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Development and Investigation of HRM Markers to Discriminate Two Ogura Cytoplasmic Male Sterility Restorer Genes in Radish

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Abstract: Ogura male sterile cytoplasm is widely used for radish breeding. In this study, high-resolution melting (HRM) markers associated with *Rft* and *Rfo*, major restorer-of-fertility genes in Ogura cytoplasmic male sterility (CMS) in radish, were developed. Genetic mapping was carried out using F_2 populations derived from crosses between male-sterile Ogura CMS lines and male-fertile lines. Identification of the *Rft* and *Rfo* loci was achieved through SNP-based genotyping and linkage grouping. HRM markers were subsequently developed based on flanking sequences of SNPs linked to these loci. For the *Rft* gene, a set of 117 SNPs was selected within a candidate region on chromosome 5, and 14 HRM markers were successfully developed. Genotyping of F_2 showed high correlation between three markers and the phenotype. Regarding the *Rfo* gene, a set of 27 HRM markers was designed based on flanking sequences of SNPs located on chromosomes 9 and 0. Genotyping in the *Rfo* segregating population identified a single marker, RSRF27, that accurately distinguished the male sterility phenotype. Validation of the developed markers was performed in populations containing both *Rft* and *Rfo* genes, confirming their utility for genotyping and demonstrating that these two genes independently contribute to male sterility recovery. Overall, this study provides HRM markers that can be used for genotyping *Rft* and *Rfo* and contributes to a deeper understanding of male sterility restoration mechanisms in Ogura CMS.

Keywords: radish; restorer-of-fertility; molecular marker; Ogura CMS

Citation: Ahn, H.-I.; Han, K.; Yang, H.-B.; Lee, E.S.; Lee, Y.-R.; Kim, J.; Park, H.Y.; Kim, D.-S. Development and Investigation of HRM Markers to Discriminate Two Ogura Cytoplasmic Male Sterility Restorer Genes in Radish. *Agronomy* **2024**, *14*, 43. https://doi.org/10.3390/agronomy14010043

Academic Editor: Ainong Shi

Received: 13 November 2023
Revised: 11 December 2023
Accepted: 19 December 2023
Published: 22 December 2023

1. Introduction

The history of research and development on Ogura cytoplasmic male sterility (CMS) and restorer-of-fertility (*Rf*) genes has been an important topic in the fields of agriculture and plant genetics. These genes play a crucial role in crop production and have been used in research on F1 hybrid seed production in cruciferous crops, including cabbage. Research on Ogura CMS began in the 1960s, and since then, various researchers have made efforts to elucidate the functions and regulatory mechanisms of these genes in *Brassica* genus [1,2]. In the case of radish, genes known to induce CMS include mitochondrial *orf138* in the case of Ogura CMS [3–5], *orf125* in Kosena CMS [6,7], *apt6* in NWB CMS [8,9], *orf463* in DCGMS [10,11], and *trnD—trnY* genes in DBRMF1, 2 [12]. The *Rf* gene interacts with Ogura CMS, restoring male fertility and, thereby, enhancing the productivity of CMS plants. Research and development related to the *Rf* gene have been conducted in conjunction with Ogura CMS research, and understanding the interaction mechanism between these two genes has been an important component. Such research is a fundamental aspect of

crop breeding and improvement to increase food production and enhance valuable traits in crops. The first case of a cloned *Rf* gene in cruciferous crops was PGI-2 in rapeseed (*Brassica napus*) by Delourme and Eber in 1992 [13], and in the case of European radishes, the first cloned *Rf* (*Rfo*) gene was identified by Brown et al. in 2003 [14]. A similar gene, *Rfk1*, was discovered by Koizuka et al. in 2003 [7], and *D81Rfo* was reported by Desloire et al. [15]. Another *Rf* gene named *Rft* was identified in wild radishes in Japan in 2009 [16], and its mechanism mediated by the *Rfob* gene was studied by Wang et al. in 2013 [17]. Different from the nuclear genes of Ogura CMS and known to be distinct, the DCMGS gene was previously identified as a single *RF* gene named *Rfd1*, and a high-density linkage map of the *Rfd1* gene locus was developed using the molecular markers CAPS3450 and ILP3482 [18,19]. Most restorer genes are part of the pentatricopeptide repeat (PPR) protein family, one of the most important nuclear-encoded protein families in higher plants, with more than 400 to 500 paralogous genes found in most genome-sequenced plant species [20,21]. The molecular functions and physiological roles of PPR proteins in plant growth and development have been extensively investigated. Evidence suggests that PPR proteins are involved in the post-transcriptional regulation of chloroplast and mitochondrial genes, including RNA maturation, editing, intron splicing, transcript stabilization, and translation initiation. The synergy of RNA metabolism has profound effects on the biogenesis and function of both chloroplasts and mitochondria, influencing processes such as photosynthesis, respiration, development, and environmental responses in plants [20,22,23]. The male sterility recovery gene likely evolved through numerous interactions with the host in the symbiotic relationship with mitochondria. To identify the candidate gene and understand the fertility-restoring mechanism, further studies such as fine-mapping and complementation tests with the *Rfo* and *Rft* genes are needed. The marker developed in this study will be valuable for breeding new CMS lines in radish.

2. Materials and Methods

2.1. Plant Materials and Male Sterility Evaluation

To identify the *Rft* and *Rfo* loci, 'Bokjeong' × 'Bakdal sn' and 'OharuA' × 'Bakdal' F_2 populations were generated, respectively. All maternal lines were male sterile with Ogura CMS, and the paternal lines were male fertile. 'Bokjeong' and 'Bakdal sn' are parental lines of the *Rft* segregating population, and 'Gwandong summer radish' is a commercial variety with Ogura cytoplasm and a heterozygous *Rfo* genotype. *Rft* segregating populations consisted of a total of 367 F_2 individuals. Related to the *Rfo* gene, a total of 183 F_2 individuals were used for genetic mapping. In addition, 'Bokjeong' × 'Bakdal sn' F_1 (*Rft*/*rft*) and the commercial variety 'Gwandong summer radish' (*Rfo*/*rfo*) were crossed to develop a segregating population including 199 individuals.

Seeds were sown in a 50 cell plug tray and grown in a greenhouse until three true leaves. Seedlings were kept in a 5 °C chamber for 50 days for vernalization and moved to a greenhouse with temperatures ranging from 10 to 30 °C. Male sterility was evaluated by observation of the presence and absence of pollen grains.

2.2. Total DNA Isolation and the PCR Assays

Genomic DNA was isolated from fresh leaves according to the following steps. Plant leaves were placed in a 1.5 mL microcentrifuge tube containing three 5 mm stainless beads, and 700 µL of DNA extraction buffer (CTAB) (200 mM Tris–Cl, pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS) was added to the tube. The samples were then lysed using a TissueLyser II (Qiagen, Hilden, Germany) for 3 min and centrifuged at 4 °C for 10 min at 13,000× *g* rpm using a 1730R microcentrifuge (Labogene, Seoul, Korea). After centrifugation, 600 µL of the supernatant was transferred to a new tube, and chloroform:isoamyl alcohol (24:1) was added at a 1:1 ratio and centrifuged again. The acquired supernatant was mixed with isopropanol at a 1:1 ratio in a new tube and then stored at −20 °C for 10 min. The pellet was obtained by centrifuging the above mixture at 4 °C for 5 min at 14,000× *g* rpm, and it was then washed and dried twice using 70% ethanol. The final DNA pellet was dissolved

in 100 µL of distilled water containing 0.1 µL of 10 mg mL^{-1} RNase A solution (Bio Basic Canada Inc., Markham, ON, Canada). The concentration of DNA was measured using a BioDrop µLITE (BioDrop UK Ltd., Cambridge, UK) and diluted to 30 ng μL^{-1} for further analysis [24].

To amplify Ogura-specific *orf138*, we used the following oligonucleotides as primers: orf138_F, GACATCTAGAAAGTTAAAAAT; orf138_R, AGCAATTGGGTTCACAAAGC AT. The orf138_F primer is a sense 22 mer corresponding to position 161,182 in the coding region of *orf138*, whereas the orf138_R primer is an antisense 22mer located immediately 3′ to the gene. Thirty PCR cycles were carried out in a MyCycler (Bio-Rad). Each cycle consisted of denaturation for 1 rain at 94 °C annealing for 1 min at 52 °C and extension for 2 min at 72 °C. PCR using primers D and E amplifies a 278-bp DNA fragment in plants with Ogura-type mtDNA, whereas no PCR product appears in plants with normal-type mtDNA. Amplified DNA fragments were separated by electrophoresis on a 1% agarose gel [3].

2.3. Genetic Mapping of Restorer-of-Fertility Genes

Genomic DNA was meticulously extracted using the CTAB method, ensuring quality with A260/280 values 1.8 and concentrations $\geq$ 100 ng/µL. We conducted genotyping on all parental lines and F_2 individuals to confirm the presence of Ogura-type CMS [3].

Genetic positions of the restorer-of-fertility genes were determined using a set of radish SNP-based markers. We employed the SNPtype Assay system (Fluidigm®, South San Francisco, CA, USA) using 288 radish probes developed from a previous study by Kim et al. (2019) [25]. Genotyping was conducted for 92 and 183 individuals for *Rft* and *Rfo* segregating populations, respectively. We excluded cases with either a parent having a heterozygous genotype (XY) or both parents having the same genotype. Linkage mapping was performed using JoinMap® ver. 4.1 software (Van Ooijen 2006) [26]. The markers were mapped at LOD $\geq$ 3.0 with a maximum distance of 30 cM. Map distances were calculated using the Kosambi mapping function (Kosambi 1944) [27]. The population option of type CP (outbreeder full-sib family) was used because the linkage phases were originally unknown in the F1 segregating population resulting from a cross between two heterogeneously heterozygous and homozygous parents (Van Ooijen 2006) [26]. Final linkage maps were drawn using MapChart ver. 2.2 software (Voorrips 2002) [28].

2.4. Development of Molecular Markers

The flanking sequences of SNP probes linked to the *Rft* locus were BLAST searched against the radish reference genome 'RSAskr_r1.0' accessible at https://plantgarden.jp, accessed on 2 March 2023 [29]. Based on the physical position, 451 SNPs were previously selected from variant calling data (Table S2) [25]. Molecular markers were developed using the 120bp flanking sequences adjacent to each SNP.

Molecular markers related to the *Rfo* gene were developed by analyzing known sequences AY285674–6, EU163282–3, AJ535623–4, and AJ550021.2. These markers were applied to the *Rfo* segregating population, comprising 183 individuals, using 160 bp contiguous sequences of 820 SNPs located on chromosomes 9 and 0 that showed relevance to the reference genome through BLAST (Table S2).

The primer design was conducted using the web-based Primer 3 program. The primer length was set to an average of 22 bp within the range of 18–26 bp, based on the upper and lower sequences flanking the SNP. The melting temperature (Tm) was set within the range of 57–62 °C with an average of 60 °C, and the GC ratio was set within the range of 30–70% with an average of 50%. Other parameters were set to the default values provided by the program. In cases where Primer 3 could not design primers, manual composition was performed by directly examining the nucleotide sequences [30].

2.5. HRM Analysis for Fine-Mapping

Primers were designed for HRM analysis using the flanking sequence of SNPs. Real-time PCR-based HRM analysis was conducted using the LightCycler 96 (Roche®, Basel, Switzerland) instrument following the manufacturer's protocol. The total reaction volume for HRM analysis was 20 µL, containing 10 ng of genomic DNA, 2.0 µL of 10 × Easy Taq buffer (TransGen Biotech, Beijing, China), 1.0 µL of 2.5 mM dNTP mixture (TransGen Biotech, Beijing, China), 0.1 µL of Easy Taq DNA polymerase (Transgen Biotech, Beijing, China), 1.0 µL of SYTO®9 green fluorescent nucleic acid stain (Life Technologies™, Carlsbad, CA, USA), 0.5 µL each of 10 pmol μL^{-1} of a pair of primers (Supplementary Table S2), and autoclaved distilled water for the remainder of the volume. PCR was performed using the Biometra TAdvanced (Biometra GmbH, Göttingen, Germany) as follows: initial denaturation at 95 °C for 5 min; denaturation at 95 °C for 10 s, and annealing and elongation at 60 °C for 20 s, repeated 40 times; and final denaturation at 95 °C for 10 s. HRM was analyzed at each temperature during a rise of 0.3% from 60 to 90 °C using the LightCycler® 96 Real-Time PCR System (Roche, Basel, Switzerland). The HRM graphs were drawn by LightCycler® 96 software ver. 1.1 (Roche, Basel, Switzerland). Marker polymorphisms between parental lines were used for further analysis of F_2 individuals. By comparing the genotype and male sterility phenotype of F_2, the markers most strongly linked to *Rft* and *Rfo* were tested in populations derived from 'Bokjeong' × 'Bakdal sn' F_1 and 'Gwandong summer radish'. Candidate genes for *Rft* were selected based on the reference genome 'RSAskr_r1.0' annotation data.

3. Results

3.1. Genetic Mapping of Rft and Rfo

Male sterility of the 'Bokjeong' × 'Bakdal sn' F_2 population was evaluated by observing pollen morphology and production. A total of 367 individuals showed 3:1 segregation of male fertile and sterile phenotypes (Table S1). The paternal line and fertile individuals had normal anther morphology and produced pollen, while the maternal line and sterile individuals did not show pollen (Figure 1A). The 'OharuA' × 'Bakdal' F_2 population also showed the 3:1 segregation of male sterility. Both populations had Ogura cytoplasm genotypes (Figure 1B). To identify the locus associated with fertility restoration, we conducted genotyping on two populations using 288 SNP markers previously reported and designed for the Fluidigm assay in our earlier study [25].

By linkage mapping, 10 linkage groups were constructed for the 'Bokjeong' × 'Bakdal sn' F_2 population. As shown in Figure 2A, male sterility of this population was strongly associated with the NRS160 assay of linkage group 5, which is chromosome 5. We predicted the locus as the *Rft* locus, a restorer of the fertility gene for Ogura CMS, identified in a previous study [16]. The restorer-of-fertility gene of the 'OharuA' × 'Bakdal' F_2 population was mapped on chromosome 9 and closely linked to the NRS286 assay, where *Rfo* is located (Figure 2B). Therefore, we used the 'Bokjeong' × 'Bakdal sn' (hereafter *Rft* segregating population) and 'OharuA' × 'Bakdal' (hereafter *Rfo* segregating population) F_2 populations for marker development for *Rft* and *Rfo*, respectively.

3.2. Development of Rft Gene-Related HRM Molecular Markers

Based on the NRS160 assay, RS017 markers were designed and tested for the *Rft* segregating population. Three recombinants were identified, and these individuals were self-pollinated to develop the F_3 population. For fine-mapping the *Rft* locus, 451 candidate SNPs located 30.4–36.8 Mbp from chromosome 5 were identified (Table 1). Among them, 117 SNPs excluding the case of homologous bases of A/T and C/G were selected for construction as high-resolution melting (HRM) primers (Table S7). HRM was performed using 117 markers and DNA samples of the parents and F_1 and F_2 individuals of the *Rft* segregating population. As a result, primers capable of significant discrimination were identified in all 14 pairs of markers (Figure S1). We genotyped 183 F_2 individuals with the 14 markers (Table S3). The genotypes of the three markers, RSc15, RSb13, and RSc17,

and the male sterility phenotype were highly cosegregated (Table 2; Figure 3A). Of these markers, RSc17 showed the least number of recombinants.

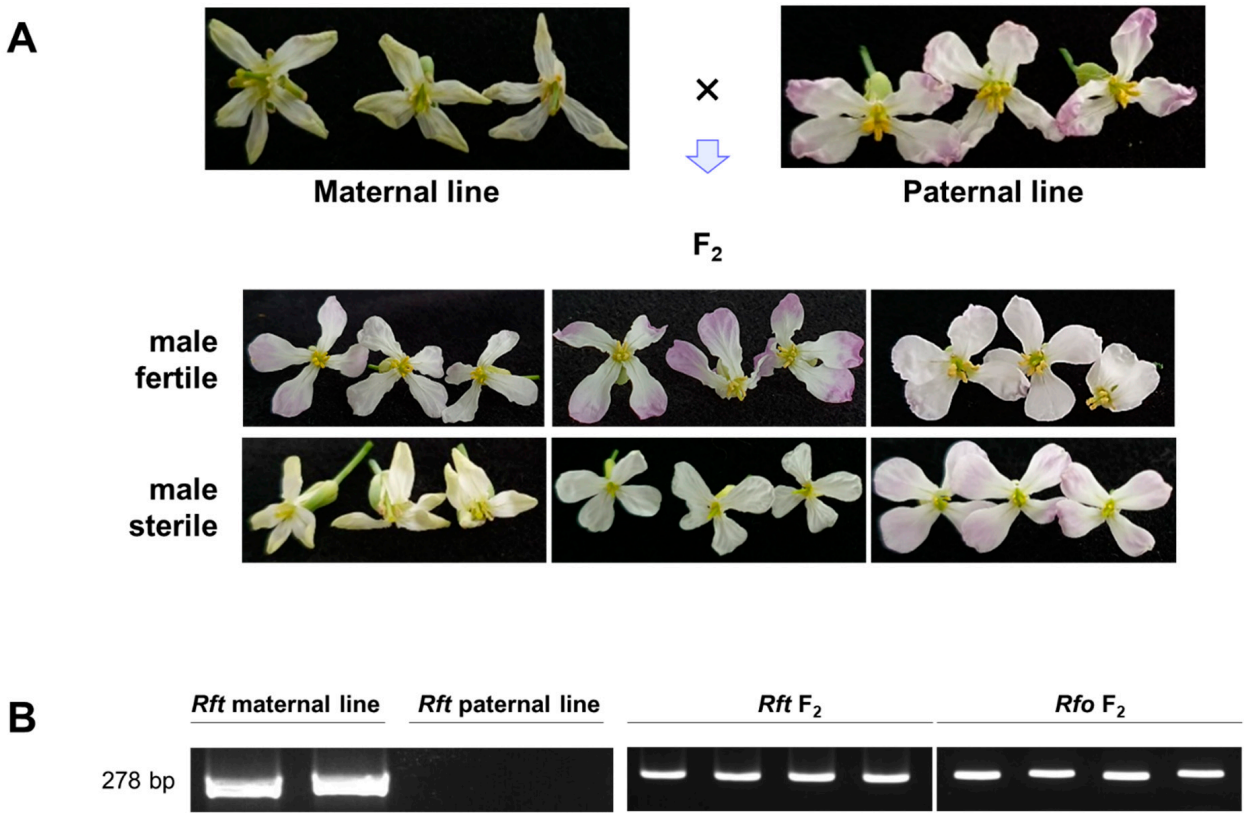

Figure 1. Male sterility phenotype and genotype of Ogura cytoplasm. (**A**) Flower morphology of parental line and F_2 individuals of *Rft* segregating population. (**B**) Ogura male sterile cytoplasm test using markers from Yamagishi and Terachi (1996) [3].

Table 1. Design of HRM markers for the confirmation of *Rft* and *Rfo*.

Locus.	Chr.	Number of SNPs	Position (Mbp)	Designed Marker	Average Distance between Markers
Rft	R5	451	30.4–36.8	18	355 kbp
			32.8–35.2	79	30 kbp
			33.0–33.5	20	25 kbp
Rfo	R9	510	11.39–11.41	17	1 kbp
	R0	310	28.45–28.46	10	1 kbp

3.3. Development of HRM Molecular Markers Related to the Rfo Gene

To develop molecular markers related to the *Rfo* gene, the nucleotide sequences of the AY285674, EU163282–3, AJ535623–4, and AJ550021 genes, known as previously published *Rfo* genes, were blasted to the reference genome (RSAskr_r1.0) [29,31]. All genes were confirmed to have 99% similarity to the RSAskr_r1.0R0g01744 gene on chromosome R0 and 87% similarity to the RSAskr_r1.0R9 g87877 gene existing on chromosome R9 (Table S4). Flanking sequences of SNPs on 28.45–28.46 Mbp of chromosome R9 and 11.39–11.41 Mbp of chromosome R0, which are considered candidate male sterility recovery genes, were used to design HRM markers. By considering the average physical distance between markers, we developed 27 markers linked to *Rfo* (Table 1). The *Rfo* segregating population was genotyped using 27 HRM markers, and one primer, RSRF27, located on chromosome R9, significantly distinguished the male sterility phenotype (Figure 3B).

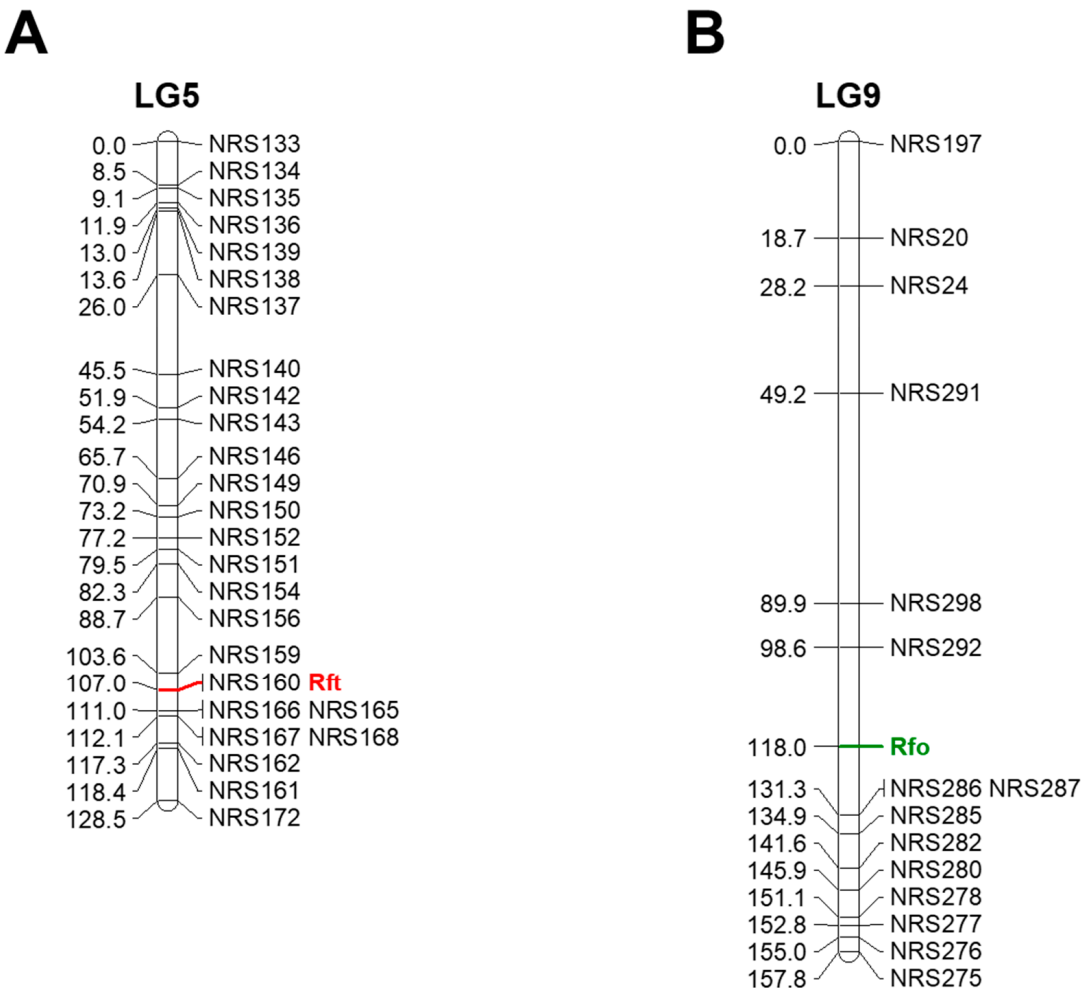

Figure 2. Genetic mapping of restorer-of-fertility genes in *Rft* (**A**) and *Rfo* (**B**) segregating populations. The red line represents the loci of the *Rft* gene, and the green line represents the loci of the *Rfo* gene.

Table 2. Co-segregation of male sterility phenotype and genotype of developed markers.

Population	No. of Individuals	Marker	Genotype	Phenotype		No. of Recombinants
				Fertile	Sterile	
Rft segregating population; 'Bokjeong' × 'Bakdal sn' F_2	180	Rsc17	A	0	43	0
			B	45	0	
			H	92	0	
			Total	137	43	
		RSRF27	A	36	13	62
			B	29	6	
			H	61	20	
			No call	11	4	
			Total	137	43	
Rfo segregating population; 'OharuA' × 'Bakdal' F_2	183	Rsc17	A	42	13	75
			B	29	15	
			H	66	18	
			Total	137	46	
		RSRF27	A	1	42	5
			B	49	1	
			H	87	3	
			Total	137	46	

Table 2. *Cont.*

Population	No. of Individuals	Marker	Genotype	Phenotype		No. of Recombinants
				Fertile	Sterile	
('Bokjeong' × 'Bakdal sn' F_1) × 'Gwandong summer radish'	199	Rsc17	A	34	12	66
			B	60	17	
			H	61	15	
			Total	155	44	
		RSRF27	A	1	41	3
			B	46	0	
			H	108	2	
			No call	0	1	
			Total	155	44	

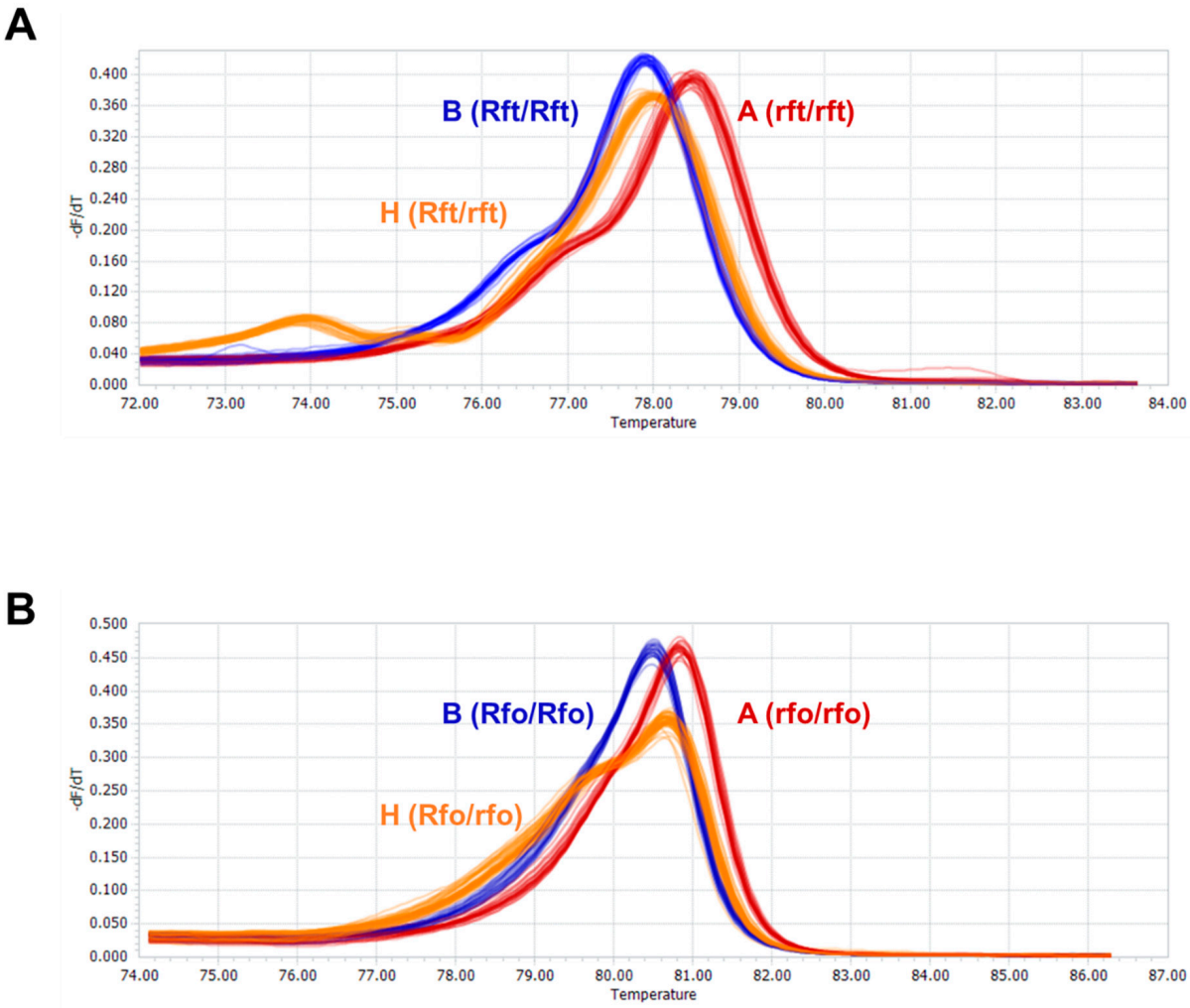

Figure 3. HRM analysis of *Rft*−linked marker Rsc17 (**A**) and *Rfo*−linked marker RSRF27 (**B**). Genotype A and B is maternal and paternal genotype, and genotype H is heterozygous fertility, respectively.

3.4. Validation of HRM Markers Linked to Rft and Rfo

From the two segregating populations for *Rft* and *Rfo*, we developed four HRM markers that can be used for genotyping restorer-of-fertility genes of Ogura CMS (Table 3). We tested the developed markers for opposite populations to validate that the two genes act independently. Genotypes of RSc15, RSb13, and RSc17, linked to *Rft*, were also polymorphic in the *Rfo* segregating population (Table S1). The RSRF27 marker linked to *Rfo* was also

segregated in the *Rft* segregating population (Table S5). However, the genotype did not match the phenotype.

Table 3. Nucleotide sequences and genes of developed HRM molecular markers for *Rfo* and *Rft* genes.

Primer		Sequence	SNP Position (bp)	Gene	Position in Gene	Function
Rft	RSc15F	TACAATCATGTGGCAAAGCACA	33,336,477	RSAskr_r1.0R5g59291	intron	PentatricoPeptide Repeat
	RSc15R	CGGAATCATCGTCTACCAGGTT				
	RSb13F	CATGAAGTGTGATTTGTATTGGT	33,340,197	RSAskr_r1.0R5	intergenic region	-
	RSb13R	GTGTCATCGTTCACTATACATTCT				
	RSc17F	ACAAGTTCGTATTGAGGAGCGT	33,366,537	RSAskr_r1.0R5g59296	exon	Leucine Rich Repeat
	RSc17R	TCAGAGAGACCATCCAAAGCTG				
	RS017F	CAATCTTGGCTGTAAACTTGTGAA	33,903,431	RSAskr_r1.0R5g59414	exon	N-acetyltransferase 9-like protein
	RS017R	TAGGAAAGGAATCTGTGTTGATGA				
Rfo	RSRF27F	TCTCAAACATACAGCTGGAAAGC	28,459,573 /28,459,588	RSAskr_r1.0R9	intergenic region	-
	RSRF27R	ACCGTCGTGTTATTGGCTACC				

These four markers were also tested in another population derived from 'Bokjeong' × 'Bakdal sn' F_1 and 'Gwandong summer radish'. This population also showed the segregation of all four markers (Table S6). *Rft*-related HRM markers were inaccurate, while the *Rfo*-related marker RSRF27 showed high concordance between phenotype and genotype (Table 2).

Although both the *Rft* and *Rfo* genes are involved in the recovery of male sterility in Ogura CMS, it was confirmed that they act independently of each other, and it was necessary to use HRM markers associated with the two genes together to accurately predict the phenotype.

4. Discussion

In previous studies, various models for fertility restoration in Ogura CMS have been proposed [4,16,32,33]. Recently, Wang et al. reported that male sterility was restored by suppressing the *orf138* gene through ORF687 of the *Rfo* gene (PPR-B) [34]. Since the first radish genome was reported in 2015 [35], it has been documented that the previously reported QTL of the *Rfo* gene exists on chromosome 9 [36], and numerous studies have been conducted, including marker development [7,14–17].

By blasting these genes, reported as *Rfo* genes of chromosome R9, against the recently published RSAskr_r1.0 whole genome, most of the genes showed high homology with the R0g01744 PPR gene of the unassembled R0 chromosome. These genes showed lower homology with the R9g87877 gene, which is close to the R9 chromosomal QTL. However, it cannot be definitively concluded that the previously reported genes are not located on the R9 chromosome. This is because the assembly of the latest genome is still ongoing, and there is the possibility of further improvements in the future. In the case of the *Rft* gene, two RAPD fragments (AB458520 and AB458521) linked to the previously reported *Rft* gene show similar homology to the R5g59372 and R5g59315 genes of the R5 chromosome [16] (Table 3). However, this also differs from the PPR gene of R5g59291 and the LLR gene of R5g59296, which were identified as *Rft* QTLs in our experiments.

The original purpose of blasting the existing *Rfo* genes against the reference genome (RSAskr_r1.0) was to search for homologous PPR genes on the R5 chromosome to identify them as *Rft* genes. Through BLAST, we confirmed the presence of PPR genes, specifically R5g59289, 59291, and 59315, on chromosome R5. However, the gene showing the highest similarity to the known *Rfo* genes was not R9g87877 on the R9 chromosome but rather R0g01744 on the unassembled R0 chromosome. We conducted SNP verification for these two regions and developed HRM markers based on them. The marker from the R0 chromosome exhibited inconclusive results, while the marker from the R9 chromosome provided precise and accurate outcomes.

The genotyping results of this study showed the independent actions of *Rfo* and *Rft* in male sterility recovery. By crossing two F_1 plants heterozygous for *Rft* and *Rfo*, it was discovered that male sterility was restored by the *Rfo* gene, regardless of the presence of

the *Rft* gene. Conversely, as seen in the *Rft* segregating population, the recovery of male sterility was determined by the *Rft* genotype regardless of the genotype of the *Rfo* gene (Tables 2 and S1). A very complex explanation was made through various hypotheses to discriminate Ogura CMS recovery. According to Yamagishi et al. [37], the interpretation of male sterility recovery in radish using only the *Rfo* and *Rft* genes is not complete. They suggest that an *Rf* gene other than *Rfo* and *Rft* is present in radishes. The new Rf gene named *Rfs* processes *orf138* mRNA at a different position from that of *Rft*.

These various interpretations are considered to result from the lack of precise markers for the *Rfo* gene, and to supplement the incomplete interpretation of results, the introduction of other male sterility recovery genes has been considered. Additionally, these efforts have provided an opportunity to discover other *Rf* genes, leading to the identification of the *Rft* gene. The *Rfo* gene markers we developed can contribute to a clearer understanding of the relationship between *Rft* and *Rfo* genes. In future research related to Ogura CMS, the marker for the *orf138* gene could be a valuable reference for conducting deeper investigations into whether it is associated with the *Rft* gene or if other genetic factors are involved.

Our primary goal was to develop markers for discriminating the restorer-of-fertility genes in Ogura CMS. We could develop four markers related to *Rft* and *Rfo* that can be used as a set. In previous studies, sequence tagged site (STS) markers were used to map the *Rft* locus [16], and cleaved amplified polymorphic sequence (CAPS) markers were developed to genotype functional *Rfo* [38]. These markers are used with gel electrophoresis and can be replaced with a marker system using fluorescent dye to increase the efficiency of genotyping. Therefore, based on the recent high-quality reference genome [29], we designed HRM markers based on SNPs.

From the fine-mapping of the *Rft* locus, we observed that the highly correlated SNP was near the PPR, LLR, and N-acetyltransferase 9-like protein genes (Table 3). As the target region consisted of repetitive sequences, further analysis will be needed to clone the gene and speculate on the mechanism underlying male sterility recovery.

5. Conclusions

This study successfully developed high-resolution melting (HRM) markers associated with the *Rft* and *Rfo* genes, major restorer-of-fertility genes in Ogura cytoplasmic male sterility (CMS) in radish. Genetic mapping using F2 populations enabled the identification of these loci through SNP-based genotyping and linkage grouping. The study presents a set of 4 HRM markers for the *Rft* gene on chromosome 5 and one HRM marker for the *Rfo* gene on chromosomes 9. These markers demonstrate high accuracy in genotyping and contribute valuable insights into male sterility restoration mechanisms in Ogura CMS, offering practical utility in radish breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14010043/s1, Table S1. Phenotype and genotype of *Rft* segregating population. Table S2. SNPs detected on the *Rft* and *Rfo* locus. Table S3. Analysis results of phenotype/genotype discrimination using HRM markers associated with the *Rft* gene. Table S4. Analysis results of phenotype/genotype discrimination using HRM markers associated with the *Rft* gene. Table S5. Phenotype and genotype of *Rft* & *Rfo* segregating population. Table S6. Validation of developed markers in segregating population derived from maternal line (*Rft/rft*) and paternal line (*Rfo/rfo*). TableS7. *Rft* primer list. TableS8. *Rfo* primer list. Figure S1. HRM analysis results using RealTime-PCR for 14 pairs of HRM markers associated with the *Rft* gene. Left peak: B(Rf/Rf), midle peak: H(Rf/rf), right peak: A(rf/rf).

Author Contributions: Conceptualization, K.H., J.K. and H.Y.P.; methodology, H.-B.Y., E.S.L. and Y.-R.L. software, H.-B.Y.; investigation, H.-I.A. and E.S.L.; resources, J.K. and H.Y.P.; data curation, K.H. and Y.-R.L.; writing—original draft, H.-I.A. and K.H.; supervision, H.Y.P. and D.-S.K.; project administration, D.-S.K. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the National Institute of Horticultural and Herbal Science, Rural Development Administration, Republic of Korea (Project No. PJ01504002).

Data Availability Statement: The data presented in this study are available in Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

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