Identification of *Ms2*, a Novel Locus Controlling Male-fertility Restoration of Cytoplasmic Male-sterility in Onion (*Allium Cepa* L.), and Development of Tightly Linked Molecular Markers

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Research Article

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Abstract

Cytoplasmic male-sterility (CMS) has been exclusively used to produce F_1 hybrid seeds of onion (*Allium cepa* L.). A single nuclear locus, *Ms*, is known to restore male-fertility of CMS in onions. Unstable male-sterile onions producing a small amount of pollen grains have been identified in a previous study. When such unstable male-sterile onions were crossed with stable male-sterile onions containing CMS-T cytoplasm, male-fertility was completely restored, although genotypes of the *Ms* locus were homozygous recessive. Inheritance patterns indicated that male-fertility restoration was controlled by a single locus designated as *Ms2*. A combined approach of bulked segregant analysis and RNA-seq was used to identify candidate genes for the *Ms2* locus. High resolution melting (HRM) markers were developed based on single nucleotide polymorphisms (SNPs) detected by RNA-Seq. Comparative mapping of the *Ms2* locus showed that *Ms2* was positioned at the end of chromosome 2 with a distance of approximately 70 cM away from the *Ms* locus. Although 38 contigs containing reliable SNPs were analyzed using recombinants selected from 1,344 individuals, no contig showed perfect linkage to *Ms2*. Interestingly, transcription levels of *orf725*, a CMS-associated gene in onions, were significantly reduced in male-fertile individuals of segregating populations. However, no significant change in its transcription level was observed in individuals of a segregating population with male-fertility phenotypes determined by the *Ms* locus, suggesting that male-fertility restoration mechanism of *Ms2* might be different from that of the *Ms* locus.

Introduction

Male-sterility is an inability of producing functional pollen grains in plants. Any critical defects from initial stages of male gametophyte development to anther dehiscence can result in male-sterility (Wan et al. 2019). Gynodioecy attributed by male-sterility is a widespread reproductive strategy in flowering plants. Approximately 7% of angiosperm species are known to show gynodioecy (McCauley and Bailey 2009). Depending on genomic positions of causal genes, male-sterility is classified as genic male-sterility (GMS) and cytoplasmic male-sterility (CMS). Genes inducing GMS and CMS are located in nuclear and mitochondrial genomes, respectively (Chen and Liu 2013).

CMS is caused by abnormal mitochondrial genes and thus is mostly maternally inherited in plants. Plant mitochondrial genomes show exceptional variations of size and structure (Chen et al. 2017). In contrast to small and circular animal mitogenomes (14–20 kb), plant mitogenomes vary in size from 66 kb for *Viscum scurruloideum* (Skippington et al. 2015) to 11.3 Mb for *Silene conica* (Sloan et al. 2012). Size variations occur even in the same species (Sloan et al. 2010). Complexity of genome structures is another feature of plant mitogenomes. Linear, circular, and branched forms of genome structures have been reported (Backert et al. 1997; Oldenburg and Bendich 2001; Sloan 2013).

Multipartite structures and sublimons are generally observed in plants. They make plant mitogenomes even more complex. A master circle of plant mitogenomes is generally divided into multipartite of subcircles. Besides a mater circle, sublimons present at low copy numbers have been reported in many

plant species (Fauron et al. 1995; Satoh et al. 2004; Allen et al. 2007; Kitazaki and Kubo 2010; Chen et al. 2017). Abundant repeat sequences distributed in plant mitogenomes are known to be responsible for dynamic rearrangements of plant mitogenomes (Small et al. 1989; Albert et al. 1998; Kmiec et al. 2006; Woloszynska and Trojanowski 2009). CMS-associated genes cloned so far are mostly created by repeat-mediated mtDNA rearrangements. Frequently, CMS-associated genes are chimeric genes composed of partial sequences of known mitochondrial genes and unknown sequences (Hanson and Bentolila 2004; Chen and Liu 2013; Chen et al. 2017).

Sublimons present at a substoichiometric level sometimes become sources of new variations (Sakai and Imamura 1993; Bellaoui et al. 1998; Janska et al. 1998; Arrieta-Montiel et al. 2001). Copy number of sublimons can be infrequently increased by a substoichiometric shifting (SSS) mechanism (Bellaoui et al. 1998; Janska et al. 1998; Sandhu et al. 2007). Although the origin and maintenance of sublimons are not clearly resolved yet, tissue culture (Kanazawa et al., 1994) and nuclear genes such as *Msh1* (Abdelnoor et al. 2003), *OSB1* (Zaegel et al. 2006), and *RecA* (Shedge et al. 2007) are known to trigger SSS. These nuclear genes are likely to control specific stoichiometry of sublimons throughout generations. CMS phenotypes can appear by SSS of sublimons harboring CMS-associated genes (Arrieta-Montiel et al. 2001; Sandhu et al. 2007).

Meanwhile, male-sterility caused by CMS-associated genes can be suppressed by nuclear restorer-of-fertility (Rf) genes. Majority of Rf genes isolated so far encode pentatricopeptide repeat (PPR) proteins (Gaborieau et al. 2016). PPR-coding genes consist of a large family in plants. Most of them are known to regulate gene expression in organelles at the transcription level (Barkan and Small 2014; Manna 2015). In addition, Rf genes encoding other proteins such as aldehyde dehydrogenase (Cui et al. 1996), putative acyl-carrier proteins (Fujii and Toriyama 2009), glycine-rich proteins (Hu et al. 2012), and peptidases (Kitazaki et al. 2015) have been reported.

CMS is an ideal topic for studying commutation between nucleus and mitochondrion. It has also been utilized in commercial production of F_1 hybrid seeds in many crops (Bohra et al. 2016). In the case of onion, CMS is a unique emasculation tool for F_1 hybrid breeding. Two major types of CMS have been reported in onions. A male-sterile (MS) onion named as CMS-S was first discovered by Jones and Emsweller (1936). Inheritance patterns indicated that male-fertility of CMS-S could be restored by a single Rf locus designated as *Ms* (Jones and Clarke 1943). Another type of CMS called CMS-T was reported by Berninger (1965). Three independent Rf loci have been assumed to be involved in male-fertility restoration (Schweisguth 1973).

A chimeric mitochondrial gene, *orf725*, composed of partial sequences of *coxI* and unknown sequences has been suggested as a CMS-associated gene in onions (Kim et al. 2009). Comparative analysis of complete mitogenomes of normal, CMS-S, and CMS-T supports a role of *orf725* in CMS induction. *orf725* was present in both CMS-S and CMS-T cytoplasm (Kim et al. 2016, 2019b). In conflict with Schweisguth (1973), our previous study (Kim 2014) has shown that male-fertility of both CMS-S and CMS-T can be restored by the common *Ms* locus. Many molecular markers tagging the *Ms* locus have been developed

(Gökçe and Havey 2002; Bang et al. 2013; Park et al. 2013; Yang et al. 2013; Havey 2013; Kim 2014; Huo et al. 2015; Kim et al. 2015a). Some of them show perfect linkage disequilibrium with the *Ms* locus (Kim 2014; Huo et al. 2015; Kim et al. 2015a).

In addition to the *Ms* locus, a novel Rf locus was identified in this study from onion populations in which the *Ms* locus was fixed as a homozygous recessive genotype. A combined approach of bulked segregant analysis (BSA, Michelmore et al. 1991) and RNA-seq was used to develop tightly linked molecular markers and to determine the chromosomal location of this novel Rf locus. Interaction between *Ms* and the novel loci and the mechanism of male-fertility restoration by the novel Rf locus were also discussed in this study.

Materials And Methods

Plant materials

Onions showing unstable MS phenotypes in our previous study (Kim et al. 2019a) were used. Two of them in the OP_2 population were crossed with onions showing stable MS phenotypes and containing CMS-T cytoplasm. Two resulting populations were designated as TUMS4 and TUMS9, respectively. Male-fertile (MF) plants in these two populations were self-pollinated or cross-pollinated with MS plants in the same populations to produce large-sized segregating populations. A detail pedigree of onion populations used in this study is depicted in Supplementary Fig. 1.

Seedlings germinated in 128-cell plug trays were transplanted into pots in the greenhouse or fields of Chonnam National University, Gwangju, Republic of Korea (35° 17´ N, 126° 90´ E). Floral induction was initiated naturally during winter seasons. Male-fertility phenotypes were determined by visual examination in June for three years (2018–2020). Statistics of temperatures during flowering seasons are summarized in Supplementary Table 1. Morphologies of anthers and pollen grains were examined using a dissecting microscope (Stemi 2000-C; ZEISS, Oberkochen, Germany) and a scanning electron microscope (SEM, JSM-IT300; JEOL, Tokyo, Japan), respectively.

RNA extraction, RNA-Seq analysis, and real-time RT-PCR

To perform a combined analysis of BSA and RNA-seq, flower buds of 10 MF and 10 MS individuals of the TUMS4 population were pooled, respectively. Total RNAs were extracted from each bulked sample using an RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). In the first RNA-Seq analysis, diverse sizes of flower buds larger than 3 mm were used. Flower buds smaller than 3 mm were included in the second analysis to cover early stages of male gametophyte development. The third analysis was performed using the TUMS4-S1 population after removing heterozygous MF individuals. Genotypes of heterozygous individuals were predicted using tightly linked molecular markers developed in this study.

RNA-Seq analyses were performed by a specialized company (Phyzen Genomics Institute, Seoul, Republic of Korea). Transcriptomes were sequenced using a HiSeq X Ten platform (Illumina, Hayward, CA, USA). Trimmed reads were separately mapped to two reference transcriptomes (Kim et al. 2015b; Sohn et al.

2016). Unmapped reads were pooled and *de novo* assembled into contigs using Trinity software (Haas et al. 2013). Identification of single nucleotide polymorphisms (SNPs) between bulked RNAs and quantification of transcription levels of contigs were carried out using SAMTools (Li et al. 2009) and RSEM software (Li and Dewey 2011), respectively.

To estimate transcription levels of *orf725*, real-time RT-PCRs were performed. Total RNAs were extracted from flower buds of five MF and five MS plants of the TUMS4 population using an RNeasy Plant Mini Kit (QIAGEN). Total RNAs extracted in the previous study (Kim et al. 2015a) from MF and MS F_2 individuals with male-fertility phenotypes controlled by the *Ms* locus were also used for comparison of transcription levels. cDNAs were synthesized using a cDNA synthesis kit (SuperScript™ III first-strand synthesis system for RT-PCR, Invitrogen, Carlsbad, CA, USA).

Real-time RT-PCRs were carried out using SYBR® Green Realtime PCR Master Mix (Toyobo Co. Ltd, Osaka, Japan) and a LightCycler®96 Real-Time PCR system (Roche Molecular Systems, Pleasanton, CA, USA) according to manufacturers' instructions with four technical replicates. cDNAs diluted 100-fold were then used as templates. Onion *nad6* gene positioned in the master circle of mitochondrial genome (GenBank accession: KU318712) was used as an internal control. Primer sequences used for real-time RT-PCR are shown in Supplementary Table 2.

DNA extraction, PCR amplification, and high-resolution melting (HRM) marker analysis

Total genomic DNAs of segregating populations were extracted from leaves or flower peduncle tissues using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). For sequencing of contigs, PCR amplifications were performed in a 25 μL reaction mixture containing 0.1 μg template, 2.5 μL 10x PCR buffer, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.5 μL dNTPs (10 mM each), and 0.25 μL polymerase mix (Advantage 2 Polymerase Mix, Takara Bio, Shiga, Japan). PCR amplification consisted of an initial denaturation step at 95°C for 4 min, 10 cycles at 95°C for 30 s, 65°C (0.8°C decrements in each cycle) for 30 s, and 72°C for 1 min, 35 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min, and a final 10 min extension at 72°C. After confirming successful PCR amplification by visualizing on 1.5% agarose gels after ethidium bromide staining, PCR products were purified for sequencing using a QIAquick PCR Purification kit (QIAGEN). Sequencing was performed by a specialized company (Macrogen, Seoul, Republic of Korea). Regarding HRM and cleaved amplified polymorphic sequence (CAPS) markers, detail procedures were described in previous studies (Kim and Kim 2019; Cho et al. 2021). Primer sequences of molecular markers are shown in Supplementary Table 2.

Results

Identification of a novel locus responsible for fertility restoration of male-sterility caused by CMS-T cytoplasm in onions

An unstable MS phenotype producing a small amount of viable pollen grains was identified from open-pollinated (OP) populations derived from PI273626 in a previous study (Kim et al. 2019a), although *Ms*

genotypes of unstable MS plants were homozygous recessive. These OP populations and PI273626 contained the CMS-Y cytoplasm designated by Kim et al. (2019a). To investigate whether such an unstable MS phenotype occurred in other types of cytoplasm, two plants (Plants #4 and #9) showing unstable MS phenotypes were crossed with stable MS onions containing the CMS-T cytoplasm (Supplementary Fig. 1).

MF and MS plants segregated at a ratio of one to one in progeny populations (TUMS4 and TUMS9) of these two crosses (Table 1). In the case of MF onions (Figs. 1A and 1C), male-fertility phenotypes were completely normal with plenty of pollen grains. However, no pollen was detected in MS plants (Figs. 1B and 1D). Pollen shapes of MF plants were also normal with few deformed pollen grains (Supplementary Fig. 2), whereas many deformed pollen grains were observed in unstable MS plants (Kim et al. 2019a). Genotypes of six molecular markers showed almost perfect linkage disequilibrium with the *Ms* locus, confirming that genotypes of both MF and MS plants were all homozygous recessive for the *Ms* locus (Supplementary Fig. 3). These results indicated that male-fertility of these populations was restored by another novel Rf locus, not the *Ms* locus. Single-gene inheritance patterns of male-fertility restoration were also observed in successive generations (Table 1). This novel Rf locus identified in this study is designated as *Ms2*.

Table 1
Inheritance patterns of male-fertility phenotypes controlled by the *Ms2* locus in segregating populations

Population[a]	Expected genotype	Male-fertility phenotype[b]		Total	χ^2	*P*
		MF	MS			
TUMS4	*ms2ms2* × *Ms2ms2*	20	19	39	0.026	0.87
TUMS9	*ms2ms2* × *Ms2ms2*	104	114	218	0.46	0.50
TUMS4-S1	*Ms2ms2* selfing	164	64	228	1.15	0.28
TUMS4-S3	*Ms2ms2* selfing	309	98	407	0.18	0.67
TUMS4-C1	*ms2ms2* × *Ms2ms2*	148	157	305	0.27	0.61
TUMS4-C9	*ms2ms2* × *Ms2ms2*	204	200	404	0.040	0.84

[a]Pedigree of populations is depicted in Supplementary Fig. 1.

[b]MF: Male-fertile; MS: Male-sterile.

Identification of candidate genes responsible for the Ms2 **locus and development of molecular markers tightly linked to the** Ms2 **locus**

To identify candidate genes responsible for the *Ms2* locus, combined analyses of BSA and RNA-seq were carried out using bulked RNAs of MF and MS plants of the TUMS4 population. RNA-seq analyses were performed twice using the same population for repetition. Approximately 6 Gb raw reads were produced from each bulked RNA. Sequences of raw reads were deposited to the SRA database (Supplementary Table 3). After trimming low quality sequences, trimmed reads were mapped to the reference transcriptome (Supplementary Table 4). Approximately 80% trimmed reads were mapped to the reference transcriptome (Supplementary Table 5).

Using a customized screening process (Supplementary Table 6), 60 contigs containing SNPs between MF and MS bulked RNAs were selected after visual investigation of SNPs using an IGV viewer software (Robinson et al. 2011). Among these contigs, one contig (AC.Combine.Locus_14688.6) was mapped to the end of chromosome 2 in a previous study (Cho et al. 2021). In addition, four contigs showed high homologies with corresponding contigs located at the chromosome 2 of another linkage map (Supplementary Table 7). These results suggested that the *Ms2* locus was likely to be positioned at the end of chromosome 2. Eight HRM markers were developed to construct an *Ms2*-flanking linkage map using contigs screened in this study or contigs showing high homologies with the loci mapped by Duangjit et al. (2013). Since the *Ms* locus was also positioned at chromosome 2, previously developed markers linked to the *Ms* were included to compare their chromosomal positions. Linkage maps indicated that the *Ms2* locus was positioned at the end of chromosome 2 and linked to the *Ms* locus with a distance of approximately 70 cM (Fig. 2).

To identify more candidate genes, additional RNA-seq analysis was performed after removing heterozygous MF individuals from MF bulked RNA (Supplementary Table 3). Trimmed reads produced from three RNA-seq analyses were also mapped to another reference transcriptome (Sohn et al. 2016). Trimmed reads were also mapped to the *de novo* assembled transcriptome (Supplementary Tables 4 and 5). Pooled reads which were unmapped to the reference transcriptome (Kim et al. 2015b) from three RNA-Seq analyses were used for *de novo* assembly of contigs.

Using a less stringent screening process (Supplementary Table 8), contigs containing homozygous SNPs between bulked RNAs were selected. Among 164 screened contigs, 38 contigs in which SNPs were clearly supported by sufficient read depths were selected for developing HRM markers and identifying candidate genes (Supplementary Table 9). To determine linkage relationship of these selected contigs and the *Ms2* locus, a total of 30 recombinants between H89571 and H56318 were identified from 1,344 individuals of four segregating populations (Supplementary Table 10). H53996 and H36898 markers flanked the *Ms2* locus most tightly. However, no contig showing perfect linkage to the *Ms2* locus was identified (Supplementary Table 10).

Analysis of differentially expressed genes (DEGs) between bulked RNAs and effect of dominant Ms2 allele on transcription of orf725

Overall transcription levels of all contigs in three types of transcriptomes were similar between MF and MS bulked RNAs, showing correlation coefficients of more than 0.8 in all cases (Supplementary Fig. 4).

Using a stepwise screening process (Supplementary Table 11), a total of 204 contigs were selected as DEGs between bulked RNAs (Supplementary Table 12). No plausible candidate genes such as PPR-coding genes were identified. Many contigs seemed to be involved in general processes of male gametophyte development. For this reason, the majority of DEGs selected in this study showed reduced transcription in MS bulked RNA previously isolated from a F_2 population (Kim et al. 2015a) in which male-fertility phenotypes were determined by the *Ms* locus (Fig. 3).

Interestingly, amounts of transcripts of *orf725*, the causal gene for CMS induction in onions, were reduced in MF bulked RNAs of all three RNA-Seq analyses, whereas a higher amount of *orf725* transcripts was observed in MF bulked RNAs isolated from the F_2 population controlled by the *Ms* locus (Fig. 4A). Real-time RT-PCRs were carried out to confirm reduced transcription of *orf725* in MF plants. Reduced transcripts of *orf725* were observed in all five MF individuals, while no significant difference was observed in F_2 individuals in which male-fertility was controlled by the *Ms* locus (Figs. 4B and 4C). These results suggested that male-fertility might be restored by different mechanisms depending on Rf loci in onions.

Discussion

Discovery of a novel Rf locus in onion and its implication in onion F_1 hybrid breeding

A novel Rf locus was identified from OP populations derived from PI273626 in this study. Clear single-gene inheritance patterns and reliable positioning of the *Ms2* locus at the end of chromosome 2 proved existence of a novel Rf locus in addition to the *Ms* locus. Based on complex segregation ratios, Schweisguth (1973) has suggested involvement of three independent Rf loci in fertility restoration of male-sterility conferred by CMS-T cytoplasm. However, this is the first report about the discovery of the second onion Rf locus with specific chromosomal position revealed by constructing a linkage map. Since genotypes of the *Ms* locus were fixed as homozygous recessive in all segregating populations used in this study and their genotypes could be confirmed by multiple molecular markers tagging the *Ms* locus (Supplementary Fig. 3), segregation of male-fertility controlled by the *Ms2* locus could be successfully identified in this study.

Considering the fact that male-fertility phenotypes of numerous onion breeding lines in Korea and probably other countries could be perfectly predicted by molecular markers showing linkage disequilibrium with the *Ms* locus (Kim 2014; Kim et al. 2015a), existence of the *Ms2* locus was somewhat unexpected. Since male-fertility phenotypes of the S_1 population produced by self-pollination of PI273626 were perfectly matched with genotypes of molecular markers tagging the *Ms* locus and that unstable male-sterility was identified from a single plant in the OP_1 population (Kim et al. 2019a), a dominant *Ms2* allele might have been introduced from other unknown exotic germplasm.

Unlike breeding lines used in Korea, it was assumed that frequency of dominant *Ms2* allele might be higher in exotic onion germplasm. Khar and Saini (2016) and Ferreira and Santos (2018) have reported

that some populations show discrepancy between genotypes of molecular markers tagging the *Ms* locus and phenotypes of male-fertility in Indian and Brazilian onion germplasm, respectively. Such discrepancies observed in Indian and Brazilian accessions might be caused by presence of the *Ms2* locus. Further studies are needed to clarify this. Considering the fact that unstable male-sterility was attributable to a relatively low expressivity of the *Ms2* locus as observed in a previous study (Kim et al. 2019a), it is desirable to eradicate the dominant *Ms2* allele among breeding lines for efficient deployment of marker-assisted selection of the *Ms* locus, the major Rf locus in onions. Molecular markers tagging the *Ms2* locus developed in this study could be efficiently used for this purpose.

Mechanism underlying male-fertility restoration by the Ms2 locus and factors affecting the function of Ms2

Inheritance patterns of male-fertility in segregating populations showed that both *Ms* and *Ms2* loci were redundantly involved in fertility restoration in onions. When genotypes of both Rf loci were homozygous recessive, stable male-sterility was observed, although the efficacy of the *Ms* locus might be higher than that of *Ms2* locus. In a previous study (Kim et al. 2019a), stable male-fertility was observed when at least one dominant *Ms* allele was present. However, an unstable MS phenotype was detected when *Ms* and *Ms2* genotypes were homozygous recessive and heterozygous, respectively.

Genetic redundancy is typically arisen by gene duplication. It is widespread in higher organisms (Pickett and Meeks-Wagner 1995; Nowak et al. 1997; Kafri et al. 2009). Whether causal genes for *Ms* and *Ms2* loci are paralogs is not resolved yet. Mechanisms by which male-fertility is restored might be different from each other. Unlike the *Ms* locus which did not affect transcription of *orf725*, transcripts of *orf725* were significantly reduced in MF F_2 individuals (Fig. 4). The causal gene for *Ms2* might be able to restore male-fertility by suppressing transcription of *orf725* or degrading transcripts. Suppression of transcription of CMS-associated genes by Rf genes is frequently observed in other crops (Kennell and Pring 1989; Gagliardi and Leaver 1999; Menassa et al. 1999; Tang et al. 1999; Wang et al. 2006).

One interesting feature of male-fertility restored by the *Ms2* locus was that the stability of male-fertility might be affected by cytoplasm types. Unstable male-sterility was observed in plants of OP_1 and OP_2 populations containing the CMS-Y cytoplasm. Many deformed pollen grains were observed in these unstable MS plants (Kim et al. 2019a). However, male-fertility of plants containing the CMS-T cytoplasm and homozygous recessive *Ms* genotype was stably restored by the *Ms2* locus. Since male-sterility conferred by the CMS-S cytoplasm was known to be more stable and more commonly used in F_1 hybrid breeding than CMS-T male-sterility (Havey 2000), the effect of the *Ms2* locus on male-fertility phenotypes of plants containing CMS-S cytoplasm needs to be analyzed in the future. In addition, more diverse accessions need to be analyzed to elucidate whether stability of male-fertility is caused by types of cytoplasm or other nuclear gene(s).

In addition, effects of environmental factors cannot be excluded. In particular, temperature might affect the stability of male-fertility controlled by the *Ms2* locus. The effect of temperature on male-fertility

phenotypes of onions has been previously reported (Barham and Munger 1950; van der Meer and van Bennekom 1969). Stability of male-sterility was high at a low temperature (14°C). However, viable pollen grains appeared at a high temperature (23°C). The degree of stability varied depending on populations. Male-sterility of two hybrids introduced from the United States was hardly affected by temperature in comparison to European accessions (van der Meer and van Bennekom 1969). Since vulnerability to temperature might vary depending on populations, genetic factors such as *Ms2* locus might be related to temperature sensitivity of male-sterility in onions.

Effects of interaction between genetic factors and temperature on male-fertility have been reported in rice (Chen et al. 2007; Zhou et al. 2012) and wheat (Song et al. 2013). They have been utilized to implement a two-line hybrid system. Although most thermo-sensitive male-sterility are GMS, a thermo-sensitive CMS line has also been developed in wheat (Song et al. 2013). Similarly, if interaction between *Ms2* and temperature was elucidated, it could be used to establish a two-line system in onions. However, it would be an ideal strategy to eradicate the dominant *Ms2* allele from breeding populations since other unknown factors might also affect the stability of male-fertility controlled by the *Ms2* locus. Indeed, seven plants showing unstable MF or MS phenotypes were observed in populations analyzed in this study. These plants showing unstable phenotypes were identified by discrepancy of phenotypes and genotypes of molecular markers tightly flanking the *Ms2* locus (data not shown). Taken together, results presented in this study could be used to develop an optimal strategy for F_1 hybrid breeding of onions.

Declarations

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Authors' contribution

Nari Yu performed experiments and drafted the manuscript. Sunggil Kim organized and coordinated this research project and edited the final manuscript.

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Ethics approval

All experiments were performed in compliance with current laws of the Republic of Korea.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interest.

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Figures

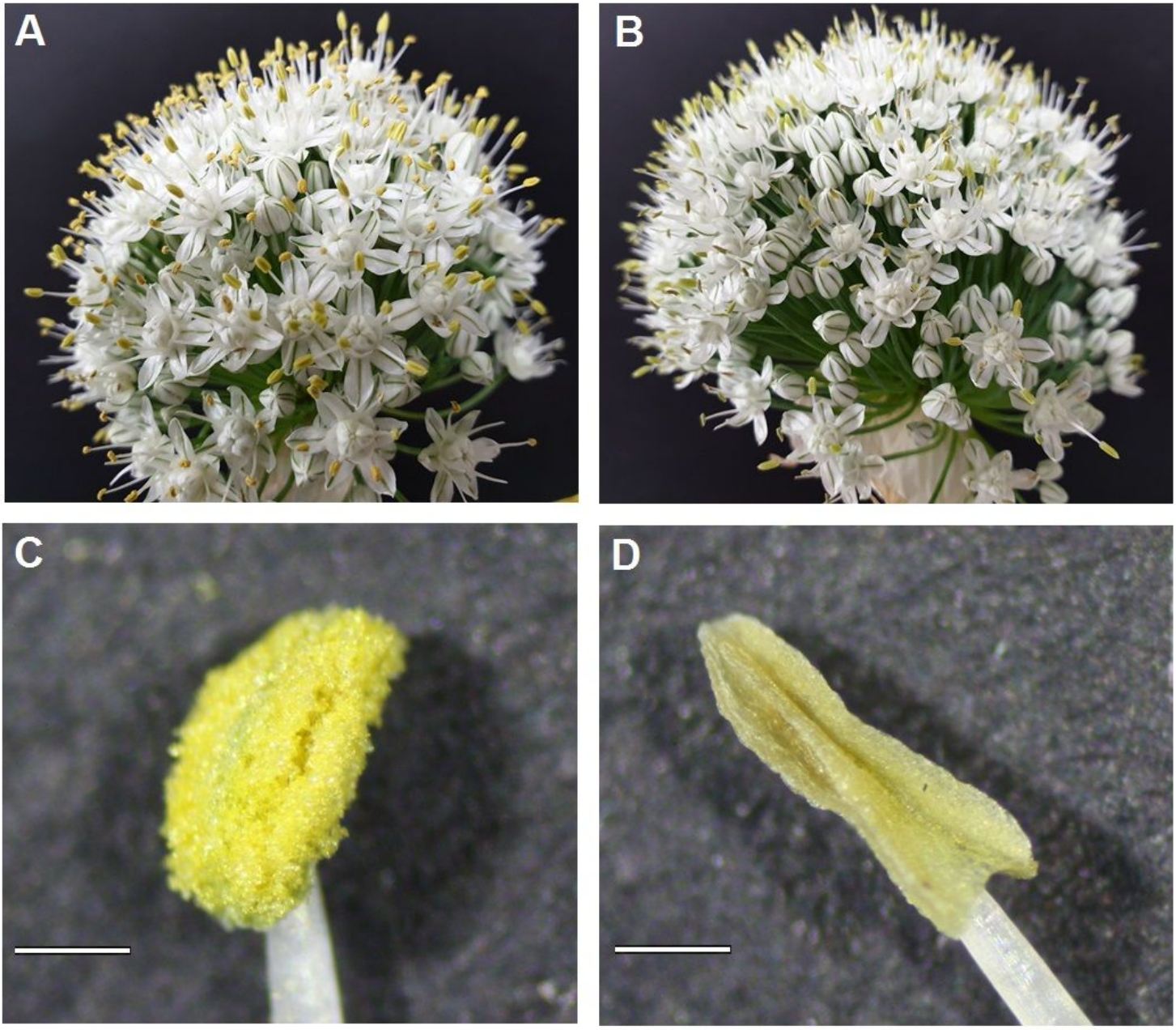

Figure 1

Morphologies of umbels and anthers of male-fertile and male-sterile onions in the TUMS4 population. A. Male-fertile umbel, B. Male-sterile umbel, C. Male-fertile anther, D. Male-sterile anther. Scale bars in images of anthers indicate 0.5 mm.

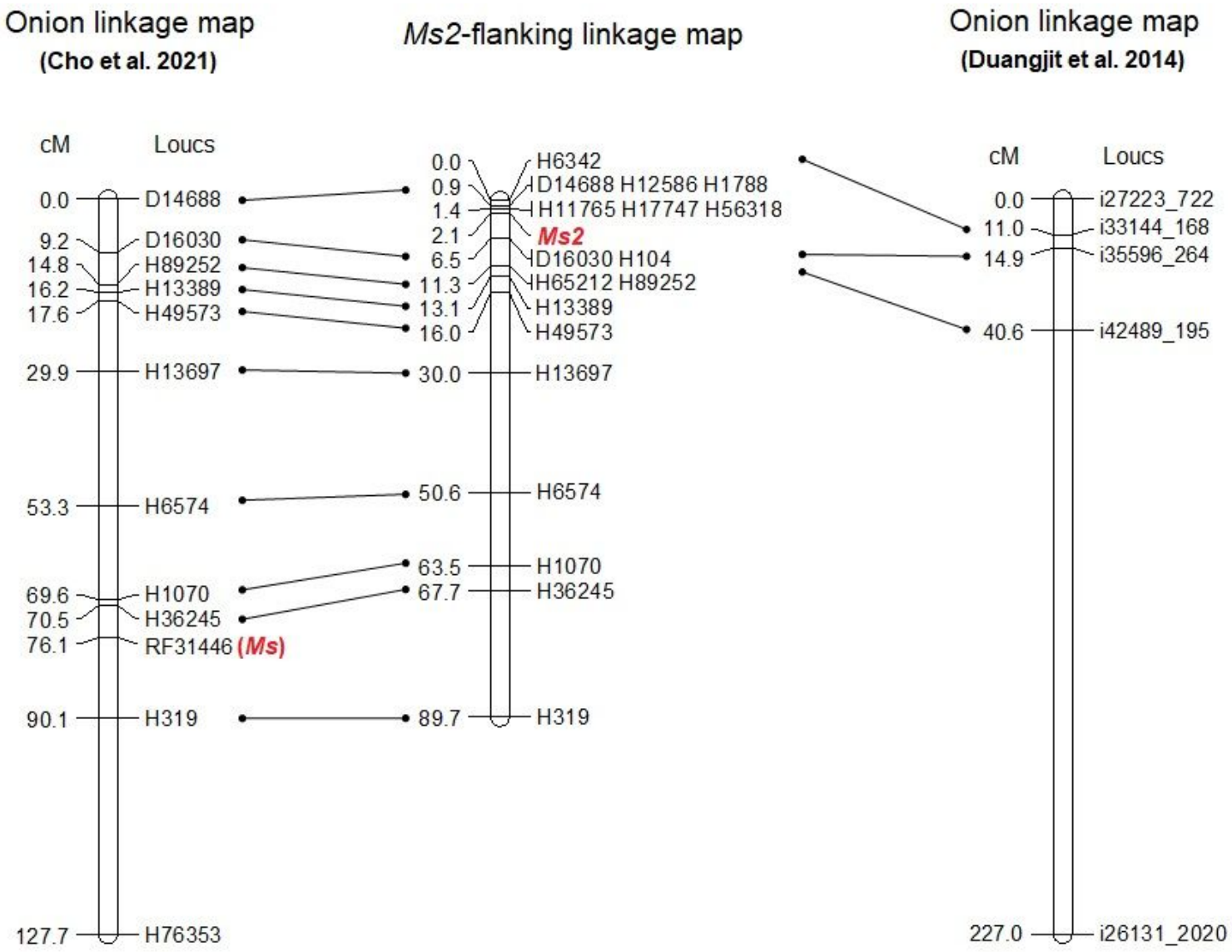

Figure 2

Onion linkage maps flanking the Ms2 locus. Left: Linkage map of chromosome 2 constructed by Cho et al. (2021); Center: Ms2-flanking linkage map constructed in this study; Right: Linkage map of chromosome 2 constructed by Duangjit et al. (2013). Homologous and identical contigs or markers are connected with horizontal lines. Ms and Ms2 loci are shown as red boldfaces.

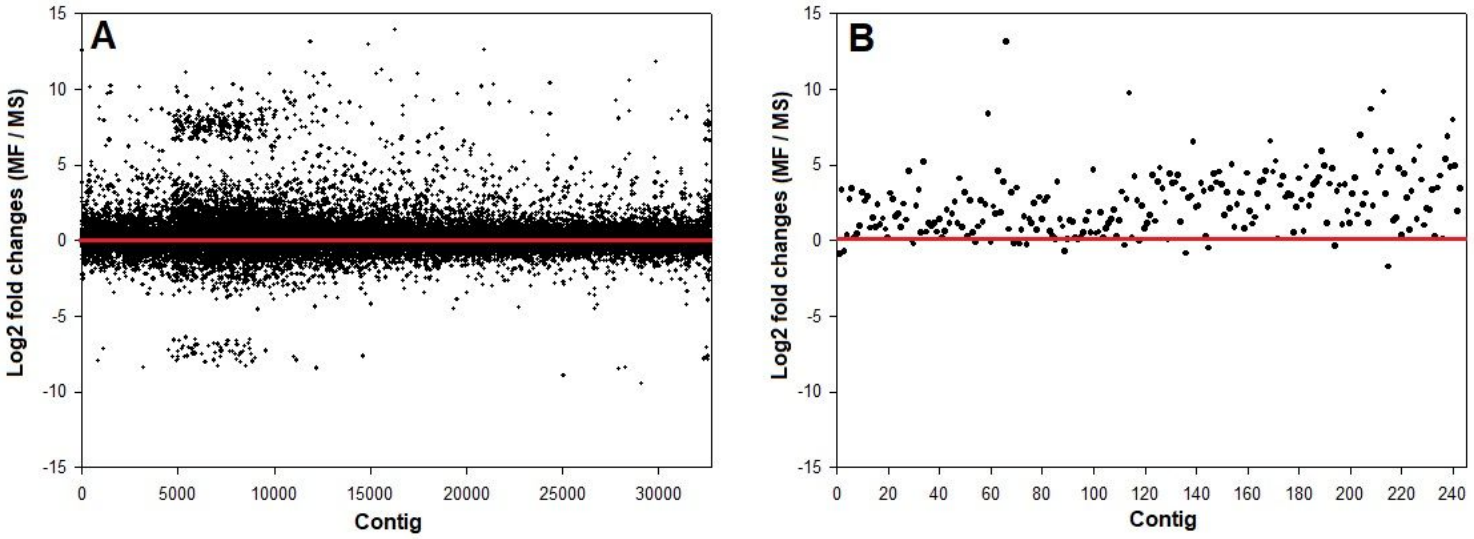

Figure 3

Fold changes of transcription levels of contigs between male-fertile and male-sterile bulked RNAs isolated in a previous study (Kim et al. 2015a) from the F2 population in which male-fertility phenotypes were controlled by the Ms locus. A. Fold changes of all contigs. These contigs were de novo assembled by Kim et al. (2015a). B. Fold changes of selected contigs showing high homologies with DEGs selected in this study (Supplementary Table 12). Red lines indicate equal levels of transcription between male-fertile and male-sterile bulked RNAs.

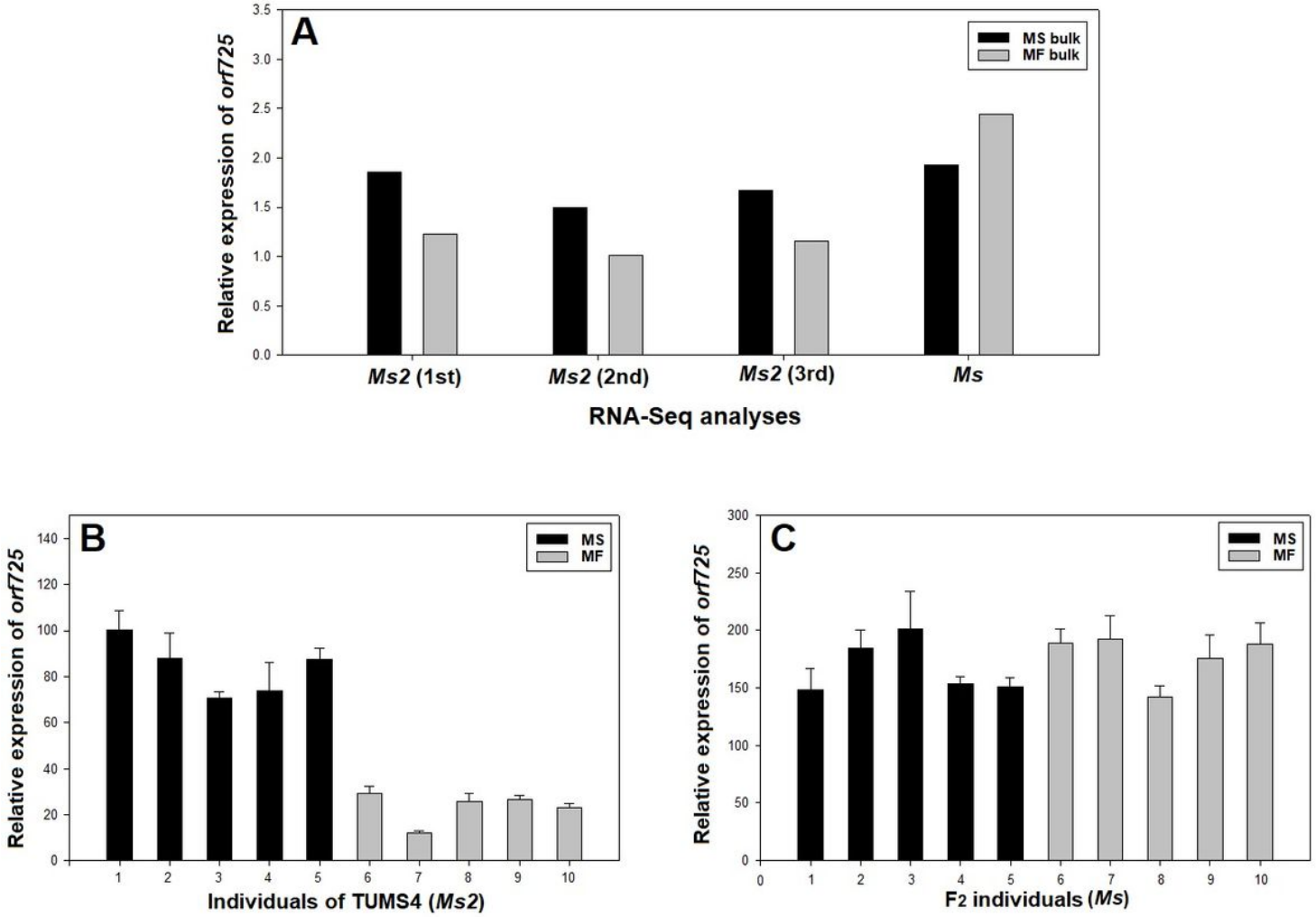

Figure 4

Comparison of relative expression levels of orf725 between male-fertile and male-sterile individuals of populations in which male-fertility phenotypes were determined by Ms and Ms2 loci, respectively. Expression levels of nad6, a mitochondrial gene, were used as internal controls. A. Relative expression of orf725 estimated using data from RNA-Seq analyses. B. Relative expression levels of orf725 in individuals of the TUMS4 population. C. Relative expression levels of orf725 in F2 individuals of the population produced in a previous study (Kim et al. 2015a).

Supplementary Files

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- SupplementaryFig.1.Pedigree.tif
- SupplementaryFig.2.SEMofpollengrains.tif
- SupplementaryFig.3.GenotypesofMslinkedmarkers.tif
- SupplementaryFig.4.Correlationofexpressionlevels.tif

- SupplementaryTable1.Statisticsoftemperatures.xlsx
- SupplementaryTable2.Primersequenceusedinthisstudy.xlsx
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- SupplementaryTable12.ListofDEGs.xlsx