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ORIGINAL PAPER

Linkage analysis between the partial restoration (*pr*) and the restorer-of-fertility (*Rf*) loci in pepper cytoplasmic male sterility

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Abstract Cytoplasmic male sterility (CMS) in chili pepper is restored by one major dominant nuclear gene, *restorer-of-fertility* (*Rf*), together with some modifier genes and is also affected by temperature. As a result, male fertility was identified as having several phenotypes. That identified and used in the present study allowed partial restoration of fertility, producing plants that simultaneously produce normal and aborted pollen grains, with most grains stuck to the anther wall, even after dehiscence, resulting in low seed set per fruit. The trait was visible only in the presence of Paterson's sterile cytoplasm and was controlled by a recessive nuclear gene, *partial restoration* (*pr*). A CAPS marker, PR-CAPS, closely linked to the trait, has been developed by Lee et al. (2008). In this study, linkage analysis was performed in 205 F_2 individuals derived from the 'Buja' Korean commercial F_1 chili pepper variety using the PR-CAPS marker and the three *Rf*-linked markers (OPP13-CAPS, AFRF8-CAPS, and CRF-SCAR) previously reported. Consequently, we found that these four markers were tightly linked. This result means that the *pr* gene might be tightly linked to the *Rf* locus or the third allele of *Rf* locus. The sequence diversity of the *pr*- and *Rf*-linked markers was also analyzed. The internal sequences of OPP13-CAPS (1,180 bp) and PR-CAPS (640 bp) markers in 91 Korean inbred lines were clearly divided into three haplotypes. According to the sequencing results, a new PR-CAPS (*Mse*I or *Sph*I digestion) marker was designed to distinguish the three haplotypes. This marker will be useful for marker-assisted selection to develop new maintainers and restorers in commercial hybrid pepper breeding using CMS.

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Introduction

Cytoplasmic male sterility (CMS) is important in many crops for F_1 hybrid seed production as well as for research on nuclear–mitochondrial interactions (Duvick 1959; Budar et al. 2003; Hanson and Bentolila 2004; Chase 2007). CMS is a maternally inherited inability to produce functional pollen grains due to the presence of a novel chimeric mitochondrial open-reading frame that results from mitochondrial DNA rearrangements (Hanson 1991; Linke and Börner 2005). Male fertility has often been restored in cases of CMS by a specific nuclear gene, termed restorer-of-fertility (*Rf*), that alters expression of CMS-associated genes in many plants (Schnable and Wise 1998; Hanson and Bentolila 2004; Horn 2006).

CMS in chili peppers (*Capsicum annuum* L.) was first found by Peterson (1958) in USDA accession PI 164835. This CMS pepper was thought to be the only usable source for F_1 hybrid seed production using a cytoplasmic-genic male sterility system (Shifriss 1997). Several molecular studies have been performed to reveal the cause of the pepper CMS. RFLP analysis of male-fertile and CMS peppers revealed that the *coxII* and *atp6-2* regions of both lines have different DNA structures (Kim et al. 2001). In addition, based on sequences of these regions, two CMS-specific SCAR markers were developed (Kim and Kim 2005). Recently, two candidate genes, *atp6-2* (Kim and Kim 2006) and *orf456* (Kim et al. 2007), of CMS in chili pepper were characterized to be the determinant.

It has generally been reported that pepper CMS could be restored by one major dominant *Rf* gene (Peterson 1958). However, several test crosses exhibited a duplicate gene ratio (3 fertile:1 sterile), suggesting the existence of an additional duplicate *Rf* gene (Peterson 1958). In addition, fertility restoration in sweet pepper was found to be controlled by two complementary genes, based on the 1:3 and 9:7 (fertile:sterile) ratios in the BC_1 and F_2 populations, respectively (Novak et al. 1971). CMS can also be temporarily restored by low temperatures, indicating the presence of sterility-modifier genes that are affected by temperature (Peterson 1958; Shifriss and Guri 1979; Shifriss 1997). Hence, fertility restoration in pepper CMS is likely controlled in a complex manner. According to these results, developing molecular markers linked to the genes related to fertility restoration are required for marker-assisted selection (MAS). Two RAPD markers, $OP13_{1400}$ (0.37 cM) and $OW18_{800}$ (8.12 cM), linked to the major *Rf* gene were firstly reported by Zhang et al. (2000). Lee et al. (2004) and Gulyas et al. (2006) also developed an STS marker (CRF-SCAR) from an *Rf*-linked RAPD marker, OPT-02/570 (5 cM). Kim et al. (2006) identified a breeder-friendly codominant CAPS marker, AFRF8-CAPS (1.8 cM), that was converted from an AFLP marker E + ACT/M + GAC. Furthermore, QTL analysis for fertility restoration has been performed to determine the effect of the major *Rf* gene, some minor genes, and environment-related genes. The major *Rf* locus was mapped on the chromosome P6, and four additional minor QTLs were also positioned on chromosomes P5 and P2 and on linkage groups PY3 and PY1, respectively (Wang et al. 2004).

Rf alleles are mainly distributed in several hot and small-fruited pepper genotypes, whereas many sweet or large-fruited genotypes possess *rf* alleles (Peterson 1958; Novak et al. 1971; Yoo 1990; Zhang et al. 2000; Lee 2001; Kumar et al. 2007). According to the male fertility of F_1 plants, Yoo (1990) classified pepper lines into three groups: maintainer, restorer, and uncertain (so-called unstable) types. Moreover, the unstable lines could be divided into two different phenotypes (Lee 2001). One phenotype consists of unstable male-sterile plants, which are usually sterile but whose fertility can be temporarily restored at low temperatures. This is the unstable phenotype, which was previously reported as the instability of pepper CMS (Peterson 1958; Shifriss and Guri 1979; Lee 2001; Pákozdi et al. 2002). The other phenotype consists of partially restored plants that simultaneously produce normal and aborted pollen grains that remain stuck to the anther wall, even after dehiscence, resulting in low seed setting (Lee et al. 2008). The inheritance of these two unstable traits was reported to be controlled by nuclear genes, which differ from the *Rf* gene (Lee 2001). Recently, this latter gene responsible for the partially restored phenotype was named *partial restoration* (*pr*), and a CAPS marker, PR-CAPS (1.8 cM), closely linked to the *pr* locus was developed (Lee et al. 2008).

In this study, we aimed to determine the relationship between *pr* and *Rf* loci by using molecular analysis of one *pr*-linked marker (PR-CAPS) and three *Rf*-linked markers (OPP13-CAPS, AFRF8-CAPS, and CRF-SCAR). Based on the results of linkage and sequencing analysis between markers, we suggest that the *pr* gene might be tightly linked to the *Rf* locus or the third allele of *Rf* locus.

Materials and methods

Plant materials

A total of 205 F_2 individuals of the 'Buja' (Dongbu Hitek Co.) chili pepper (*Capsicum annuum* L.), a Korean commercial F_1 variety (S*, Rf/rf*) developed using the Peterson's CMS, were used for linkage analysis of *pr*-linked or *Rf*-linked markers. To investigate the diversity of sequences in the *pr*-linked and *Rf*-linked regions, a total of 91 inbred lines, including 22 male-sterile (A), 28 maintainer (B), and 41 restorer (C) lines, were used. To determine any relationships between the *pr* and the *Rf* or *rf* genes, the (S) *pr/pr* line was crossed with each of two maintainers (B1 and B2) and two restorers (C1 and C2), and male fertility of their F_1 hybrids was assessed.

Assessment of male fertility

Pepper plants were grown in a greenhouse, under natural conditions, from February to June. Phenotypes were visually scored as one of three different types: completely male-sterile (*rf/rf*), fully restored (*Rf/Rf*), or partially restored (*pr/pr*), according to the method of Lee et al. (2008). The partially restored plants (*pr/pr*) were identified by the simultaneous presence of normal and aborted pollen grains that did not separate easily from the anther wall after dehiscence.

Analysis of DNA markers linked to the *pr* or *Rf* gene

The PR-CAPS marker converted from the E-AGC/M-GCA_{122} AFLP marker was used to analyze the linkage relationship between the *pr* and *Rf* genes (Lee et al. 2008, GenBank accession EU121861). In addition, three *Rf*-linked markers, OPP13-CAPS (Zhang et al. 2000; Kim 2005), AFRF8-CAPS (Kim et al. 2006), and CRF-SCAR (Lee et al. 2004; Gulyas et al. 2006), were also analyzed in the 205 F_2 individuals. All of these markers were prepared using the following PCR protocol: an initial denaturation at 95°C for 5 min; 40 cycles of amplification, each consisting of 95°C for 45 s, annealing for 45 s, and 72°C for 90 s; and a final extension at 72°C for 10 min. Each primer and

Table 1 Primers and annealing temperatures of the markers used in this study

Marker name (enzyme)	Primer (5′ to 3′)		Annealing temperature (°C)	Reference
	Forward	Reverse		
PR-CAPS (*Mse*I)	ATGTCACCCCCACA-CACTCCTTCACCT	TCCCATCTAGCCTCT-GCCTTCTCAAATG	56	Lee et al. 2008
OPP13-CAPS (*Hin*fI)	TACAGCTTCAAAGT-AAACACAACC	ATTCGGGTCCCAAG-AAGGTTCTAT	58	Kim 2005
AFRF8-CAPS (*Rsa*I)	GTTGATGCTCTATG-GTTGGAGAAC	GCACTATTCCTATTG-GCTTTCTG	56	Kim et al. 2006
CRF-SCAR	GTACACACCACTCG-TCGCTCCT	TTCTTGGGTCCCTTT-CTTCCAA	62	Lee et al. 2004; Gulyas et al. 2006

annealing temperature is listed in Table 1. The PCR products of PR-CAPS, OPP13-CAPS, and AFRF8-CAPS were then digested with the restriction enzymes *Mse*I, *Hin*fI, and *Rsa*I, respectively (New England BioLabs, USA). Finally, the digested PCR products and the CRF-SCAR PCR product were separated on a 1.5% agarose gel.

Linkage analysis

Linkage analysis of the four markers was performed using 205 F_2 individuals and the program MapMaker 3.0b. The criteria for linkage grouping were an LOD of 4.0, a maximum distance of 30 cM, and the mapping function of Kosambi (1944).

Sequencing of internal regions of the PR-CAPS and OPP13-CAPS markers

To investigate the sequence diversity of internal regions of the OPP13-CAPS (1,180 bp) and PR-CAPS (640 bp) markers, each PCR product was eluted from the agarose gel using the QIAquick Gel Extraction kit (Promega, USA) and directly sequenced. Sequencing was performed using the Big-Dye Terminator v. 3.1 Cycle Sequencing kit and an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems, USA).

Results

Linkage analysis between PR-CAPS and three *Rf*-linked markers

In 205 'Buja' F_2 individuals segregating male fertile (S, *Rf*/_) and male sterile (S, *rf*/*rf*) peppers, all of the markers used in this study were polymorphic (Fig. 1). The PR-CAPS marker has been reported to be associated with partial restoration of male fertility (Lee et al. 2008). When digesting the PR-CAPS PCR product with the *Mse*I enzyme, Lee et al. (2008) reported that lower band was linked to the *Pr* allele, but it also appeared to be linked to the *Rf* allele in the present study (Fig. 1a). OPP13-CAPS, AFRF8-CAPS, and

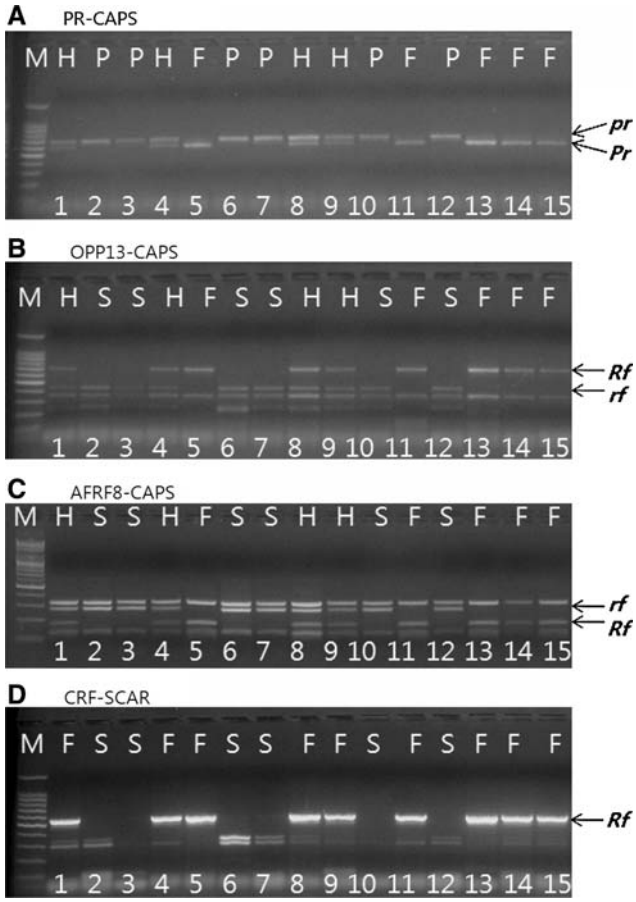

Fig. 1 Tight linkages between the PR-CAPS marker and three *Rf*-linked markers, OPP13-CAPS, AFRF8-CAPS, and CRF-SCAR. *Black arrows* on the *right* indicate the linked allele of each marker. *Numbers* indicate individuals of F_2 populations derived from a 'Buja' Korean commercial F_1 variety. The *M lane* is a 100-bp ladder (Promega, USA). **a** PR-CAPS marker performed by *Mse*I digestion after PCR (Lee et al. 2008). F, fully fertile (*Pr*/*Pr*); P, partially fertile (*pr*/*pr*); H, heterozygous (*Pr*/*pr*). **b** OPP13-CAPS marker performed by *Hin*fI digestion after PCR (Kim 2005). F, fertile (*Rf*/*Rf*); S, sterile (*rf*/*rf*); H, heterozygous (*Rf*/*rf*). **c** AFRF8-CAPS marker performed by *Rsa*I digestion after PCR (Kim et al. 2006). F, fertile (*Rf*/*Rf*); S, sterile (*rf*/*rf*); H, heterozygous (*Rf*/*rf*). **d** CRF-SCAR marker performed only by PCR amplification (Gulyas et al. 2006). F, fertile (*Rf*/_); S, sterile (*rf*/*rf*)

CRF-SCAR markers were reported to be linked to the major *Rf* locus at distances of 0.7, 1.8, and 5.3 cM, respectively (Kim 2005; Gulyas et al. 2006; Kim et al. 2006). The

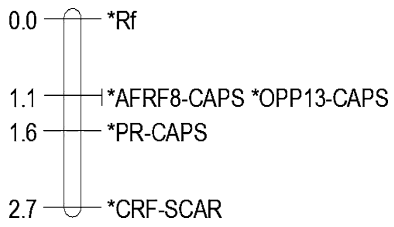

Fig. 2 The genetic map near the *Rf* locus using one *pr*-linked marker (PR-CAPS) and three *Rf*-linked markers (OPP13-CAPS, AFRF8-CAPS, and CRF-SCAR). The *numbers* on the *left* are genetic distances (Kosambi, cM); the locus or marker names are on the *right*: PR-CAPS (Lee et al. 2008); OPP13-CAPS (Kim 2005); AFRF8-CAPS (Kim et al. 2006); CRF-SCAR (Gulyas et al. 2006)

upper band of the *Hin*fI-digested OPP13-CAPS PCR product, the lower band of the *Rsa*I-digested ARFR8-CAPS PCR product, and the presence of the CRF-SCAR PCR product were also associated with the *Rf* allele (Fig. 1b–d). AFRF8-CAPS (1.1 cM), OPP13-CAPS (1.1 cM), PR-CAPS (1.6 cM), and CRF-SCAR (2.7 cM) were linked to the *Rf* locus in close order (Fig. 2). The four markers were tightly linked to each other (Fig. 2).

Internal sequences of OPP13-CAPS and PR-CAPS markers

To analyze the diversity of *pr*-linked and *Rf*-linked sequences, the internal regions of the OPP13-CAPS (1,180 bp) and PR-CAPS (640 bp) markers in 91 inbreds were sequenced. A total of 30 SNPs and three indels were found within the internal regions of OPP13-CAPS, and the polymorphism at 780 bp in Fig. 1b resulted in the size difference of the OPP13-CAPS marker between male-fertile and male-sterile peppers (Fig. 3). In the analysis of the 91 inbred lines, OPP-Haplotypes 1, 2, and 3 were found in 27 (all *Rf/Rf*), 25 (21 *rf/rf* and 4 *Rf/Rf*), and 39 (29 *rf/rf* and 10 *Rf/Rf*) lines. OPP-Haplotype 1 was mostly linked to the *Rf* allele. Within the internal regions of the PR-CAPS marker, there were 11 SNPs and one indel site (Fig. 4). The PR-CAPS marker was developed from a polymorphic sequence at 95 bp (Figs. 1a, 4). The PR-Haplotypes 1, 2-1, and 3 were distributed in 42 (10 *rf/rf* and 32 *Rf/Rf*), 32 (24 *rf/rf* and 8 *Rf/Rf*), and 13 (12 *rf/rf* and 1 *Rf/Rf*) lines, respectively. The PR-Haplotypes 2-2 and 2-3 may have originated from the PR-Haplotype 2-1 through point mutations at 114 and 483 bp, respectively (Fig. 4). PR-Haplotypes 2-1 and 3 were mostly linked to the *rf* allele. Sequences of the internal regions of the PR-CAPS and OPP13-CAPS markers were clearly classified into three different haplotypes (Figs. 3, 4).

The PR-CAPS marker to distinguish the three haplotypes

Based on the above results, we successfully developed a CAPS marker, PR-CAPS (*Mse*I or *Sph*I digestion), that distinguished the three haplotypes (Fig. 5). When the

Fig. 3 Three haplotypes based on the internal sequences of the OPP13-CAPS marker in the 96 Korean inbreds. *Black boxes* indicate single nucleotide polymorphisms or insertion/deletion sites. *Underlines* indicate *Hin*fI-digested sites

Fig. 4 Three haplotypes based on the internal sequences of the PR-CAPS marker in the 96 Korean inbreds. *Black boxes* indicate single nucleotide polymorphisms or insertion/deletion sites. *Underlines* indicate *Mse*I- or *Sph*I-digested sites. PR-Haplotypes 2-2 and 2-3 might be derived from PR-Haplotype 2-1 through a single point mutation on the 114th and 483rd sequence, respectively

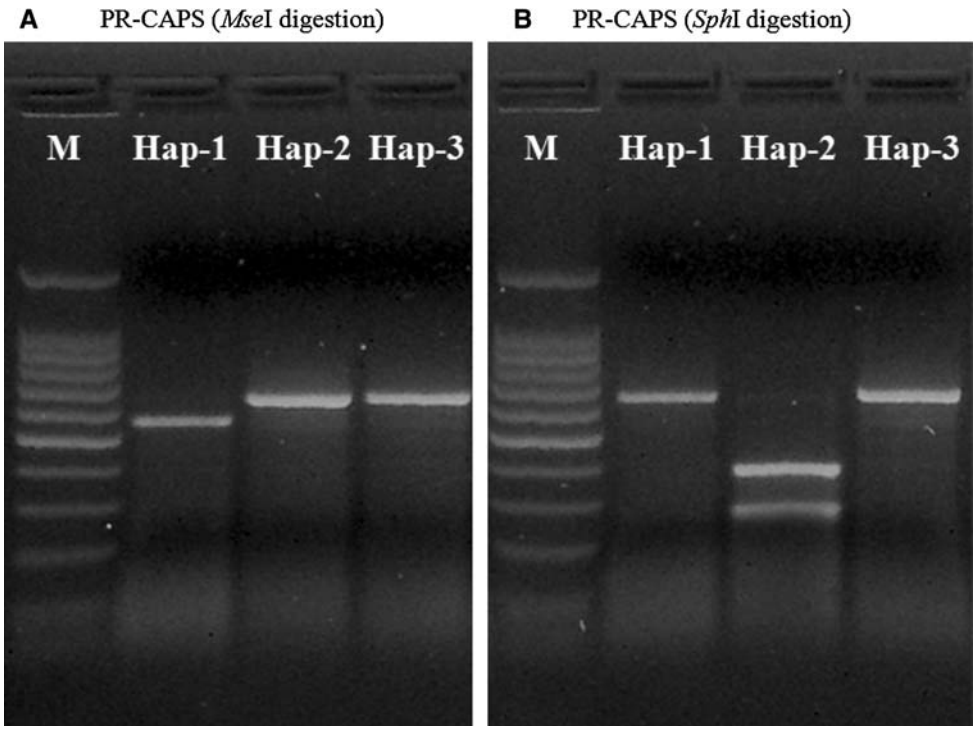

Fig. 5 The newly-designed PR-CAPS marker distinguished among three haplotypes. Hap-1, Hap-2, and Hap-3 mean PR-Haplotypes 1, 2, and 3, respectively. PR-Haplotype 1 and 2 were cut by *Mse*I and *Sph*I restriction enzymes, respectively, whereas PR-Haplotype 3 was not cut by either *Mse*I or *Sph*I enzyme

PCR product of PR-CAPS marker was digested with an *Mse*I enzyme, only PR-Haplotype1 was cut, showing the lower band (Fig. 5a), while only PR-Haplotype2 was digested with an *Sph*I enzyme (Fig. 5b). Both enzymes were unable to cut the PCR product of PR-Haplotype 3 (Fig. 5a, b).

Relationship between the *pr* and the *Rf* genes

To analyze the relationships between the *pr* and the *Rf* genes, a partially restored line (S, *pr/pr*) was crossed with each of two maintainers (*rf/rf*) and two restorers (*Rf/Rf*). In the F_1 progenies crossed with maintainers, male fertility

was partially restored, whereas it was fully restored in the F_1 progenies crossed with the restorers (Table 2).

Discussion

Tight linkage between the *pr* and the *Rf* loci

Linkage analysis of one *pr*-linked and three *Rf*-linked markers was performed (Figs. 1, 2). The four markers were tightly linked to each other. The genetic distances of the four markers were similar to those previously reported (Kim 2005; Gulyas et al. 2006; Kim et al. 2006; Lee et al. 2008). However, the AFRF8-CAPS and OPP13-CAPS markers were located in the same side in this study, whereas they were previously reported to be located oppositely (Kim et al. 2006). This might be resulted from errors in marker analysis or phenotype scoring. For map-based cloning of the *Rf* gene, this relationship needs to be investigated in detail.

Based on the results that the four markers were tightly linked, it was suggested that the *pr* and the *Rf* genes might be allelic (Fig. 2). In a similar study, mapping approaches were used for allelic analysis of the *Rf* of *pol* CMS in *Brassica napus* L. (Jean et al. 1997). In this case, both *Rfp1* and *Rfp2* were mapped using several *Rf*-linked markers, and the cRF1b-RFLP marker cosegregated with *Rfp1* and *Rfp2*, suggesting that *Rfp1* and *Rfp2* were likely to be allelic. To determine whether the *pr* and the *Rf* genes are allelic, allelism tests have to be performed by examining the segregation of fertile/sterile plants in an F_2 of several hundred individuals from a cross between *Pr/Pr* and *Rf/Rf* genotypes or between *pr/pr* and *rf/rf* genotypes. In addition, to know whether the *pr* and the *Rf* genes were the same, both *pr* and *Rf* genes have to be cloned, because in petunia, radish, and rice, several *Rf* homologous genes whose functions are not yet identified were found near the cloned *Rf* genes (Bentolila et al. 2002; Brown et al. 2003; Desloire et al. 2003; Kazama and Toriyama 2003; Koizuka et al. 2003; Akagi et al. 2004; Komori et al. 2004; Wang et al. 2006).

Table 2 Relationships between the *pr* and the *Rf* or *rf* genes

Cross combination[a]	Genotype of male plant[b]	Number of plants observed (FR:PR:MS)[c]
(S) *pr/pr* × B1	(N) *rf/rf*	0:25:0
(S) *pr/pr* × B2	(N) *rf/rf*	0:24:0
(S) *pr/pr* × C1	(N) *Rf/Rf*	25:0:0
(S) *pr/pr* × C2	(N) *Rf/Rf*	25:0:0

[a] (S) *pr/pr* is the partially restored line that has Peterson's sterile cytoplasm. B1 and B2 are maintainer lines. C1 and C2 are restorer lines

[b] (N), normal cytoplasm; *Rf*, restorer allele; *rf*, nonrestorer allele

[c] FR, fully-restored pepper; PF, partially-restored pepper; MS, male-sterile pepper

Three haplotypes of *pr*-linked and *Rf*-linked sequences and designing the CAPS markers to distinguish the haplotypes

The diversity of internal sequences of the *pr*-linked and *Rf*-linked markers (PR-CAPS and OPP13-CAPS) in 91 pepper inbreds was investigated (Figs. 3, 4). As a result, two internal sequences were clearly divided into three groups (Figs. 3, 4). The PR-Haplotype 1 and OPP-Haplotype 1 were mostly linked to *Rf* allele, while PR-Haplotypes 2 and 3 and OPP-Haplotypes 2 and 3 were seemed to be linked to *rf* allele. PR-Haplotype 3 and OPP-Haplotype 3 were first found in partially restored pepper; so, we thought it was linked to the *pr* allele. However, in this study, PR-Haplotype 3 and OPP-Haplotype 3 were also found in many *Rf/Rf* and *rf/rf* lines. This might be caused by recombination between the markers and the *Rf* gene or by scoring errors resulting from confusion between *pr/pr* and *rf/rf* phenotypes, but this has yet to be confirmed; to do so, the *Rf* gene must be cloned or cosegregated markers must be developed. However, this study is the first try to suggest that the pepper *Rf* locus might have multiallele. The two dominant RAPD markers, $OPW19_{800}$ and $OPP13_{1400}$, were inconsistent with their phenotypes. The reason might be that the *pr* gene was tightly linked to the *Rf* locus and the *pr*-linked and *Rf*-linked sequences have at least three haplotypes (Kumar et al. 2007).

Several DNA markers that distinguish between the *Rf* and *rf* alleles have been reported in chili peppers (Zhang et al. 2000; Pákozdi et al. 2002; Lee et al. 2004; Kim 2005; Gulyas et al. 2006; Kim et al. 2006; Kumar et al. 2007). These markers were closely linked to the *Rf* locus, but were often inconsistent with the phenotypes in inbred lines (Zhang et al. 2000; Kumar et al. 2007). This has usually been explained by frequent recombinations between the markers and the *Rf* gene. However, we suggest that another reason is that the *Rf*-linked sequences have three haplotypes. Therefore, the CAPS markers (OPP13-CAPS and PR-CAPS), which could distinguish the three haplotypes, will be helpful to better understand fertility restoration in chili pepper CMS (Fig. 5). In addition, these markers will increase the usable range for marker-assisted selection in commercial chili pepper breeding programs to develop new restorers and maintainers.

Relationship between the *pr* and the *Rf* genes

The partial restoration was recessive to the full restoration but dominant to the male sterility (Table 2). If the *pr* and *Rf* genes are allelic, the *pr* allele may be recessive to the *Rf* allele but dominant to the *rf* allele. On the other hand, if the *pr* and *Rf* are different loci, the *Rf* locus may have dominant epistasis to the *pr* locus, because the partial restoration appeared only in cases of crosses with maintainers. To

confirm this suggestion, allelism tests between the *pr* and the *Rf* have to be performed above all.

This study will be helpful to well understand the fertility restoration of pepper CMS, and the CAPS markers in this study will be useful for marker-assisted selection to develop new maintainers and restorers in commercial F_1 hybrid pepper breeding using CMS.

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