The Unstable Restorer-of-Fertility Locus in Pepper (*Capsicum Annum*. L) is Delimited to a Genomic Region Containing PPR Genes

Moo Chan Kang

Seoul National University

Hwa-Jeong Kang

Seoul National University

So-Young Jung

Seoul National University

Hae-Young Lee

Seoul National University

Min-Young Kang

Seoul National University

Yeong Deuk Jo

Korea Atomic Energy Research Institute <https://orcid.org/0000-0002-9018-7395>

Byoung-Cheorl Kang (✉ bk54@snu.ac.kr)

Seoul National University <https://orcid.org/0000-0002-7606-7258>

Research Article

Keywords: cytoplasmic genetic male sterility, Unstable Restorer-of-fertility, sweet pepper, pentatricopeptide repeat protein, marker-assisted breeding.

Posted Date: December 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1163811/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Theoretical and Applied Genetics on March 31st, 2022. See the published version at <https://doi.org/10.1007/s00122-022-04084-1>.

The *Unstable Restorer-of-fertility* Locus in Pepper (*Capsicum annuum*. L) is Delimited to a Genomic Region Containing PPR Genes

Moo Chan Kang¹, Hwa-Jeong Kang¹, So-Young Jung¹, Hae-Young Lee¹, Min-Young Kang¹,
Yeong Deuk Jo^{2*}, Byoung-Cheorl Kang^{1*}

¹ Department of Agriculture, Forestry and Bioresources, Research Institute of Agriculture and Life Sciences, Plant Genomics and Breeding Institute, College of Agriculture and Life Sciences, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, South Korea

² Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup, 56212, Republic of Korea

*** Correspondence:**

Byoung-Cheorl Kang (email address: bk54@snu.ac.kr; Orcid ID: 0000-0002-7606-7258)

Yeong Deuk Jo (email address: jyd@kaeri.re.kr; Orcid ID: 0000-0002-9018-7395)

Abstract

The use of cytoplasmic-genic male sterility (CGMS) systems greatly increases the efficiency of hybrid seed production. Although marker development and candidate gene isolation have been performed for the *Restorer-of-fertility* (*Rf*) gene in pepper (*Capsicum annuum* L.), the broad use of CGMS systems has been hampered by the instability of fertility restoration among pepper accessions, especially sweet peppers, due to the widespread presence of the *Unstable Restorer-of-fertility* (*Rf^u*) locus. Therefore, to investigate the genetic factors controlling unstable fertility restoration in sweet peppers, we developed a segregation population from a cross between a male-sterile line and an *Rf^u*-containing line to examine the inheritance of *Rf^u*. Individuals with unstable restoration vs. sterility segregated at a 3:1 ratio, indicating that a single dominant gene controls unstable fertility restoration. Genetic mapping delimited the *Rf^u* locus to a 479 kb genomic region on chromosome 6 flanked by two markers, which is close to but different from the previously identified *Rf*-containing region. The *Rf^u*-containing region harbors a *pentatricopeptide repeat* (*PPR*) gene, along with 13 other candidate genes. In addition, this region is syntenic to the genomic region containing the largest number of *Rf-like* PPR genes in tomato. Therefore, the dynamic evolution of PPR genes might be responsible for both the restoration and instability of fertility in pepper. During genetic mapping, we developed various molecular markers, including one that co-segregated with *Rf^u*. These markers showed higher accuracy for genotyping than previously developed markers, pointing to their possible use in marker-assisted breeding of sweet peppers.

Keywords

cytoplasmic genetic male sterility, *Unstable Restorer-of-fertility*, sweet pepper, pentatricopeptide repeat protein, marker-assisted breeding.

43 **Key Message**

44 *Unstable Restorer-of-fertility (Rf^u)*, conferring unstable fertility restoration in the pepper CGMS
45 system, was delimited to a genomic region near *Rf* and is syntenic to the *PPR-like* gene-rich
46 region in tomato.

47

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait characterized by the inability to produce functional pollen (Chen et al., 2017). The CMS phenotype is caused by the presence of aberrant mitochondrial genes in various plant species (Hanson, 1991). In the cytoplasmic-genic male sterility (CGMS) system, the *Restorer-of-fertility* (*Rf*) gene from the nuclear genome suppresses the CMS phenotype. *Rf* genes often alter the expression of proteins from CMS-associated genes and are therefore thought to reduce or remove the deleterious effects caused by CMS-associated genes. The CGMS system eliminates the need for the laborious emasculation process and thus can be used for hybrid seed production in diverse, agriculturally important crops.

To explore the mechanisms of the CGMS system, *Rf* genes have been cloned and characterized in many crop plants, including maize (Cui et al., 1996), petunia (Bentolila et al., 2002), rice (Wang et al., 2006; Fujii and Toriyama, 2009; Itabashi et al., 2011; Tang et al., 2014; Huang et al., 2015), radish (Koizuka et al., 2003), sorghum (Jordan et al., 2010), rapeseed (Uyttewaal et al., 2008; Liu et al., 2016), and sugar beet (Kitazaki et al., 2015). Most *Rf* genes examined to date encode pentatricopeptide repeat (PPR) proteins. However, there are the *Rf* genes encode non-PPR proteins: *Rf2* in maize encodes aldehyde dehydrogenase (Cui et al., 1996), *Rf17* in rice encodes acyl-carrier protein synthase (Fujii and Toriyama, 2009), and *Rf2* in rice encodes a glycine-rich domain-containing protein (Itabashi et al., 2011).

PPR proteins constitute one of the largest protein families in land plants, with more than 400 members in most species (Barkan and Small, 2014). PPR proteins contain a number of PPR motifs, including a degenerate 35 -amino acid motif repeated in tandem (Manna, 2015). The combination of the 5th and 35th amino acids from the respective PPR motifs can determine the specificity of recognition of one nucleotide of the target RNA molecule (Shen et al., 2016). PPR

proteins function in various processes in plants, including RNA editing, splicing, cleavage, and degradation. In CMS-BT rice, the proteins encoded by PPR type *Rf* genes *Rf1a* and *Rf1b* bind to and cleave dicistronic *atp6-orf79* transcripts (Wang et al., 2006). Unlike other PPR genes, *Rf-like PPR (RFL)* genes are located close to each other in a cluster. The *Rf4* locus in rice is located in a typical PPR cluster region that also includes *Rf1a* and *Rf1b* (Tang et al., 2014).

The stability of *Rf* genes in various environments is critical for the use of CGMS systems in producing F₁ hybrid seeds. However, various reports indicate that the stability of *Rf* genes is strongly affected by the external environment. In CMS-S maize, *Rf9* shows consistent restorer ability at temperatures below 28°C. However, when the daily maximum temperature is higher than 30°C, the male fertility phenotype changes to male sterility (Gabay-Laughnan et al., 2009). In rice CMS-BT, the *Rf5* or *Rf6* gene can restore normal seed setting, but lines heterozygous for *Rf6* show more stable seed setting rates than lines heterozygous for *Rf5* under heat-stress conditions (Zhang et al., 2017).

Although numerous past efforts to identify the *Rf* gene in pepper have led to the development of several molecular markers that co-segregate with *Rf* and the isolation of *Rf* candidate genes in the co-segregation regions, the identity of the *Rf* gene has not yet been confirmed through functional complementation experiments. Jo et al. (2016) delimited the genomic region of *Rf*, and identified potential candidate genes within it (*CDS1*, *CDS2*, *CDS3*, and *CaPPR6*), and chose *CaPPR6* as the candidate pepper *Rf* gene. Similarly, Cheng et al. (2020) and Zhang et al (2020) performed fine mapping for pepper *Rf* and identified several PPR genes in this region that co-segregated with *Rf*. All co-segregation regions defined in these three studies were located in a narrow area of chromosome 6, although each study identified different PPR genes as the *Rf* candidate gene. In contrast, Wei et al. (2020) chose a gene encoding a NEDD8-conjugating enzyme E2 as the candidate *Rf* gene based on bulk segregant RNA

sequencing. Further functional confirmation based on transformation or genome editing is required to confirm the identity of *Rf* genes in pepper.

In addition to complete sterility or fertility, pepper plants can exhibit unstable fertility at certain temperatures, as described in the first report of CMS and fertility restoration in peppers in 1958 (Peterson, 1958). Kim et al. (2013) reported that a pepper line with thermosensitive cytoplasmic male sterility (TCMS) was sterile at temperatures above 15°C but became fertile when the night temperature dropped below 13°C. Lee (2001) suggested that besides the major fertility restorer gene, there is an unstable dominant *Rf* (*St^u*) locus that affects male fertility. Lee et al. (2008a) observed the partial restoration phenotype, which refers to the generation of only a small quantity of pollen, in CMS cytoplasm-containing individuals and determined that the nuclear gene *partial restoration* (*pr*) is tightly linked to *Rf* or is the third allele of *Rf* based on genetic mapping. On the other hand, Min et al. (2009) demonstrated that the locus of the gene responsible for unstable male sterility is not linked to the *Rf* locus. The partial or unstable restoration phenotype is widespread among pepper accessions, especially among sweet peppers. The lack of stable restorer lines in sweet pepper has been an obstacle to using the CGMS system in this important crop. To overcome this obstacle, the stable *Rf* gene from hot pepper was successfully introduced into sweet pepper through marker-assisted backcrossing (Lin et al., 2015).

In the current study, we investigated the unstable restoration trait in sweet pepper and identified the genetic and genomic positions of the instability gene using previously and newly developed markers. Based on this information, we chose candidate genes underlying the unstable restoration trait. Our findings should facilitate the use of the CGMS system for the improvement of sweet pepper.

Materials and Methods

Plant materials

The hot pepper lines *C. annuum* ‘Chungyang A’ and ‘Chungyang C’ and the sweet pepper lines *C. annuum* ‘MSGR-A’ and ‘SPR03’ were used for inheritance analysis and to develop molecular markers linked to the unstable restoration trait (Table 1). *C. annuum* ‘MSGR-A’, with small blocky fruit and stable male sterility, was used as the stable *rf* allele donor parent, and ‘SPR03’, with blocky fruit and *Unstable Restorer-of-fertility* (hereafter designated as *Rf^u*), was used as the recurrent parent (Fig. 1). A BC₄F₅ population was developed by crossing MSGR-A and SPR03 and self-pollinating the progenies (Fig. 2). Heterozygotes were selected using two *Rf*-linked markers: PR-CAPS, which is linked to the *partial restoration* locus, and PPR6_3’UTR-SCAR, which is tightly linked to the *Rf* locus. The BC₄F₅ individuals were grown in the greenhouse at the Seoul National University farm (Suwon, Korea). Leaf samples of 12 stable male sterile lines and 11 unstable male sterile lines, which were kindly provided by Eco-Seed (Gimje, South Korea), were used to test the newly developed markers.

Evaluation of the male fertility phenotype

The male fertility phenotype was investigated at least four times per individual by observing pollen production on anthers. The presence of pollen was determined by observing anthers with the naked eye and was confirmed by dusting the pollen from anthers onto black paper. If pollen was detected at least once in an individual during the four or more observations, the individual was considered to be partially fertile. To further confirm the presence of viable pollen in the plants, pollen was collected from dehiscent flowers and stained with fluorescein diacetate (FDA, Sigma-Aldrich, USA). The stained pollen was observed under an optical microscope (Carl Zeiss, Germany). After 2 min incubation at room temperature, pollen viability

was examined under a fluorescence microscope. Pollen grains were harvested from at least five flowers and stained with FDA. This process was repeated three times.

Genomic DNA extraction

Young leaves were collected from each plant and used for genomic DNA (gDNA) extraction using the cetyltrimethylammonium bromide (CTAB) method (Park et al., 2009). The gDNA was dissolved in 1× TE buffer and diluted in triple-distilled water to a final concentration of 50 ng/μl for molecular marker analysis.

Analysis of mitotypes

The mitotypes of the lines were analyzed using a multiplex-PCR marker developed by Jo et al. (2019). This marker can be used to determine the genotypes of four mitochondrial loci in a single PCR; five mitotypes of *C. annuum* were identified when this marker was applied to diverse germplasm. The PCR was performed using four pairs of primers (5 pmol each) described by Jo et al. (2019) in a total volume of 50 μl containing 100 ng DNA, 200 μM dNTP mix, 5 μl 10× buffer, and 1.5 units Taq DNA polymerase (Takara Bio, Inc., Kusatsu, Japan). The PCR conditions were 32 cycles of 98°C (10 s), 59°C (30 s), and 72°C (100 s), followed by a final extension at 98°C (5 min).

Molecular marker analysis

For molecular marker analysis, PCR was conducted in 20 μl reaction mixture containing 100 ng of gDNA template, 2 μl of 10 mM dNTPs, 2 μl of 10× PCR buffer (Takara, Seoul, Korea), 0.5 μl of 10 pmol primers, 0.3 μl of Takara R TaqTM polymerase (Takara, Seoul, Korea), and triple-distilled water (to 20 μl). The cycling conditions were an initial cycle of 94°C for 5 min; 35 cycles of 94°C for 30 s, the annealing temperature of each marker (Table 2) for 30 s,

and 72°C for 20 s; and then a final extension of 72°C for 5 min. For Cleaved Amplified Polymorphic Sequence (CAPS) markers, the PCR product was digested with 5 units of restriction enzymes (Table 2). The digested PCR products were separated on 2% agarose gels.

Development of *Rf*-linked markers

To identify the polymorphism between MSGR-A and SPR03, direct sequencing was carried out (Macrogen, Seoul, Korea). Basic local alignment search tool (BLAST) was carried out using the pepper reference genome ‘UCD10X (v1.0)’, which was obtained from an F₁ line of a cross between CM334 and a blocky nonpungent pepper (Hulse-Kemp et al. 2018) to identify the physical locations of CAPS markers. Information about additional *Rf*-linked markers was retrieved from the literature: Co1Mod1-CAPS, 4162-SCAR, and G16-CAPS (Jo et al., 2016), PR-CAPS (Lee et al., 2008), and 13T7-SCAR (Jo et al., 2010).

Linkage analysis

Linkage analysis was performed using molecular markers and 300 BC₄F₅ individuals from an introgression between MSGR-A and SPR03. The linkage map was constructed using CarthaGene software with a LOD threshold of 3.0 and distance threshold of 30 cM (De Givry et al., 2005). Genetic distance in the linkage map was determined using the Kosambi mapping function. A linkage map was created based on the genetic distance between the markers using Map Chart 2.3 software.

Gene annotation

Annotation of the target region was performed by BLAST analysis of the DNA sequence in this region [from UCD10X (v1.0) reference genome] against coding DNA sequences (CDSs) previously annotated to the CM334 v1.55 genome (Kim et al., 2014). If the

similarity was higher than 98% in the BLAST results, the sequence block in the target region was annotated based on the matched CDS.

Screening of *Rf*-like PPR genes in tomato

The deduced protein sequences of the petunia *Rf* gene (*Rf-PPR592*) and a candidate pepper *Rf* gene (*CaPPR6*) were used as query sequences for BLAST against tomato (*Solanum lycopersicum*) protein sequences (ITAG release 2.40; <http://solgenomics.net/tools/blast/>). The *e*-value threshold was set to 10^{-10} . Proteins with sequences showing identity to *Rf*-PPR592 > 50% and longer than 100 amino acids were selected as *Rf*-like PPRs. The genomic locations of the genes encoding these proteins were determined based on the tomato genome ITAG release 2.40.

Results

Inheritance study in an Rf^u -segregating population

We obtained a BC₄F₅ population during the course of introgression of the stable rf allele from a small blocky sweet pepper line (MSGR-A) to a large blocky sweet paper line (SPR03) that was assumed to contain *Unstable restorer-of-fertility* (Rf^u) (Fig. 1). To reveal the inheritance pattern of the unstable male sterility phenotype, we evaluated the male sterility phenotype in a segregating population of 300 BC₄F₅ individuals. In the BC₄F₅ population, 228 individuals were partially fertile and 72 individuals were completely sterile. The anthers of partially-fertile individuals were larger and lighter in color than those of sterile individuals (Fig. 2). Microscopic observation of FDA-stained pollen showed that partially-fertile individuals produced a small number of viable pollen grains (Fig. S1). Partially fertile individuals were also clearly distinguished from the fully fertile recurrent parent line (SPR03) in that they produced much less pollen. The amount of pollen produced by partially fertile individuals was not consistent during the four or more observations and appeared to be affected by environmental conditions. Thus, we described this phenotype as unstable fertility restoration rather than partial restoration. The segregation ratio between individuals showing unstable fertility restoration and complete sterility phenotypes in the BC₄F₅ population was fitted to an expected ratio of 3:1 ($\chi^2 = 0.160$; $P = 0.689$), suggesting that the instability is controlled by a single dominant gene (Table 3).

Analysis of mitotypes

Since the subgenomic structure of the mitochondrial genome may affect the stability or fertility of plants (Janska et al., 1998), we investigated the mitotypes of pepper lines with unstable fertility using a multiplex-PCR marker that can classify five mitotypes in *C. annuum*.

The amplification pattern in lines with unstable fertility could not be distinguished from that of lines with stable sterility (mitotype 4; Fig. 3). Therefore, the unstable lines contained mtDNA structures that are characteristic of typical CMS lines, implying that mtDNA rearrangements are not involve in the unstable fertility of these lines. Among parental lines used for population development, MSGR-A (with stable *rf*) carried mitotype 4, as did other sterile lines. The parental line SPR03 (with Rf^u) carried mitotype 3, which is predominantly detected in domesticated pepper lines from Asia. Because BC₄F₅ individuals contain cytoplasm from MSGR-A, unstable fertility is determined by the presence or absence of nuclear Rf^u . In addition, Rf^u induces instability only in the CMS cytoplasm background, as SPR03, which contains Rf^u in the non-CMS cytoplasm background, was fully fertile (Fig. 2, Fig. 3).

Polymorphism survey of previously developed markers

To test previously developed markers in the segregating population used in this study, we first surveyed the polymorphism of markers between hot and sweet pepper parental lines, including 4162-SCAR, 120kb-end-SCAR, PPR12-SCAR, 3336-last-SCAR, Co1mod1-CAPS, and 4940-CAPS. These markers showed polymorphisms in hot pepper lines Chungyang C (*RfRf*) and Chungyang A (*rfrf*), as described previously (Jo et al., 2016), but they did not distinguish the sweet pepper lines used in this study, MSGR-A (*rfrf*) and SPR03 (Rf^uRf^u) (Fig. S2a). Therefore, we developed the new molecular markers 4162-CAPS and G16-SCAR based on the previously detected SNPs in 4162-SCAR and G16-CAPS, respectively (Fig. S2b; Table 2). PPR12-SCAR and 4940-CAPS showed polymorphisms between SPR03 and MSGR-A, but inconsistent PCR products were produced. We therefore surveyed polymorphisms in 13T7-SCAR, G16-SCAR, PR-CAPS, PPR6_3'UTR-SCAR, and 4162-CAPS between the sweet pepper lines. When 31 BC₄F₄ individuals were tested, 4162-CAPS showed two recombinants, G16-SCAR and PR-CAPS showed one recombinant, and 13T7-SCAR co-segregated with the

male sterility phenotype (Fig. 4). These results indicate that the Rf^u locus is not located between 4162-CAPS and G16-SCAR and is located close to 13T7-SCAR. In a previous study, the Rf gene was mapped between 4162-SCAR and G16-CAPS (Jo et al., 2016). Therefore, the location of the Rf^u locus revealed by genetic mapping in the current study is different from that of the Rf locus.

Development of additional markers linked to unstable male sterility

Since 13T7-SCAR was located closer to Rf^u compared to the other markers examined, we performed further mapping by developing additional markers around 13T7-SCAR. We developed four CAPS markers within the 1.2 Mbp region around the 13T7-SCAR marker, including 214.14MB CAPS, 213.85MB CAPS, 213.3MB CAPS, and 212.9MB CAPS (Table 2). When we applied the newly developed markers to the hot and sweet pepper parental lines, 212.9MB-CAPS and 214.14MB-CAPS showed polymorphisms in both types of parental lines, whereas 213.3MB-CAPS and 213.85MB-CAPS showed polymorphisms only in sweet pepper parental lines (Fig. S2b).

Genetic mapping and genomic analysis of the Rf^u locus

We constructed a local linkage map using the BC₄F₅ population and seven molecular markers developed in this study (Fig. 5a). The PPR6_3'UTR-SCAR and G16-SCAR markers were linked to that Rf^u locus at distances of 2.4 and 0.9 cM, respectively. Jo et al. (2010) reported that the 13T7-SCAR marker was linked to the Rf locus at a distance of 1.4 cM; however, the genetic distance of 13T7-SCAR from the unstable male sterility locus was 0.4 cM. The markers 213.3MB-CAPS, 212.9MB-CAPS, and 213.85MB-CAPS were linked to the unstable male sterility locus at a genetic distance of 2.2, 4.3, and 8.9 cM, respectively. When we tested the 214.14 MB-CAPS marker in this population, we did not observe any recombinants,

demonstrating that the 214.14 MB-CAPS marker is tightly linked to the *Rf^u* locus. G16-SCAR and 13T7-SCAR were closest to 214.14 MB-CAPS on two different sides; thus, the *Rf^u* locus was delimited by these two flanking markers. Integrative analysis by combining our results with genetic map information obtained from a previous study (Jo et al., 2016) clearly showed that the *Rf^u* locus is separated from the *Rf* locus. The genetic distance between the *Rf^u* and *Rf* loci was determined to be 1.2cM based on a genetic map developed using the Chungyang F₂ population (Jo et al., 2016; Fig. 5a). These results indicate that *Rf^u* is not an allele of *Rf* but is a separate gene that is genetically linked to *Rf*.

Annotation and characterization of genes in the *Rf^u*-containing DNA region

The DNA region between two markers (G16-SCAR and 13T7-SCAR) flanking the *Rf^u* locus corresponded to the 4,575-5,054 kb region of chromosome 6 in the UCD10X (v1.0) pepper reference genome (Fig. 5b). Therefore, we were able to delimit the *Rf^u*-containing DNA region within a 479 kb sequence. A total of 14 genes were annotated in this region based on CDSs previously annotated in the CM334 reference genome (v1.55; Kim et al., 2014). These genes encode three NBS-LRR resistance proteins, two adenylyl isopentenyltransferases, an ER lumen retaining receptor family-like protein, a Gypsy/Ty-3 retroelement polyprotein, a PPR protein, a RING finger protein, a glycosyl group transferase, a xyloglucan endotransglucosylase, a RING/U-box domain-containing protein, and two proteins of unknown function (Table 4). The PPR gene (CA00g30080) was positioned 25 kb away from the 214.14MB-CAPS marker. This gene encodes a 248 amino acid protein. We searched for the gene sequence that matched CA00g30080 among the CDSs of the blocky-type nonpungent pepper accession ‘Early Calwonder’ (ECW), for which *de novo* genome assembly information is available (Kim et al., 2021). The sequence of the protein encoded by the best-matched CDS of ECW (ECW.scaffold897.110) shows 97% identity and an insertion of one amino acid compared to

the sequence of the protein encoded by CA00g30080 (Fig. S3). We aligned the protein sequences of CA00g30080 and ECW.scaffold897.110 on an array of the PPR motifs in Rf-PPR592, the petunia Rf, and CaPPR6 (a candidate Rf of pepper). Inside this alignment, three gaps were detected in the protein sequences of CA00g30080 and ECW.scaffold897.110. Notably, two of these gaps were 35 amino acids in size, which is identical to the length of a PPR motif (Fig. S3).

Characterization of a tomato genomic region syntenic to the pepper *Rf'*-containing region

We previously developed the G05G1-HRM marker from an expressed sequence tag (EST) in pepper with high similarity to a tomato gene near a PPR gene, which was mapped to the same position as 13T7-SCAR (Jo et al., 2010). The G50G1-HRM sequence is located inside the *Rf'*-containing DNA region identified in the present study and only 4.6 kb away from the 214.14 MB-CAPS marker that co-segregated with *Rf'*. We developed four markers (including G05G1-HRM) by comparative mapping using the tomato genome sequence, which could be used to link the DNA region surrounding *Rf* and *Rf'* to the corresponding genomic region of tomato. The corresponding tomato DNA region locates on 1.7-1.9 Mb region on chromosome 6 (Fig. 5a). More detailed analysis using the annotated genes showed that five genes in the *Rf'*-containing DNA region were orthologous to tomato genes dispersed along 1.74-1.79 Mb region on chromosome 6 (Fig. 5b). Interestingly, three PPR genes were detected in this region, and two more were identified in a surrounding region. We examined the *Rf-like PPR (RFL)* genes in tomato to determine whether they are specifically clustered in this region. Among the 249 and 248 tomato PPR proteins (>100 amino acids long) that showed similarity to petunia Rf-PPR592 and pepper CaPPR6, respectively, only nine shared >50% identity with Rf-PPR592 or CaPPR6 (Table 5). The identity detected for these nine PPR proteins was clearly higher than that of other PPR proteins (~20-30%; Fig. 6). These results indicate that the nine tomato PPR

proteins are RFL proteins. Five of these protein are encoded by five genes inside the 1.72-1.77 Mb (46 kb in size) region on chromosome 6. However, the four other genes are scattered on different regions of chromosome 6 or on different chromosomes. Therefore, we conclude that the tomato DNA region syntenic to the pepper *Rf'* locus is the region with the highest density of *RFL* genes in the tomato genome.

Application of *Rf*-linked markers to stable and unstable breeding lines

We used six markers, including two newly developed markers (212.9MB-CAPS, 214.14MB-CAPS), two markers modified from previously developed markers (G16-SCAR, 4162-CAPS), and two previously developed markers (13T7-SCAR, Co1Mod1-CAPS), to genotype breeding lines containing CMS cytoplasm (Fig. 3) that showed stable sterility or unstable fertility based on the presence of a nuclear unstable restorer (Table 6). Five markers showed polymorphism between SPR03 and MSGR-A. However, the Co1Mod1-CAPS marker that co-segregated with *Rf* in a previous study (Jo et al., 2016) did not show polymorphism in any of the sweet pepper lines examined. The 212.9MB-CAPS and 4162-CAPS markers, which are more distant from *Rf'* than the other markers, failed to correctly genotype most pepper lines harboring *Rf'*. However, 214.14MB-CAPS, which co-segregated with *Rf'*, and G16-SCAR, which is located on a boarder of an *Rf'*-containing DNA region, showed high accuracy in predicting the genotypes of pepper lines carrying *Rf'* (10 out of 11 lines for 214.14MB-CAPS, 9 out of 11 lines for G16-SCAR). The rate of successful genotyping in all lines examined was the highest using G16-SCAR (82.7%; Table 6). Finally, we generated a phylogenetic tree based on the marker genotypes from all breeding lines. Most lines showing stable sterility were grouped together in the tree, as were most lines showing unstable fertility (Fig. 7). These results indicate that several markers can be used for phylogenetic analysis of breeding lines based on *Rf* or *Rf'* genotypes.

Discussion

Localization of the *Rf'* locus in the pepper genome

The CGMS system is useful for producing F₁ hybrid seeds in pepper and other crops. Even though sweet pepper is an economically important crop, the CGMS system is not being used efficiently in this species. The presence of an unstable restorer that partially restores fertility or acts inconsistently depending on environmental conditions poses an obstacle to the use of the CGMS system in sweet peppers. Although unstable fertility or sterility in peppers has been repeatedly reported since Peterson (1958) first described this trait, there have been few genetic or genomic analyses of unstable fertility restoration, presumably due to the difficulty of phenotyping this trait, even though the pepper reference genome has been available since 2014 (Kim et al., 2014). Lee et al. (2008a, 2008b) reported the first genetic mapping of the *partial restoration (pr)* locus. Although the authors identified markers for *pr* close to *Rf*, they could not determine whether *pr* was an allele of *Rf* or another gene that is tightly linked to *Rf*. On the other hand, Wang et al. (2004) performed quantitative trait locus (QTL) analysis of fertility restoration in pepper and identified one major QTL on chromosome 6 and additional minor QTLs located on other chromosomes. The unstable *Rf* was considered to be related to the minor QTLs. Min et al. (2009) determined, based on segregation analysis in a large F₂ population, that the locus for unstable sterility was independent from the *Rf* locus. However, none of these studies identified the genomic location of the gene causing instability.

Here, we delimited the location of *Rf'* in the pepper reference genome. Based on this analysis, we demonstrated that the *Rf'* gene is located close to *Rf*. The *Rf'* gene identified in the current study might be identical to *pr* based on a comparison of the phenotypes caused by *Rf'* and *pr* and the genetic locations of these two genes (Lee et al., 2008a; 2008b). Lee et al. (2008b) developed a segregation population by crossing lines containing *pr* and CMS cytoplasm with a

line containing *Rf*. The authors obtained partially restored lines only when *pr* was homozygous in the CMS cytoplasm background, demonstrating that *pr* is a recessive gene. By contrast, in the current study, we used a line containing *rf* as one of the parental lines and determined that *Rf^u* acts as a dominant gene over the *rf* allele. If we assume that *pr* and *Rf^u* are the same gene, the results of these two studies indicate that *Rf* is epistatic to *Rf^u* (or *pr*). Except for partial restoration, temperature-sensitive instability of fertility have been reported (Kim et al., 2013; Peterson, 1958; Shifriss, 1997). For example, Kim et al. (2013) reported that the fertility of a TCMS line can be restored to almost normal levels below a critical temperature (13°C). Although minor fluctuations in pollen levels, presumably depending on environmental conditions, were detected in the current study, the observed phenotype was different from that in Kim et al. (2013) since the amount of pollen did not reach normal levels. Therefore, it appears that additional genes affect the stability of fertility restoration in pepper besides *Rf^u* characterized in the current study.

Implications for the evolution of PPR genes in the *Rf* and *Rf^u* loci in pepper

The *Rf^u* locus has been delimited to a DNA region containing a *PPR* gene on chromosome 6. All three fine-mapping-based approaches to isolate pepper *Rf* genes localized the *Rf* loci to *PPR*-gene-containing DNA regions on chromosome 6 (Cheng et al., 2020; Jo et al., 2016; Zhang et al., 2020). The *Rf^u* locus and three *Rf* loci are located near each other on chromosome 6, and thus this region has been hypothesized to be the genomic region where *RFL* genes are clustered together. Among *PPR* genes, which number more than 400 in most plant species, the *RFL* genes, which show high similarity to *Rf* and share a very close evolutionary relationship, are clustered in narrow genomic regions (Fujii et al., 2011). For example, 19 *RFL* genes are clustered within an ~1 Mb region on chromosome 1 in Arabidopsis (Lurin et al., 2004). Jo et al. (2010) screened a pepper BAC library using an EST with high similarity to the

petunia *Rf* gene as a probe. The markers developed from three major groups of the 74 selected BAC clones, including 13T7 SCAR, which is located 0.4 cM away from *Rf^u* (identified in the current study), were shown to be linked to *Rf*. This finding confirms that *RFL* genes are densely located on the DNA region surrounding the *Rf* and *Rf^u* loci. Among *Rf^u* and the three *Rf* loci, only the *Rf^u* locus is syntenic to the tomato DNA region where the *RFL* genes are clustered. This finding suggests that the pepper *Rf^u* locus and the tomato genomic region containing the *RFL* gene cluster might have evolved from a common ancestral sequence, whereas the *Rf* loci might have evolved later in pepper independently. The *RFL* genes evolved under diversifying selection, presumably to help plants cope with the evolution of new CMS genes (Fujii et al., 2011). Since CMS cytoplasm has not been found in tomato, the co-evolution of *Rf* loci following the emergence of the pepper CMS gene might have led to the generation of the pepper-specific *Rf* locus. The sequences in the *Rf^u* locus might have been used as ancestral sequences during this process, considering that PPR genes likely spread by retroposition from the original gene (O'Toole et al., 2008).

The PPR protein encoded by the *PPR* gene in the *Rf^u*-containing region is much shorter (248 amino acids) than the petunia *Rf* protein *Rf*-PPR592 (592 amino acids) and the pepper *Rf*-candidate *CaPPR6* (589 amino acids). We suggest three possibilities for the relationship between this short PPR protein and unstable fertility restoration. First, the incomplete structure might provide this protein with partially impaired function, leading to incomplete restoration. The evolution of *Rf* with higher functionality might have led to the accumulation of mutations in the *Rf^u*-candidate *PPR* gene, thus decreasing the functionality of the protein product. Second, there might be another functional PPR gene in an unassembled sequence in the reference genome used in this study. The sequence around *CaPPR6* is highly complex and thus was not successfully assembled in the main chromosome of the CM334 reference genome. Jo et al. (2016) obtained the target region only by chromosome walking. The dynamic nature of the

DNA region containing *RFL* genes might have hampered its precise assembly in the reference genome. Finally, there might be substantial differences in the structure of the *Rf^u*-containing region or sequence of the *Rf^u*-candidate *PPR* gene between the reference genome and the genome of SPR03, the *Rf^u* donor. The short *PPR* gene in the reference genome could be a nonfunctional recessive allele of *Rf^u* rather than the dominant allele, as is present in the genome of SPR03. Further studies are needed to functionally characterize the candidate *PPR* protein and to assemble the sequences in the target region in sweet peppers.

Feasibility of marker-assisted breeding using developed markers

We used previously and newly developed markers to examine breeding lines with or without unstable restorers to evaluate the feasibility of marker-assisted breeding using these markers. Although three markers developed inside or on the flanking sequences of the defined *Rf^u*-containing DNA region showed relatively high accuracy for genotyping of unstable restorer-containing lines, there were still substantial differences in their accuracy. For example, G16-SCAR, which is located 0.9 cM from *Rf^u*, showed ~20% higher accuracy than 214.14MB-CAPS, which co-segregated with *Rf^u*. This may reflect the existence of unstable restorers other than *Rf^u* (as discussed above) and/or subdivided haplotypes of markers. The markers developed from the DNA regions surrounding the *Rf* locus often contained haplotypes that were not classified as *Rf* or *rf*, presumably due to the structural dynamics in these regions (Min et al., 2009; Lee et al., 2008a; Jo et al., 2010). Therefore, perhaps additional polymorphisms that could not be detected by cleavage with a restriction enzyme or based on amplicon length are also present in the *Rf^u*-linked marker sequence. The haplotypes of the markers might be further classified based on additional polymorphisms.

To breed stable maintainer or restorer lines, lines with *rfrf* or *RfRf* without *Rf^u* should be selected. Therefore, it is important to be able to discriminate *Rf^u* from *Rf* using markers. G16-

459 SCAR showed high accuracy for determining the Rf^u genotype in the current study. On the
460 other hand, Co1Mod1-CAPS co-segregated with Rf and showed the highest accuracy for
461 predicting the Rf genotype among Rf -linked markers in a previous study (Jo et al., 2016). In this
462 study, all breeding lines containing Rf^u were genotyped as $rfrf$ using Co1Mod1-CAPS. Since
463 the Rf^u -containing breeding lines in our panel showed unstable fertility, and Rf is likely epistatic
464 to Rf^u , as discussed above, it is highly likely that this genotyping result is correct and that
465 Co1Mod1-CAPS is useful for discriminating the Rf genotype from the Rf^u genotype. Therefore,
466 the combined use of G16-SCAR and Co1Mod1-CAPS is likely to provide high accuracy in
467 marker-assisted breeding of stable maintainer or restore lines.

Acknowledgements

This work was carried out with the support of the Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ015881), Rural Development Administration, Republic of Korea. This work was also supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2021R1C1C1007883).

References

- Barkan A, Small I (2014) Pentatricopeptide repeat proteins in plants. *Annu Rev Plant Biol* 65:415-442. <https://doi.org/10.1007/s00122-019-03513-y>
- Bentolila S, Alfonso AA, Hanson MR (2002) A pentatricopeptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. *Proc Natl Acad Sci U S A* 99:10887-10892. <https://doi.org/10.1073/pnas.102301599>
- Chen Z, Zhao N, Li S, Grover CE, Nie H, Wendel JF, Hua J (2017) Plant mitochondrial genome evolution and cytoplasmic male sterility. *Critical reviews in plant sciences* 36:55-69. <https://doi.org/10.1080/07352689.2017.1327762>
- Cheng J, Chen Y, Hu Y, Zhou Z, Hu F, Dong J, Chen W, Cui J, Wu Z, Hu K (2020) Fine mapping of restorer-of-fertility gene based on high-density genetic mapping and collinearity analysis in pepper (*Capsicum annuum* L.). *Theor Appl Genet* 133:889-902. <https://doi.org/10.1007/s00122-019-03513-y>
- Cui X, Wise RP, Schnable PS (1996) The *rf2* nuclear restorer gene of male-sterile T-cytoplasm maize. *Science* 272:1334-1336. <https://doi.org/10.1126/science.272.5266.1334>
- de Givry S, Bouchez M, Chabrier P, Milan D, Schiex T (2005) CARHTA GENE: multipopulation integrated genetic and radiation hybrid mapping. *Bioinformatics* 21:1703-1704. <https://doi.org/10.1093/bioinformatics/bti222>
- Fujii S, Bond CS, Small ID (2011) Selection patterns on restorer-like genes reveal a conflict between nuclear and mitochondrial genomes throughout angiosperm evolution. *Proc Natl Acad Sci U S A* 108:1723-1728. <https://doi.org/10.1073/pnas.1007667108>

497 Fujii S, Toriyama K (2009) Suppressed expression of Retrograde-Regulated Male Sterility
 498 restores pollen fertility in cytoplasmic male sterile rice plants. Proc Natl Acad Sci U S A
 499 106:9513-9518. <https://doi.org/10.1073/pnas.0901860106>

500 Gabay-Laughnan S, Kuzmin EV, Monroe J, Roark L, Newton KJ (2009) Characterization of a
 501 novel thermosensitive restorer of fertility for cytoplasmic male sterility in maize. Genetics
 502 182:91-103. <https://doi.org/10.1534/genetics.108.099895>

503 Ha Y (2017) Candidate Gene Analysis of the Restorer-of-fertility Gene in Pepper and
 504 Transgenic Expression of the Pepper CMS Associated Gene *orf507* in *Nicotiana benthamiana*.
 505 Dissertation, Seoul National University

506 Han K, Jeong H-J, Sung J, Keum YS, Cho M-C, Kim J-H, Kwon J-K, Kim B-D, Kang B-C
 507 (2013) Biosynthesis of capsinoid is controlled by the *Pun1* locus in pepper. Molecular
 508 Breeding 31:537-548. <https://doi.org/10.1007/s11032-012-9811-y>

509 Hanson MR (1991) Plant mitochondrial mutations and male sterility. Annu Rev Genet
 510 25:461-486. <https://doi.org/10.1146/annurev.ge.25.120191.002333>

511 Huang W, Yu C, Hu J, Wang L, Dan Z, Zhou W, He C, Zeng Y, Yao G, Qi J, Zhang Z, Zhu
 512 R, Chen X, Zhu Y (2015) Pentatricopeptide-repeat family protein RF6 functions with
 513 hexokinase 6 to rescue rice cytoplasmic male sterility. Proc Natl Acad Sci U S A 112:14984-
 514 14989. <https://doi.org/10.1073/pnas.1511748112>

515 Hulse-Kemp AM, Maheshwari S, Stoffel K, Hill TA, Jaffe D, Williams SR, Weisenfeld N,
 516 Ramakrishnan S, Kumar V, Shah P, Schatz MC, Church DM, Van Deynze A (2018)
 517 Reference quality assembly of the 3.5-Gb genome of *Capsicum annuum* from a single linked-
 518 read library. Hortic Res 5:4. <https://doi.org/10.1038/s41438-017-0011-0>

519 Itabashi E, Iwata N, Fujii S, Kazama T, Toriyama K (2011) The fertility restorer gene, *Rf2*,
520 for Lead Rice-type cytoplasmic male sterility of rice encodes a mitochondrial glycine-rich
521 protein. *Plant J* 65:359-367. <https://doi.org/10.1111/j.1365-313X.2010.04427.x>

522 Janska H, Sarria R, Woloszynska M, Arrieta-Montiel M, Mackenzie SA (1998)
523 Stoichiometric shifts in the common bean mitochondrial genome leading to male sterility and
524 spontaneous reversion to fertility. *Plant Cell* 10:1163-1180.
525 <https://doi.org/10.1105/tpc.10.7.1163>

526 Jo YD, Ha Y, Lee JH, Park M, Bergsma AC, Choi HI, Goritschnig S, Kloosterman B, van
527 Dijk PJ, Choi D, Kang BC (2016) Fine mapping of Restorer-of-fertility in pepper (*Capsicum*
528 *annuum* L.) identified a candidate gene encoding a pentatricopeptide repeat (PPR)-containing
529 protein. *Theor Appl Genet* 129:2003-2017. <https://doi.org/10.1007/s00122-016-2755-6>

530 Jo YD, Kim Y-M, Park M-N, Yoo J-H, Park M, Kim B-D, Kang B-C (2010) Development
531 and evaluation of broadly applicable markers for *Restorer-of-fertility* in pepper. *Molecular*
532 *Breeding* 25:187-201. <https://doi.org/10.1007/s11032-009-9318-3>

533 Jo YD, Lee HY, Ro NY, Kim SH, Kim JB, Kang BC, Kang SY (2019) Mitotypes Based on
534 Structural Variation of Mitochondrial Genomes Imply Relationships With Morphological
535 Phenotypes and Cytoplasmic Male Sterility in Peppers. *Front Plant Sci* 10:1343.
536 <https://doi.org/10.3389/fpls.2019.01343>

537 Jordan DR, Mace ES, Henzell RG, Klein PE, Klein RR (2010) Molecular mapping and
538 candidate gene identification of the *Rf2* gene for pollen fertility restoration in sorghum
539 [*Sorghum bicolor* (L.) Moench]. *Theor Appl Genet* 120:1279-1287.
540 <https://doi.org/10.1007/s00122-009-1255-3>

541 Kim MS, Chae GY, Oh S, Kim J, Mang H, Kim S, Choi D (2021) Comparative analysis of de
 542 novo genomes reveals dynamic intra-species divergence of NLRs in pepper. BMC Plant Biol
 543 21:247. <https://doi.org/10.1186/s12870-021-03057-8>

544 Kim S, Park M, Yeom SI, Kim YM, Lee JM, Lee HA, Seo E, Choi J, Cheong K, Kim KT,
 545 Jung K, Lee GW, Oh SK, Bae C, Kim SB, Lee HY, Kim SY, Kim MS, Kang BC, Jo YD,
 546 Yang HB, Jeong HJ, Kang WH, Kwon JK, Shin C, Lim JY, Park JH, Huh JH, Kim JS, Kim
 547 BD, Cohen O, Paran I, Suh MC, Lee SB, Kim YK, Shin Y, Noh SJ, Park J, Seo YS, Kwon
 548 SY, Kim HA, Park JM, Kim HJ, Choi SB, Bosland PW, Reeves G, Jo SH, Lee BW, Cho HT,
 549 Choi HS, Lee MS, Yu Y, Do Choi Y, Park BS, van Deynze A, Ashrafi H, Hill T, Kim WT,
 550 Pai HS, Ahn HK, Yeam I, Giovannoni JJ, Rose JK, Sørensen I, Lee SJ, Kim RW, Choi IY,
 551 Choi BS, Lim JS, Lee YH, Choi D (2014) Genome sequence of the hot pepper provides
 552 insights into the evolution of pungency in *Capsicum* species. Nat Genet 46:270-278.
 553 <https://doi.org/10.1038/ng.2877>

554 Kim YM, Jo YD, Kwon J-K, Kim IT, Kang B-C (2013) Characterization and inheritance of a
 555 novel thermo-sensitive restoration of cytoplasmic male sterility in *Capsicum annuum*.
 556 Scientia Horticulturae 164:512-520. <https://doi.org/10.1016/j.scienta.2013.10.014>

557 Kitazaki K, Arakawa T, Matsunaga M, Yui-Kurino R, Matsuhira H, Mikami T, Kubo T
 558 (2015) Post-translational mechanisms are associated with fertility restoration of cytoplasmic
 559 male sterility in sugar beet (*Beta vulgaris*). Plant J 83:290-299.
 560 <https://doi.org/10.1111/tpj.12888>

561 Koizuka N, Imai R, Fujimoto H, Hayakawa T, Kimura Y, Kohno-Murase J, Sakai T,
 562 Kawasaki S, Imamura J (2003) Genetic characterization of a pentatricopeptide repeat protein

563 gene, *orf687*, that restores fertility in the cytoplasmic male-sterile Kosena radish. Plant J
564 34:407-415. <https://doi.org/10.1046/j.1365-313x.2003.01735.x>

565 Lee D, (2001) Studies on unstable fertility of CGMS (Cytoplasmic-genic male sterility) in
566 *Capsicum annuum* L. Dissertation, Seoul National University

567 Lee J, Yoon JB, Park HG (2008) A CAPS marker associated with the partial restoration of
568 cytoplasmic male sterility in chili pepper (*Capsicum annuum* L.). Molecular Breeding 21:95-
569 104. <https://doi.org/10.1007/s11032-007-9111-0>

570 Lee J, Yoon JB, Park HG (2008) Linkage analysis between the *partial restoration* (*pr*) and
571 the *restorer-of-fertility* (*Rf*) loci in pepper cytoplasmic male sterility. Theor Appl Genet
572 117:383-389. <https://doi.org/10.1007/s00122-008-0782-7>

573 Lin S-W, Shieh H-c, Wang Y-W, Tan C-W, Schafleitner R, Yang W-J, Kumar S (2015)
574 Restorer breeding in sweet pepper: Introgressing *Rf* allele from hot pepper through marker-
575 assisted backcrossing. Scientia Horticulturae 197:170-175.
576 <https://doi.org/https://doi.org/10.1016/j.scienta.2015.09.036>

577 Liu Z, Yang Z, Wang X, Li K, An H, Liu J, Yang G, Fu T, Yi B, Hong D (2016) A
578 Mitochondria-Targeted PPR Protein Restores pol Cytoplasmic Male Sterility by Reducing
579 *orf224* Transcript Levels in Oilseed Rape. Mol Plant 9:1082-1084.
580 <https://doi.org/10.1016/j.molp.2016.04.004>

581 Lurin C, Andrés C, Aubourg S, Bellaoui M, Bitton F, Bruyère C, Caboche M, Debast C,
582 Gualberto J, Hoffmann B, Lecharny A, Le Ret M, Martin-Magniette ML, Mireau H, Peeters
583 N, Renou JP, Szurek B, Taconnat L, Small I (2004) Genome-wide analysis of Arabidopsis
584 pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell
585 16:2089-2103. <https://doi.org/10.1105/tpc.104.022236>

586 Manna S (2015) An overview of pentatricopeptide repeat proteins and their applications.
587 Biochimie 113:93-99. <https://doi.org/10.1016/j.biochi.2015.04.004>

588 Min WK, Kim S, Sung SK, Kim BD, Lee S (2009) Allelic discrimination of the Restorer-of-
589 fertility gene and its inheritance in peppers (*Capsicum annuum* L.). Theor Appl Genet
590 119:1289-1299. <https://doi.org/10.1007/s00122-009-1134-y>

591 O'Toole N, Hattori M, Andres C, Iida K, Lurin C, Schmitz-Linneweber C, Sugita M, Small I
592 (2008) On the Expansion of the Pentatricopeptide Repeat Gene Family in Plants. Molecular
593 Biology and Evolution 25:1120-1128. <https://doi.org/10.1093/molbev/msn057>

594 Peterson PA (1958) Cytoplasmically Inherited Male Sterility in Capsicum. The American
595 Naturalist 92:111-119. <https://doi.org/10.1086/282017>

596 Shen C, Zhang D, Guan Z, Liu Y, Yang Z, Yang Y, Wang X, Wang Q, Zhang Q, Fan S, Zou
597 T, Yin P (2016) Structural basis for specific single-stranded RNA recognition by designer
598 pentatricopeptide repeat proteins. Nat Commun 7:11285.
599 <https://doi.org/10.1038/ncomms11285>

600 Shifriss C (1997) Male sterility in pepper (*Capsicum annuum* L.). Euphytica 93:83-88.
601 <https://doi.org/10.1023/A:1002947907046>

602 Tang H, Luo D, Zhou D, Zhang Q, Tian D, Zheng X, Chen L, Liu YG (2014) The rice
603 restorer Rf4 for wild-abortive cytoplasmic male sterility encodes a mitochondrial-localized
604 PPR protein that functions in reduction of WA352 transcripts. Mol Plant 7:1497-1500.
605 <https://doi.org/10.1093/mp/ssu047>

606 Uyttewaal M, Arnal N, Quadrado M, Martin-Canadell A, Vrielynck N, Hiard S, Gherbi H,
607 Bendahmane A, Budar F, Mireau H (2008) Characterization of *Raphanus sativus*

608 pentatricopeptide repeat proteins encoded by the fertility restorer locus for Ogura cytoplasmic
609 male sterility. *Plant Cell* 20:3331-3345. <https://doi.org/10.1105/tpc.107.057208>

610 Wang LH, Zhang BX, Lefebvre V, Huang SW, Daubèze AM, Palloix A (2004) QTL analysis
611 of fertility restoration in cytoplasmic male sterile pepper. *Theor Appl Genet* 109:1058-1063.
612 <https://doi.org/10.1007/s00122-004-1715-8>

613 Wang Z, Zou Y, Li X, Zhang Q, Chen L, Wu H, Su D, Chen Y, Guo J, Luo D, Long Y,
614 Zhong Y, Liu YG (2006) Cytoplasmic male sterility of rice with boro II cytoplasm is caused
615 by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of
616 mRNA silencing. *Plant Cell* 18:676-687. <https://doi.org/10.1105/tpc.105.038240>

617 Wei B, Bosland PW, Zhang Z, Wang Y, Zhang G, Wang L, Yu J (2020) A predicted NEDD8
618 conjugating enzyme gene identified as a *Capsicum* candidate *Rf* gene using bulk segregant
619 RNA sequencing. *Horticulture Research* 7:210. <https://doi.org/10.1038/s41438-020-00425-7>

620 Zhang H, Che J, Ge Y, Pei Y, Zhang L, Liu Q, Gu M, Tang S (2017) Ability of Rf5 and Rf6
621 to Restore Fertility of Chinsurah Boro II-type Cytoplasmic Male Sterile *Oryza Sativa* (ssp.
622 Japonica) Lines. *Rice (N Y)* 10:2. <https://doi.org/10.1186/s12284-017-0142-9>

623 Zhang Z, Zhu Y, Cao Y, Yu H, Bai R, Zhao H, Zhang B, Wang L (2020) Fine mapping of the
624 male fertility restoration gene *CaRf032* in *Capsicum annuum* L. *Theor Appl Genet* 133:1177-
625 1187. <https://doi.org/10.1007/s00122-020-03540-0>

626

627 Table 1. Hot pepper and sweet pepper lines used for marker development

Name	Pungency	Fruit shape	CGMS characteristics
Chungyang A	Pungent	Elongated	Stable CMS line
Chungyang C	Pungent	Elongated	Stable restorer line
MSGR-A	Nonpungent	Small blocky	Stable CMS line
SPR03	Nonpungent	Large blocky	Unstable restorer line

628

629

630 Table 2. Molecular markers developed in this study

Marker name	Primer sequence (5'-3')	Annealing temperature (°C)	Marker type / restriction enzyme	Reference
214.14MB CAPS	F : AAAGCTAAAACAGGAGCCTGAG ^z R : ATGTTTCTTTGGGCTGTTGAGA	60°C	CAPS / <i>NaeI</i>	Developed in this study
213.85MB CAPS	F : GGAAACCAATCACATGTTGAAGA R : ACCCGCTGGATATGTCTTGTC	62°C	CAPS / <i>FokI</i>	Developed in this study
213.3MB CAPS	F : GAGCGGTTTTGATCCAAGTAACA R : ACTCTGATATACGCCACAAGGAC	60°C	CAPS / <i>BsrI</i>	Developed in this study
212.9MB CAPS	F : GGAAACCAATCACATGTTGAAGA R : ACCCGCTGGATATGTCTTGTC	60°C	CAPS / <i>MseI</i>	Developed in this study
13T7 SCAR	F : CACTAAGCCCGATGTATGAATC R : GAAGTAGGCCAAAACCTTATCACG	53°C	SCAR	Jo et al. 2010
G16 SCAR	F : GATTTTACGATGCTCAACCC R : AAGTTGAACAATCTTCGCTG	60°C	SCAR	Jo et al. 2016
4162 CAPS	F : GCAGTTCGGTTTTACGGAGTTAC R : CCATTGGACAAAAGGGGATC	56°C	CAPS / <i>EcoRI</i>	Jo et al. 2016
PR CAPS	F : ATGTCACCCCCACACACTCCTTCACC R : TCCCATCTAGCCTCTGCCTTCTCAAATG	56°C	CAPS / <i>MseI</i>	Lee et al. 2008

631

632 ^zF, forward; R, reverse.

633 Table 3. Chi-squared test for Mendelian segregation (3:1) of partially fertile and completely
 634 sterile lines in the BC₄F₅ population

Number of individuals			Expected ratio	χ^2	<i>P</i> -value
Total	Partially fertile	Completely sterile			
300	228	72	3:1	0.160	0.689

635

636 Table 4. Predicted genes in the mapped DNA region containing *Rj^{ai}*

Gene model ID ^z	Length of CDS	Location on chromosome 6	
		in the UCD 10× genome (kb)	Predicted function
CA00g30010	3,408	5,031-5,034 (–)	NBS-LRR resistance protein-like protein
CA00g30020	3,666	5,005-5,009 (–)	Root-knot nematode resistance protein
CA00g30030	3,792	4,981-4,984 (–)	Root-knot nematode resistance protein
CA00g30040	669	4,830-4,831 (+)	Adenylate isopentenyltransferase
CA00g30050	864	4,791-4,792 (+)	Adenylate isopentenyltransferase
CA00g30060	816	4,732-4,735 (+)	ER lumen retaining receptor family-like protein
CA00g30070	1,248	4,724-4,726 (–)	Gypsy/Ty-3 retroelement polypeptide
CA00g30080	747	4,698-4,699 (+)	PPR protein
CA00g30090	555	4,675-4,676 (–)	Unknown
CA00g30100	648	4,671-4,673 (+)	RING finger protein
CA00g30110	504	4,661 (+)	Transferase, transferring glycosyl groups
CA00g30120	300	4,643 (+)	Unknown
CA00g30130	630	4,624-4,635 (+)	Xyloglucan endotransglucosylase/hydrolase protein 2
CA00g30140	645	4,616-4,624 (–)	RING/U-box domain-containing protein

637 ^z The gene model IDs and CDS lengths were obtained from the CDS database of the CM334
638 v1.55 genome.

Table 5. Rf-like PPRs in tomato screened by BLAST analysis against petunia Rf (Rf-PPR592) and the Rf candidate in pepper (CaPPR6)

Gene ID ^z	Protein length (aa)	Location (chromosome)	Results of BLASTP against:					
			Rf-PPR592 (petunia Rf)			CaPPR6 (Rf candidate in pepper)		
			Identity	Positive	Bit score	Identity	Positive	Bit score
Solyc06g007820	164	1,762,162-1,762,965 (6)	71	82	228	73	86	240
Solyc06g007810	268	1,761,299-1,762,105 (6)	65	80	350	68	81	362
Solyc06g007850	435	1,766,163-1,768,701 (6)	64	76	556	71	83	635
Solyc06g005220	406	260,259-262,393 (6)	64	78	529	72	82	599
Solyc05g009253	545	3,442,275-34,44,051 (5)	63	75	677	70	82	770
Solyc06g007730	141	1,723,197-1,723,541 (6)	62	72	140	69	80	166
Solyc06g007740	590	1,726,521-1,728,293 (6)	61	76	733	71	81	833
Solyc06g007300	592	1,340,738-1,342,516 (6)	61	75	731	71	81	825
Solyc04g080120	612	62,355,663-62,357,501 (4)	52	71	612	52	72	595

^z The gene model IDs and protein lengths (number of amino acids, aa) were obtained from the tomato reference genome database (ITAG release 2.40).

645 Table 6. Genotypes of *Rf*- and *Rf^u*-linked markers in breeding lines with stable and unstable
646 fertility

Breeding line	Type of restorer	Marker genotype ^z					
		212.9MB-CAPS	13T7-SCAR	214.14 MB-CAPS	G16-SCAR	ColMod1-CAPS	4162-CAPS
SPR03	Unstable <i>Rf</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>rfrf</i>	<i>Rfu^uRfu^u</i>
MSGR-A	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>rfu2rfu2</i>
Chungyang C	Stable <i>Rf</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>rf^urf^u</i>	<i>RfRf</i>	<i>Rfu^uRfu^u</i>
Chungyang A	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>Rf^uRf^u</i>	<i>rfrf</i>	<i>rfurfu</i>
UA-2	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>rf^u2rf^u2</i>
UA-3	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>rf^u2rf^u2</i>
UA-4	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>rf^u2rf^u2</i>
A-138	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>rf^u2rf^u2</i>
A-178	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>rfrf</i>	<i>rf^urf^u</i>
A-85	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>rfrf</i>	<i>rf^u2rf^u2</i>
A-136	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>Rf^uRf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>rf^u2rf^u2</i>
A-76	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>Rf^uRf^u</i>
A-81	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>Rf^uRf^u</i>
A-82	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>Rf^uRf^u</i>
A-83	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>Rf^uRf^u</i>
A-84	Stable <i>rf</i>	<i>rf^urf^u</i>	<i>Rf^uRf^u</i>	<i>Rf^uRf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>Rf^uRf^u</i>
A-148	Unstable <i>Rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>Rf^uRf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>rf^u2rf^u2</i>
A-176	Unstable <i>Rf</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rf^urf^u</i>	<i>rfrf</i>	<i>rf^u2rf^u2</i>

A-1	Unstable <i>Rf</i>	<i>r^{f^u}r^{f^u}</i>	<i>r^{f^u}r^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>r^{f^r}r^{f^r}</i>	<i>r^{f^u}2r^{f^u}2</i>
A-2	Unstable <i>Rf</i>	<i>r^{f^u}r^{f^u}</i>	<i>r^{f^u}r^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>r^{f^r}r^{f^r}</i>	<i>r^{f^u}2r^{f^u}2</i>
A-4	Unstable <i>Rf</i>	<i>r^{f^u}r^{f^u}</i>	<i>r^{f^u}r^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>r^{f^r}r^{f^r}</i>	<i>r^{f^u}2r^{f^u}2</i>
A-5	Unstable <i>Rf</i>	<i>r^{f^u}r^{f^u}</i>	<i>r^{f^u}r^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>r^{f^r}r^{f^r}</i>	<i>r^{f^u}2r^{f^u}2</i>
A-72	Unstable <i>Rf</i>	<i>r^{f^u}r^{f^u}</i>	<i>r^{f^u}r^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>r^{f^r}r^{f^r}</i>	<i>r^{f^u}2r^{f^u}2</i>
A-73	Unstable <i>Rf</i>	<i>r^{f^u}r^{f^u}</i>	<i>r^{f^u}r^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>r^{f^r}r^{f^r}</i>	<i>r^{f^u}2r^{f^u}2</i>
A-74	Unstable <i>Rf</i>	<i>r^{f^u}r^{f^u}</i>	<i>r^{f^u}r^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>r^{f^r}r^{f^r}</i>	<i>r^{f^u}2r^{f^u}2</i>
A-75	Unstable <i>Rf</i>	<i>r^{f^u}r^{f^u}</i>	<i>r^{f^u}r^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>r^{f^r}r^{f^r}</i>	<i>r^{f^u}2r^{f^u}2</i>
A-79	Unstable <i>Rf</i>	<i>r^{f^u}r^{f^u}</i>	<i>r^{f^u}r^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>R^{f^u}R^{f^u}</i>	<i>r^{f^r}r^{f^r}</i>	<i>R^{f^u}u^uR^{f^u}u^u</i>
Rate of successful		52.2	30.4	60.9	82.7	52.2	34.8
genotyping (%)							

^z The genotypes of markers that were mapped in this study are represented as *R^{f^u}* or *r^{f^u}*, while the genotypes of Co1Mod1-CAPS, which co-segregated with *Rf* (Jo et al., 2016), are designated as *Rf* or *rf*.

Figure Legends

Fig. 1. Procedure used to develop the segregation population used for genetic mapping.

Fig. 2. Phenotypes of anthers in the parental lines and BC₄F₅ lines used for genetic mapping.

Fig. 3. Application of the multiplex-PCR marker MCM1 to the pepper breeding lines used in this study. Mitotypes were classified as described in Jo et al. (2019).

Fig. 4. Genotypes of recombinants found among BC₄F₄ individuals determined using *Rf^u*-like markers.

Fig. 5. Genetic and physical maps of the *Rf^u* locus. **(A)** Integrative representation of the *Rf^u* and *Rf* genetic maps of pepper and physical map of tomato created using marker sequences. **(B)** Physical maps of the *Rf^u*-containing DNA region in pepper and the corresponding region in tomato. *PPR* genes are indicated in red. Genes or markers that are identical or syntenic to each other are linked by dotted lines.

Fig. 6. Distribution of tomato PPR proteins based on their identity with petunia Rf-PPR592 and pepper CaPPR6.

Fig. 7. Phylogenetic tree based on marker genotypes from breeding lines showing unstable fertility restoration or stable sterility.

Supplementary Figure Legends

Fig. S1. Fluorescein diacetate (FDA)-stained pollen from BC₄F₅ lines that showed partial fertility or complete sterility.

Fig. S2. Application of molecular markers to hot pepper and sweet pepper parental lines. **(A)** Application of markers developed in a previous study (Jo et al., 2016). **(B)** Application of markers that were developed in this study or modified from previously developed markers.

Fig. S3. ClustalW analysis of the sequences of PPR proteins encoded by genes in the *R^f'*-containing DNA region from two pepper reference genomes (CM334 v1.55 and ECW v1.0), Rf-PPR592 from petunia, and CaPPR6 from pepper.

Statements & Declaration

Funding

This work was carried out with the support of the Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ015881), Rural Development Administration, Republic of Korea. This work was also supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2021R1C1C1007883).

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

MCK participated in population generation, phenotypic analysis, marker development, and manuscript writing. HJK participated in phenotypic and genotypic analysis. SYJ participated in phenotypic analysis. HYL participated in genotyping of breeding lines. MYK managed the project that supported this research. YDJ participated in data curation and manuscript writing. BCK participated in the design of the study and editing of the manuscript.

Data availability

The reference genome sequences used in this study are available at the Sol Genomics Network (<http://solgenomics.net>).

Figures

Figure 1

Procedure used to develop the segregation population used for genetic mapping.

Parental lines

MSGR-A
(completely sterile)

SPR03
(fully fertile)

Individuals in a BC_4F_5
segregation population

BC_4F_5 individuals
(partially fertile)

BC_4F_5 individuals
(completely sterile)

Figure 2

Phenotypes of anthers in the parental lines and BC_4F_5 lines used for genetic mapping.

Figure 3

Application of the multiplex-PCR marker MCM1 to the pepper breeding lines used in this study. Mitotypes were classified as described in Jo et al. (2019).

Figure 4

Genotypes of recombinants found among BC₄F₄ individuals determined using Rfu-like markers.

Figure 5

Genetic and physical maps of the *Rfu* locus. (A) Integrative representation of the *Rfu* and *Rf* genetic maps of pepper and physical map of tomato created using marker sequences. (B) Physical maps of the *Rfu* -containing DNA region in pepper and the corresponding region in tomato. PPR genes are indicated in red. Genes or markers that are identical or syntenic to each other are linked by dotted lines.

Figure 6

Distribution of tomato PPR proteins based on their identity with petunia Rf-PPR592 and pepper CaPPR6.

Figure 7

Phylogenetic tree based on marker genotypes from breeding lines showing unstable fertility restoration or stable sterility.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigureS1Rfu.pdf](#)
- [FigureS2Rfu.pdf](#)