each F_1 cultivar, were used to investigate co-segregation of the developed markers and MS phenotypes.

MS phenotypes of flowers were evaluated visually with or without a microscope (Fig. 1). Plants lacking any visible pollen on the anthers were scored as MS. Plants with abundant pollen were scored as male-fertile. Plants with only a small quantity of abnormal sticky pollen on the surface of the anthers were scored as unstable male-sterile. All plants were grown under standard greenhouse conditions at the Dongbu Institute (20–30°C, 30–40% relative humidity, and 16-h light/8-h dark cycles).

Allelism tests

Three different tests were performed. (1) The stability of three stable male-sterile lines (S *rf*/*rf*) and three maintainer lines (N *rf*/*rf*) were examined via crossing. (2) To test for a possible relationship between unstable MS and *Rfls*^{7701}, four breeding lines homozygous for *Rfls*^{7701} with normal cytoplasm (N *Rfls*^{7701}/*Rfls*^{7701}) were crossed with three stable male-sterile lines homozygous for *rf* with sterile cytoplasm (S *rf*/*rf*). (3) To test for a relationship between low seed purity of commercial cultivars and unstable MS, two maintainer lines of commercial cultivars having *Rfls*^{7701} (N *Rfls*^{7701}/*rf*) were crossed with three stable male-sterile lines (S *rf*/*rf*). Fertility was scored as described above.

AFLP analysis of recombinant plants and linkage group construction for the *Rf* locus

To find *Rf*-linked markers residing on the side of the *Rf* locus opposite the OP13, OPP13CAPS, and CaRf-M1 markers (Kim et al. 2006; Min et al. 2008; Zhang et al. 2000), recombinants were selected from 939 F_2 plants of the Buja population. After comparing phenotypes and CaRf-M1 marker scores, the discordant plants were determined to be recombinant. The parental line of Buja, the *Rf*-pool, the *rf*-pool, and the recombinants were used for AFLP analysis. The *Rf* genotypes of some randomly selected recombinants were confirmed via segregation ratio after self-pollination.

AFLP analysis was performed, as described by Vos et al. (1995) with minor modifications. Three *Eco*RI

Fig. 1 Phenotypic differences of male fertility in pepper flowers. **a** Fully fertile flowers with abundant pollen, **b** stable male-sterile flowers with nearly no pollen on their shrinking anthers, and **c** unstable male-sterile flowers bearing a relatively small quantity of sticky pollen on their anthers

primers with three selective nucleotides and eight *Mse*I primers with three selective nucleotides were screened. Amplified fragments were separated on denaturing 6% polyacrylamide gels. The gels were stained with SILVER SEQUENCE™ staining reagents, as per the manufacturer's instructions (Promega, Madison, WI, USA).

Conversion of AFLP markers to sequence-tagged-site (STS) markers

Each polymorphic band shared between parental lines (the *Rf*-pool, the *rf*-pool, and the four recombinants) was cut from a glass plate and eluted, as described in Sambrook et al. (2000). Eluted AFLP fragments were cloned into the pGEM-T easy vector (Promega). The clones were sequenced using automated Big Dye DNA cycle sequencing (Applied Biosystems, Foster City, CA, USA) on a capillary sequencer (ABI 3700 Genetic Analyzer; Applied Biosystems). For genome walking, the flanking sequence of each AFLP fragment was obtained using the Universal Genome Walker Kit (Clontech, Mountain View, CA, USA), according to the manufacturer's instructions and sequenced as described above. If single nucleotide polymorphisms (SNPs) or insertion/deletion (IN/DEL) mutations were found, cleaved amplified polymorphic sequence (CAPS) primers or STS primers were designed. PCRs using corresponding primers were performed using genomic DNA isolated from five plants of each *Rf* genotype (*Rf*/*Rf*, *Rf*/*rf*, and *rf*/*rf*) for segregation analysis.

Linkage analysis of the molecular markers and *Rf* gene

For linkage analysis, two different F_2 segregating populations were produced by self-pollination of the Buja and Tamna F_1 cultivars. The populations consisted of 939 and 99 individual plants, respectively.

Three previously identified *Rf*-linked markers, AFRF8CAPS (Kim et al. 2006), CaRf-M1 (Min et al. 2008), and CRF3S1S (Gulyas et al. 2006) were utilized in the selection of recombinants and the calculation of genetic distances. For detection of the three different haplotypes, flanking sequences of the AFRF8CAPS and CRF3S1S markers were obtained using the Universal Genome Walker Kit, as described above (Table 1).

Linkage analysis was performed using MapMaker 3.0b. The linkage group was established with an LOD-score threshold of 4.0 and a maximum distance of 30 cM using the Kosambi mapping function (Kosambi 1944).

Genotyping of *Rf* alleles using molecular markers

Total genomic DNA was isolated using the protocol described by Kim and Kim (2005). The Tm values of all primers designed from the *Rf*-linked sequences were 65°C or greater. PCRs were performed in a 10-μL reaction mixture containing 0.025 μg template, 10 μM forward and reverse primers, 10 mM dNTPs, and 0.1 μL Advantage 2 Polymerase Mix, as per the manufacturer's instructions (Clontech, Palo Alto, CA, USA). PCR cycles were as follows: 95°C for 4 min, 40 cycles of 95°C for 30 s, 65 or 68°C for 30 s, and 72°C for 2 min, and a final 5-min

Table 1 *Rf*-linked markers used in this study

Marker[a]	Type	Sequence	Size (bp)	Distance (cM)
AFRF1	AFLP	*Eco*RI + ACT/*Mse*I + CCA	478	1.1
AFRF1CAPS	CAPS	5′-CTGCGTACCAATTCACTAAAGCACT-3′	474	1.1
		5′-TGAGTCCTGAGTAACCAGGTCATTG-3′	*Kpn*I[b]	
AFRF3	AFLP	*Eco*RI + ACT/*Mse*I + CGC	261	18.3
AFRF3CAPS	CAPS	5′-GCAATGGAGAAGGTGAAAGTGATTCTGG-3′	522	18.3
		5′-TTGGACTTCATGTCCTTTTCAGCTTCC-3′	*Eco*RV	
AFRF4	AFLP	*Eco*RI + AGG/*Mse*I + CTT	218	0.1
CaRf-FL-M2	STS	5′-ATGGATCCAAAGGACCACAAGCTAACC-3′	650	0.5
		5′-GCATTGGGACTGAAATCCCCAAATAAG-3		
CRF3S1S	SCAR	5′-ATTTTCAGATTGTGGCGACG-3′	870	1.5
		5′-CGACCATCACGACGAGG-3′		
CRFCAPS	CAPS	5′-GATTCATCCTCGACGAAAGAGGATTCAC-3′	1,534	1.5
		5′-TCAAGGAAATCCCCAAATAAGTGTGAACC-3′	*Hha*I	
OPP13CAPS	CAPS	5′-TACAGCTTCAAAGTAAACACAACC-3′	1,350	0.4
		5′-ATTCGGGTCCCAAGAAGGTTCTAT-3′	*Hinf*I	

[a] Marker names linked to the *Rf* locus

[b] Restriction enzymes for the detection of polymorphisms

extension at 72°C. PCR products were visualized on 1–2% agarose gels stained with ethidium bromide.

Fluorescein diacetate (FDA) staining

In order to assess pollen viability, pollen grains from dehisced anthers were stained with FDA, as previously described (Heslop-Harrison and Heslop-Harrison 1970). FDA was dissolved in acetone (2 mg/ml), and diluted with 10% sucrose before staining. Pollen grains were incubated with FDA/sucrose dilutions at room temperature for 5 min, then examined with a fluorescence microscope (Nikon, Tokyo, Japan) using a 465–495-nm excitation filter, a 505-nm dichroic mirror, and a 515–555-nm barrier filter.

Results

Fertility scoring of the mapping population

In the Buja F_2 population, a total of 939 individuals were examined for fertility. Of these, 693 individuals showed normal fertility, 195 plants showed complete sterility, and 51 plants showed unstable sterility (Fig. 1). The phenotype segregation ratio was close to the expected 12:3:1 ratio (male-fertile:male-sterile:unstable sterile plants) ($\chi^2 = 3.225$, $P = 0.1994$). Some of the unstable sterile plants were artificially self-pollinated and had relatively low number of seeds (4–20 seeds per fruit). In the Tamna F_2 population (99 plants), the ratio of male-fertile (77) to sterile (22) plants was roughly 3:1 ($\chi^2 = 0.407$, $P = 0.5233$) and no unstable sterile individuals were found.

AFLP analysis of recombinants flanking the *Rf* locus

To detect molecular markers of CaRf-M1, genetic mapping was performed in recombinant plants. For this purpose, the phenotypes (male sterility or fertility) and CaRf-M1-marker types of 939 Buja F_2 plants were compared and six recombinant plants were identified. From these, four recombinants were used for AFLP analysis after confirmation of the *Rf* genotypes. The progeny test of three recombinants showed the expected segregating ratio of 3:1 (male-fertile:MS). All three recombinants were heterozygous *Rf/rf* (Table 2). The fourth recombinant plant, RcB02, was male-sterile.

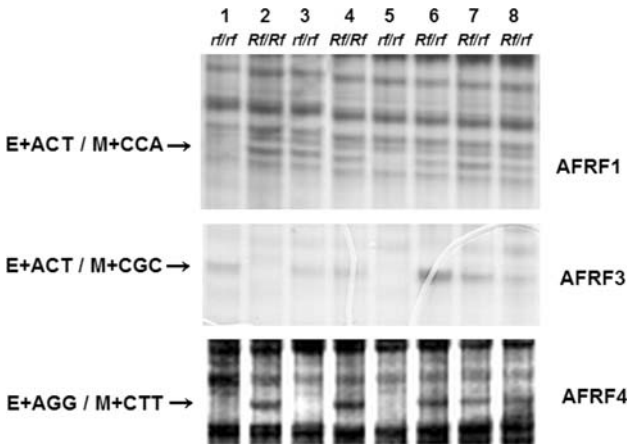

Fig. 2 AFLP analysis for the identification of *Rf*-linked markers using BSA pools and recombinants. Three polymorphic bands between parental lines, the *Rf*-/*rf*-BSA pool and/or the recombinants. *1* male-sterile parent; *2* restorer parent; *3* *rf*-BSA pool; *4* *Rf*-BSA pool; *5–8* recombinant from Buja F_2 population

For AFLP analysis, five plants, which were either homozygous dominant or recessive, were pooled to make *Rf*- or *rf*-BSA (bulked segregant analysis) pools, respectively. In addition to the *Rf*-BSA and the *rf*-BSA pools, the four recombinants were used for AFLP analysis. A total of 24 primer combinations (three *Eco*RI primers with three selective nucleotides and eight *Mse*I primers with three selective nucleotides) were used. Three primer combinations showed polymorphisms between pools and/or recombinants (Fig. 2). The polymorphic bands were cloned and sequenced.

Genome walking was used to identify primer sets for obtaining larger amplified fragments. All AFLP-derived markers were checked for co-segregation with their specific phenotypes using 15 Buja or Tamna F_2 individuals (five plants each having *Rf/Rf*, *Rf/rf*, or *rf/rf* genotypes) (Fig. 3). Three AFLP-derived markers were selected for further genetic mapping. AFRF1 and AFRF3 were easily converted to STS markers using the cloned AFLP-band or one step of genome walking.

Table 2 Segregation ratio of the progeny from self-pollinated recombinants

Recombinant (F_2)	Phenotype	CaRf-M1-linked type	Self-pollinated progeny (F_3)		*Rf* genotype
			Male-fertile plant	Male-sterile plant	
RcC05	Fertile	*rf*	13	3	*Rf/rf*
RcD04	Fertile	*rf*	20	6	*Rf/rf*
RcJ02	Fertile	*rf*	37	12	*Rf/rf*

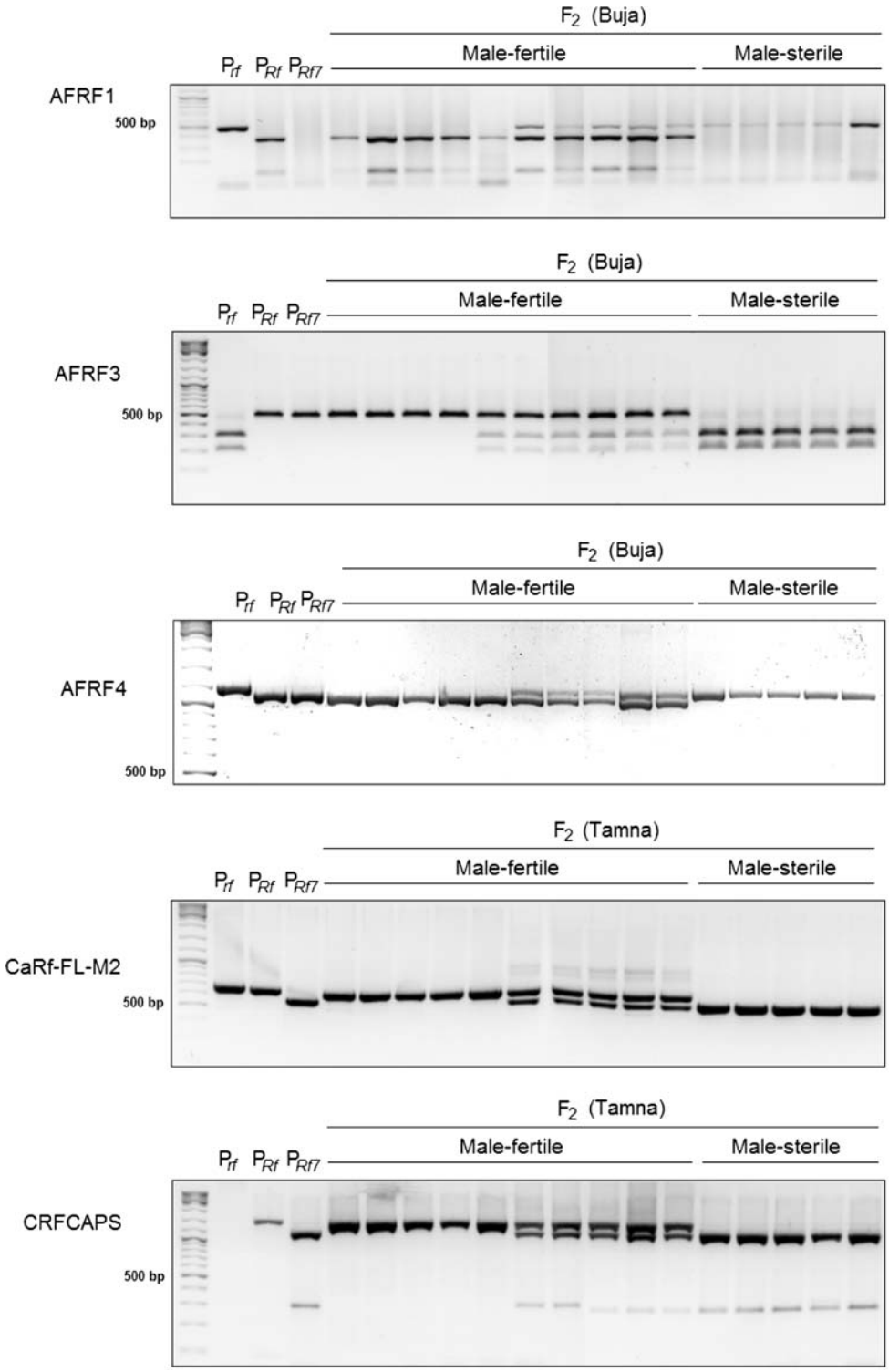

Fig. 3 Co-segregation of the PCR-based markers developed from AFLP markers and the *Rf* phenotypes in F_2-segregating populations. P_{rf} male-sterile line (*rf*/*rf*); P_{Rf} restorer line (*Rf*/*Rf*); P_{Rf7} male-sterile line ($Rfls^{7701}$/$Rfls^{7701}$)

Linkage analysis of the *Rf* locus

Three previously reported *Rf*-linked markers, CaRf-M1, AFRF8CAPS, and CRF3S1S, as well as the AFLP-derived markers, were used for linkage group construction. AFRF8CAPS was initially reported by Kim et al. (2006) to be the flanking marker of OP13 RAPD, but the marker was later shown to be located at the same site as OP13 RAPD (Min et al. 2008). Moreover, when analyzing homozygous $Rfls^{7701}$ haplotype breeding lines, PCR with the

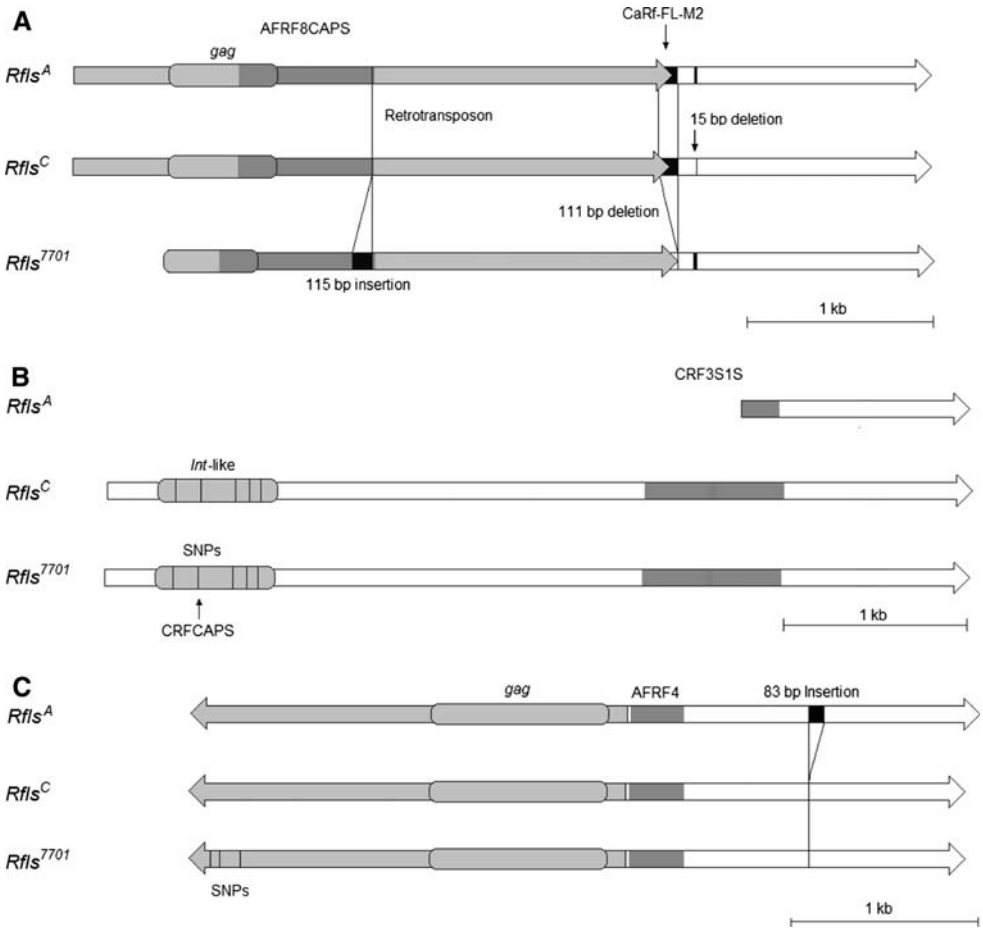

Fig. 4 Alignment of three haplotypes of *Rf*-linked sequences derived from **a** AFRF8CAPS marker, **b** CRFCAPS marker, and **c** AFRF4 marker. The *arrows* indicate 5′–3′ direction. *Dark gray boxes* represent the fragment used as an initial *Rf*-linked sequence for genome walking. *Light gray boxes* indicate putative retrotransposon sequences. *gag gag* domain; *Int* integrase domain; $Rfls^A$, $Rfls^C$, and $Rfls^{7701}$ *Rf*-linked sequences

AFRF8CAPS primers failed because these breeding lines have 115 bp of inserted sequence that prohibits primer binding. To utilize AFRF8CAPS marker information, the other marker CaRf-FL-M2 was designed from 4.7 kb of AFRF8CAPS-flanking sequence (Fig. 4a).

Another *Rf*-linked marker, CRF3S1S SCAR (Gulyas et al. 2006), generated a single monomorphic band for $Rfls^C/Rfls^{7701}$ heterozygous breeding lines, which was not applicable to the Tamna F_2 population (data not shown). About 4.6 kb of flanking sequence of the CRF3S1S marker showed various SNPs between the two haplotypes. One SNP was converted to a CAPS marker, CRFCAPS, and was co-segregated in the Tamna F_2 population and used for further genetic mapping (Fig. 3).

Two linkage groups were constructed from the Buja and Tamna F_2 populations (Fig. 5). They shared four common markers (AFRF1, CaRf-M1, CaRf-FL-M2, and CRF). In the genetic analysis, the *Rf* locus was delimited to a genetic interval of 0.5 cM between CaRf-M1 and AFRF4 in the Buja F_2 population (Table 3). The CRF3S1S marker resided on the opposite side of the *Rf* locus as did CaRf-M1 in the Buja F_2 map (1.5 cM). However, CRFCAPS co-segregated with the *Rf* locus in the

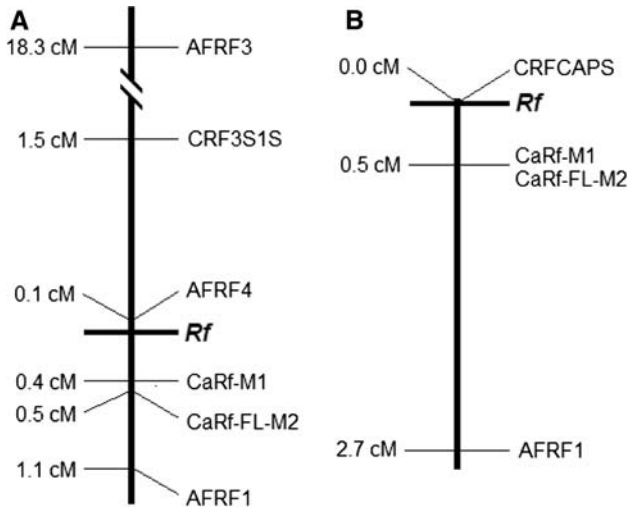

Fig. 5 Linkage maps showing genetic distances of the pepper *Rf* gene and molecular markers from **a** Buja and **b** Tamna F_2 populations. The marker names and distances (Kosambi, cM) are presented on the *right* and *left* sides of the *central bar*, respectively

Tamna F_2 population linkage map. The small Tamna F_2 mapping population may be the cause this contradictory result.

Table 3 Recombinant genotypes used for genetic mapping of the Buja F_2 *Rf* locus

Recombinant	Plant phenotype	Molecular marker			
		AFRF4	CaRf- M1	CaRf-FL-M2	AFRF1
RcC10	S[a]	*F*[b]	S	S	S
RcJ03	S	*F*	S	S	S
RcB02	F	F	*S*	*S*	*S*
RcC05	F	F	*S*	*S*	*S*
RcD02	F	F	*S*	*S*	*S*
RcD04	F	F	*S*	*S*	*S*
RcJ02	F	F	*S*	*S*	*S*
RcC02	F	F	*S*	*S*	*S*
RcC03	F	F	F	*S*	*S*
RcC01	F	F	F	F	*S*
RcB01	S	S	S	S	*F*
RcC04	S	S	S	S	*F*
RcD03	S	S	S	S	*F*
RcF01	S	S	S	S	*F*
RcI02	S	S	S	S	*F*

S sterile; *F* fertile

[a] Only plants with recombination events tightly linked to *Rf* are shown

[b] Italicized plants denoted discrepancy between marker-linked genotype and phenotype indicating their recombinant nature

A comparison between phenotype and AFRF4 marker type of three recombinants from the Buja F_2 population was performed to determine the accurate location of AFRF4 within the Buja linkage group. AFRF4 resides on the opposite side of the *Rf* locus as the CaRf-M1 generated monomorphic bands, regardless of their phenotype. Therefore, AFRF4 marker-based genotypes co-segregated with their phenotypes (Fig. 6).

Relationship between unstable sterility/fertility and the $Rfls^{7701}$ haplotype

Three stable maintainer lines were crossed with three stable MS lines (category I), resulting in progeny that produced no pollen and confirming the MS stability in the three stable MS lines. Four homozygous $Rfls^{7701}$ breeding lines with normal cytoplasm were crossed with three stable MS lines (category II). The resulting progeny also showed stable MS phenotypes (data not shown).

A previous report had shown that some breeding lines that have both a sterile cytoplasm and the $Rfls^{7701}$ haplotype have unstable MS (Min et al. 2008). Two maintainer lines of commercial cultivars with the $Rfls^{7701}$ haplotype were crossed with stable male-sterile lines, because these cultivars showed relatively low seed purity (category III). Though a few of their progeny showed unstable MS (Table 4; Fig. 7), the $Rfls^{7701}/Rfls^{A}$ and $Rfls^{A}/Rfls^{A}$ genotypes were represented by a 1:1 ratio. Therefore, no relationship between MS instability and the $Rfls^{7701}$ haplotype was observed.

Discussion

Recombinant selection and development of markers linked to the pepper *Rf* locus

Flanking markers that are tightly linked to the *Rf* locus have practical applications within breeding programs. In this study, we identified markers that are more closely linked to the *Rf* locus than those previously reported, and that reside on the opposite side of the *Rf* locus. Several recombinant plants from the 939 Buja F_2 individuals were selected for comparison with the closest known linked marker (CaRf-M1) and their fertility/sterility phenotypes. A total of six recombinants were identified from that comparison. One male-sterile and three male-fertile recombinants were used for further AFLP analysis. To define the genotype of the recombinants, the three fertile recombinants were self-pollinated, and the segregation ratios of the F_3 individuals were observed. All three fertile recombinants showed segregated phenotypes and contained heterozygous *Rf* alleles (Table 2).

Rf-BSA and *rf*-BSA pools were also used for AFLP analysis. Of the 24 primer combinations tested, three primer combinations resulted in polymorphic bands and a single primer combination (AFRF4) co-segregated with the *Rf* genotypes from both BSA pools and all recombinants (Fig. 2). From 939 Buja F_2 plants, only two recombinants were observed using the AFRF4-derived marker. This indicates that AFRF4 is the closest marker to the *Rf* locus (Table 3). Thus, AFLP analysis of recombinants would be an effective way to find additional tightly linked markers of the *Rf* locus.

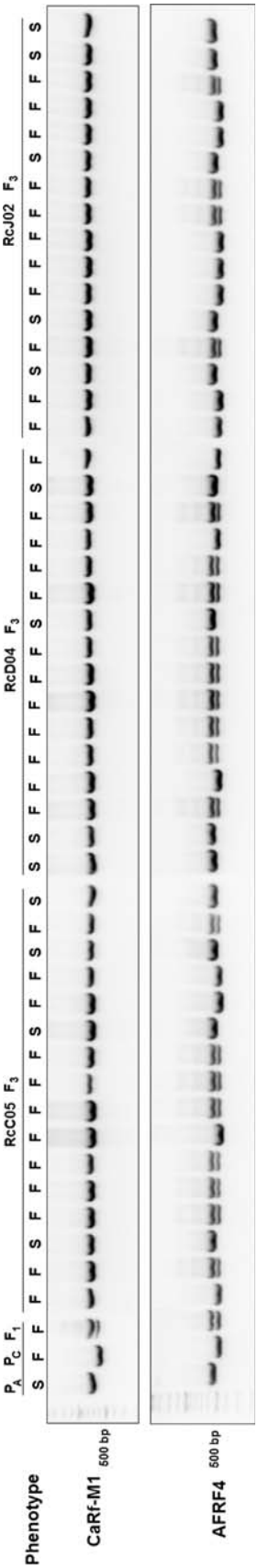

Fig. 6 Co-segregation of AFRF4 marker and the phenotypes of progeny from self-pollinated F_2 plants. P_A male-sterile parent (*rf/rf*); P_C restorer parent (*Rf/Rf*); F_1 F_1 hybrid

In this study, we analyzed three previously reported *Rf*-linked markers, CaRf-M1, AFRF8CAPS, and CRF3S1S. Of these, two markers were modified to identify each *Rf*-linked haplotype. AFRF8CAPS has been reported to be the closest flanking marker of the *Rf* locus (Kim et al. 2006), but it resides on the same side of the *Rf* locus as the OP13 RAPD and OPP13CAPS markers (Min et al. 2008). The AFRF8CAPS-derived CaRf-FL-M2 marker is positioned 0.1 cM further away than CaRf-M1. The AFRF8CAPS marker is not applicable for many breeding lines containing a third haplotype, *Rfls*^{7701}, because PCR amplification is not possible (data not shown). Additional sequences obtained via genome walking revealed a 115-bp insertion in the *Rfls*^{7701} haplotype, which disturbed primer binding of the AFRF8CAPS marker.

The CRF3S1S marker has been reported to be located approximately 5.3 or 4.8 cM away from the *Rf* locus by examining F_3 and F_4 populations, respectively (Gulyas et al. 2006). However, our study indicates that CRF3S1S is closer to the *Rf* locus than was reported by Gulyas. CRF3S1S marker could not differentiate between the *Rf and Rfls*^{7701} haplotypes, since identical bands were amplified. However, some SNPs specific to the third haplotype were detected by extending flanking sequences. The CaRf-M1 marker was generated from OPP13CAPS (Min et al. 2008), but the reported genetic distances of the related markers varied between studies. Previous work suggests that OP13 RAPD is 0.37 cM (Zhang et al. 2000) and OPP13CAPS is 1.1 cM (Kim et al. 2006; Min et al. 2008) away from the *Rf* locus, but the present study showed a result (0.4 cM) more similar to the initial report (0.37 cM) of Zhang et al. (2000).

Sequences of CaRf-M1 and AFRF8CAPS showed significant similarity to retrotransposons (Kim et al. 2006; Min et al. 2008). Our results support this finding. For example, BlastX searches indicated that a 3.2-kb region from a 4.7-kb flanking sequence of AFRF8CAPS included a *gag*-like domain and belonged to a retrotransposon (Fig. 4a). The 4.6-kb flanking sequence of CRF3S1S showed partial similarity with a retrotransposon by having an *integrase*-like domain (Fig. 4b). Half of the 3.4-kb flanking sequence of AFRF4 showed similarity to retrotransposons and contained a *gag*-like domain, as well (Fig. 4c). In plants, the long terminal repeat (LTR) and the non-LTR retrotransposons are commonly found in high copy numbers and their proportions are highly variable depending on genome size. For example, as little as 4–10% of the *Arabidopsis* genome consists of retrotransposons, whereas they make up 70–85% of the maize genome (reviewed by Kumar and Bennetzen 1999). The sequences of closely linked markers indicate that the flanking sequences of the pepper *Rf* locus is probably composed of highly duplicated retrotransposon

Table 4 Frequency of stable or unstable MS plants from allelism test

Cross combination		F_1 hybrid			
Maternal line	Paternal line	Genotype	Phenotype		
			Male-fertile	Male-sterile	Unstable sterile
1201 (S $Rfls^A/Rfls^A$)	AS702 (N $Rfls^{7701}/Rfls^A$)	$Rfls^A/Rfls^A$	0[a]	8	0
		$Rfls^{7701}Rfls^A$	0	9	1
1221 (S $Rfls^A/Rfls^A$)	AS702 (N $Rfls^{7701}/Rfls^A$)	$Rfls^A/Rfls^A$	0	22	2
		$Rfls^{7701}Rfls^A$	0	20	4
1221 (S $Rfls^A/Rfls^A$)	AS703 (N $Rfls^{7701}/Rfls^A$)	$Rfls^A/Rfls^A$	0	5	2
		$Rfls^{7701}Rfls^A$	0	10	0

[a] Numbers indicate occurrence of specific phenotypes in allelism tests

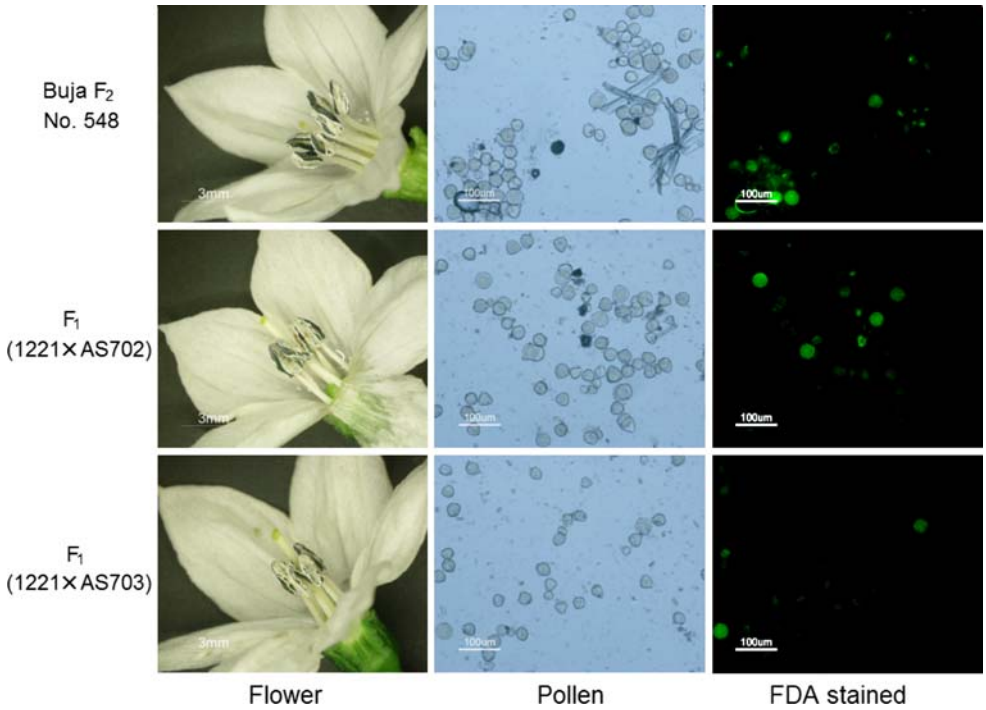

Fig. 7 Morphological characteristics of pepper flowers showing unstable MS from different lineages. Pollen grains were observed using bright field and fluorescence microscopy after staining with FDA for the fluorescent microscopic observation

sequences, which prohibits acquiring appropriate flanking sequences of the *Rf* locus. These retrotransposon sequences may explain the disparity of reported distances for *Rf* markers.

Recently, the major QTL for fertility restoration was positioned at the upper terminus of P6 (Wang et al. 2004), and OPP13CAPS was mapped to the upper terminus of LG6 (chromosome 6; Lee et al. 2009). Since the latter was constructed by joining intra-specific (*C. annuum* × *C. annuum*) and inter-specific (*C. annuum* × *C. chinense*) genetic maps, it is difficult to use these data for marker-assisted backcrosses (MAB) in commercial breeding programs. Therefore, the linkage group presented in this study will be useful for background and recombinant selection in commercial MAB programs.

Allelism and relationship of $Rfls^{7701}$ and unstable MS

MS of peppers was originally reported by Peterson (1958) and, since then, several hypotheses have been proposed to explain this phenomenon. It has been suggested that another locus, *St,* present in the nucleus might participate in unstable MS (Lee 2001). Three alleles, St^2, St^U, and St^1, constitute the *St* locus. St^U is dominant to St^1, which affects MS and its restoration, resulting in MS instability. However, others have suggested that a single major QTL present on chromosome P6 is responsible for fertility restoration, and four minor QTLs in other chromosomes contribute to complete sterility and fertility (Wang et al. 2004).

A third haplotype, $Rfls^{7701}$, linked to the *Rf* allele was recently identified (Min et al. 2008). $Rfls^{7701}$ has been

implicated in the instability of MS because it has been observed at a relatively low frequency in Korean breeding lines, mainly cytoplasmic-genic male sterility (CGMS) F_1 cultivars, but dominantly observed in foreign originated-breeding lines (Min et al. 2008). Production of non-pungent peppers, such as bell peppers, usually uses genic male sterility GMS lines for F_1-hybrid seed production, because of the relatively high frequency of unstable MS lines and rareness of stable restorer lines. Therefore, converting to stable CGMS lines would be difficult (Lee 2001; Wang et al. 2004; Yu 1990; Zhang et al. 2000).

The frequency of allelism tests indicated that the presence of $Rfls^{7701}$ haplotype did not indicate MS instability, rather other loci contributed to the instability. Approximately 1/16 of the Buja F_2 population was unstable MS plants, though their maternal lines contained the recessive *rf* allele ($Rfls^{A}$ haplotype; Fig. 7). When these unstable MS plants were self-pollinated, we observed a relatively low seed setting (4–20 seeds per fruit, unpublished data). Phenotypes of these progeny are under investigation.

Several studies have shown that temperature and seasonal variation affect unstable MS of peppers. It has been reported that MS becomes unstable at low temperatures (Peterson 1958), but stable at relatively high temperatures (Shifriss and Guri 1979). When the temperature dropped below 24°C, meiotic breakdown and microspore abortion were observed (Shifriss 1997). On the contrary, Lee (2001) reported that unstable MS plants were identified and self-pollinated under relatively high temperatures (32/25°C day/night) and could not bear fruit under relatively cold conditions (25/17°C day/night). Moreover, most of the unstable sterile plants showed seasonal variation in their instability. A greater number of unstable MS flowers appeared during the summer season (June–August); however, most of the stable MS flowers appeared during the cooler season (fall to spring) (unpublished data). If two different loci participate in MS instability, each locus might independently react to temperature. Further experiments will be needed to clarify the unstable MS inheritance and to develop molecular markers of genes that induce unstable MS. The discovery of molecular markers for MS cytoplasm, *Rf*, and unstable male-sterility will facilitate marker-assisted CGMS breeding systems.

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