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Candidate Gene Analysis of the
*Restorer-of-fertility* Gene in Pepper and
Transgenic Expression of the Pepper
CMS Associated Gene *orf507* in
*Nicotiana benthamiana*

고추의 임성 회복 후보 유전자 분석 및
웅성불임 후보 유전자의 담배 내 발현

February, 2017

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DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY
Candidate Gene Analysis of the *Restorer-of-fertility* Gene in Pepper and Transgenic Expression of the Pepper CMS Associated Gene *orf507* in *Nicotiana benthamiana*

UNDER THE DIRECTION OF DR. BYOUNG-CHEORL KANG
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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Cytoplasmic-genic male sterility (CGMS) has been widely used to produce hybrid seeds. Plant CGMS is due to genetic incompatibility between mitochondria and nucleus and is typically associated with the abnormal chimeric gene of mitochondria. Although the candidates for CMS-associated mitochondrial gene and Restorer-of-fertility (Rf) gene were proposed, direct evidence of true Rf gene identifying is still lacking. Thus, development of Rf candidate markers and transgenic expression of CMS related gene in tobacco were performed in this study. In the first chapter, expression and sequence of Rf candidates, CDS1, CDS2, CDS3 and CaPPr6 were conducted in chili pepper Bukang and Chungyang CMS and restorer lines and sweet pepper MSGR-A and SPR03. Among candidate genes, only CDS3 showed specific expression in fertile lines. Molecular markers were developed based on SNPs and InDel of each candidate and applied to Rf segregation population of sweet pepper. As a result, newly developed markers
showed reverse results between chili pepper and sweet pepper lines. This result suggests that MSGR-A and SPR03 have different type of $Rf$ co-segregated region compared to chili pepper. In the second chapter, to check the hypothesis that ORF507 is sufficient to induce male sterility in plants, the cDNA of $orf507$ was cloned in frame with mitochondria transit sequence, $coxIV$, and was expressed under tapetum-specific promoter TA29 in tobacco plants. But transgenic tobacco plants carrying $orf507$ cloned under the tapetum specific promoter with mitochondrial transit sequence did not show male sterility.

**Keyword**: *Capsicum annuum*, cytoplasmic male sterility, restoration of fertility, plant transformation

**Student Number**: 2015-21497
CONTENTS

ABSTRACT .........................................................................................................................1

CONTENTS .....................................................................................................................3

LIST OF TABLES .............................................................................................................6

LIST OF FIGURES ..........................................................................................................7

LIST OF ABBREVIATIONS .............................................................................................8

GENERAL INTRODUCTION ............................................................................................9

LITERATURE REVIEW

Cytoplasmic male sterility .............................................................................................. 11

Restoration of fertility .................................................................................................... 12

Cytoplasmic-genic male sterility in pepper ................................................................. 13
Chapter 1 Genetic and expression analysis of candidate genes for the pepper $Rf$ gene

**ABSTRACT** ........................................................................................................19

**INTRODUCTION** ..............................................................................................21

**MATERIALS AND METHODS**

Plant materials ......................................................................................................24

Genomic DNA extraction ....................................................................................26

DNA sequence analysis ........................................................................................26

HRM analysis ..........................................................................................................26

RT-PCR analysis ..................................................................................................27

**RESULTS**

Characterization of fertility restoration in a CMS, maintainer and restorer line  .29

Expression analysis of $Rf$ candidate genes ..........................................................31

Sequence analysis of $Rf$ candidate genes .............................................................34

Marker development and cosegregation analysis with the $Rf$ phenotype ..........39

**DISCUSSION** .....................................................................................................44

**REFERENCES** ................................................................................................48
Chapter 2 Transgenic expression of the pepper CMS associated gene orf507 in Nicotiana benthamiana

ABSTRACT ..................................................................................................................................................51

INTRODUCTION ........................................................................................................................................53

MATERIALS AND METHODS

Plant material ..............................................................................................................................................56

DNA extraction and analysis .......................................................................................................................56

Vector Construction .....................................................................................................................................56

Plant Transformation .....................................................................................................................................57

RT-PCR analysis ..........................................................................................................................................58

Pollen analysis .............................................................................................................................................58

RESULTS

Vector construction of ORF507 ...................................................................................................................60

Transformation of N. benthamiana with the constructs ..............................................................................62

Analysis of N. benthamiana transgenic plants over expressing ORF507 .................................................65

DISCUSSION ..............................................................................................................................................68

REFERENCES ............................................................................................................................................71

ABSTRACT IN KOREAN ..............................................................................................................................74
LIST OF TABLES

Chapter 1

Table I-1. List of hot and sweet pepper CGMS lines used in this study

Table I-2. Details of primers from direct sequencing

Table I-3. Characteristics of candidate genes from co-segregation region

Table I-4. Nucleotide and amino acid variations among Rf-CDS1, CDS2 and CaPPR6 between restorer and CMS lines

Table I-5. Application of Rf related markers to a panel of pepper breeding lines from Enza Zaden

Chapter 2

Table II-1. Summary of N. benthamiana transformation
LIST OF FIGURES

Chapter 1

Figure I-1. Expression of $R_f$ candidates in the mature anthers of CMS line and fertility restorer line.

Figure I-2. Expression of $R_f$ candidates in paprika $R_f$ segregation population.

Figure I-3. Structures of the $R_f$ candidate genes, CDS1, CDS2 and CDS3.

Figure I-4. HRM analysis of markers derived from $R_f$ candidates.

Chapter 2

Figure II-1. Schematic illustration of gene constructs developed and used.

Figure II-2. Steps in the transformation of $N. benthamiana$ with Agrobacterium.

Figure II-3. Molecular analyses of $T_0$ transgenic plants

Figure II-4. Comparison of the floral morphology, pollen viability and germination rate in transformants and wild type control plant
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>CMS</td>
<td>Cytoplasmic male sterility</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>HRM</td>
<td>High resolution melt</td>
</tr>
<tr>
<td>InDel</td>
<td>Insertion-deletion</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PPR</td>
<td>Pentatricopeptide repeat protein</td>
</tr>
<tr>
<td>Rf</td>
<td>Restorer-of-fertility</td>
</tr>
<tr>
<td>SCAR</td>
<td>Sequence-characterized amplified region</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

Hybrid vigor, or heterosis, refers to the phenomenon in which the progeny derived from a cross of two inbred lines outperform the parent lines. Hybrid crops can produce 15–50% higher yields than inbred varieties (Tester and Langridge, 2010). The utilization of heterosis in various crops has produced tremendous economic benefits in worldwide. More than half of the production of major crops such as maize, rice, sorghum, rapeseed, and sunflower are being produced from hybrid varieties (Li et al., 2007). Thus, hybrid breeding contributes significantly to the food supply in the world. Producing hybrid seeds of self-pollinating plants requires emasculation (the removal of functional pollen grains) to prevent self-pollination. Before 1950s, emasculation in hybrid seed production involved manual labor, machines, or chemical treatments and thus was costly, inefficient, and even damaging to the environment. CMS and EGMS lines do not require emasculation and therefore are ideal female lines for hybrid seed production. In the 1950s, the maize CMS-T system was first used for hybrid corn, greatly increased the efficiency of hybrid seed production and improved maize yields. Later, CMS-based hybrid technology was developed in many other crops. Commercial hybrid rice, which increases the grain yield by over 20%, was first released in 1976 in China, and it has accounted for approximately 55% of the total rice planting area in China since the late 1980s (Cheng et al., 2007). CMS-based hybrid seed technology uses a three-line system, which requires three different breeding lines: the CMS line, the maintainer line, and the restorer line. The CMS line has male-sterile cytoplasm with a CMS-causing gene and lacks a functional nuclear restorer of fertility \textit{(Rf)} gene or genes (Schnable and Wise, 1998), and is used as the female parent. The
maintainer line has normal fertile cytoplasm but contains the same nuclear genome as the CMS line, and thus serves as the male parent in crosses for the propagation of the CMS line. The restorer line possesses a functional $Rf$ gene or genes, and thus serves as the male parent to cross with the CMS line to produce $F_1$ hybrid seeds. In the $F_1$ plants, the $Rf$ gene restores male fertility, and the combination of nuclear genomes from the CMS line and the restorer line produces hybrid vigor.

In pepper (*Capsicum annuum* L.), CMS was first isolated from an Indian *C.annuum* accession (PI164835) (Peterson, 1958). Molecular investigations revealed that male sterility in many CMS lines is associated with the expression of *orf456*, which is located downstream and co-transcribed with the mitochondrial *coxII* gene (Kim et al., 2007), and the pseudogene $Ψatp6-2$, 3′-truncated form of *atp6-2* in maintainer line (Kim and Kim, 2006). Later, *orf456* was revised to *orf507* by correcting (Gulyas et al., 2010). Markers related to *orf507* were developed and used for pepper CGMS breeding. But direct evidence that ORF507 is enough to induce male sterility in pepper is not sufficient. A genomic region co-segregating with the restoration-of-fertility phenotype was identified and candidates for the $Rf$ gene were proposed. Among the candidates, one PPR type gene, *CaPPR6* was assumed as a strong candidate for the $Rf$ gene, and several molecular markers related to *CaPPR6* were developed (Jo et al., 2016).

In this study, expression and sequence analysis of $Rf$ candidate genes were performed and related molecular markers were developed in chapter one. In the second chapter, the cDNA of *orf507* was cloned in frame with mitochondria transit sequence, *coxIV*, and was expressed under tapetum-specific promoter TA29 in tobacco plants.
LITERATURE REVIEW

Cytoplasmic male sterility

Many CMS genes have been described so far. However, few genes have characterized. Based on this information, most researchers examined the orfs associated with functional genes to identify differences between CMS and F₁. Regarding the origin of CMS genes, these chimeric orfs are generally considered to be due to mitochondrial genomic rearrangements (Tuteja et al., 2013). Because hybrid crops contribute significantly to food security, abundant germplasm was found and CMS types have been investigated. In rice, orf79 derived from CMS-BT (derived from Indica rice varieties Boro II) and CMS-HL (derived from wild rice accession Hong-Lian), a small protein with N-terminal similar to COX1 was encoded and the remaining SUO (sequence of unknown origin) (Wang et al., 2006). In sorghum CMS-A3, chimera orf107 encodes a fragment of ATP9 at the N-terminus and a protein with a residue similar to ORF79 (Tang et al., 1996). In wheat, the CMS-AP line, which contains the nuclear genome of Triticum aestivum and the cytoplasm of Triticum timopheevii, is associated with orf256 (Rathburn and Hedgcoth, 1991). The 5’ franking region of orf256 and the first 11 amino acid coding sequence are identical to cox1. The atp8 sequences form parts of the CMS gene in dicot species. The Brassica CMS genes orf138 and orf125 derived from radish encode atp8-like proteins. The Brassica CMS-Pol and CMS-Nap genes orf224 and orf222, which encode a membrane protein with 79% sequence similarity, contain an atp8-derived sequence.

Emerging studies on CMS systems of various crops support four models of
mechanisms that cause CMS. These suggest disruption of mitochondrial membrane (Sabar et al., 2000), ATP synthase defects (Bergman et al., 2000), tapetal cell programmed cell death (Balk and Leaver, 2001) and alteration of the expression patterns of floral development genes (Carlsson et al., 2008). Interaction of mitochondrial proteins with CMS-related genes has been reported in rice. WA352 of CMS-WA rice interacted with nuclear encoded COX11. WA352 inhibited the function of COX11 in peroxide metabolism, leading to premature programmed cell death of the injured cells (Luo et al., 2013)

**Restoration of fertility**

Restorer-of-fertility (Rf) is a nucleus-encoded gene which suppresses the induction of CMS caused by CMS-associated genes located on mitochondrial genome. Nine Rf genes have been isolated in seven plant species: Rf2 (maize) (Cui et al., 1996; Liu et al., 2001), Rf-PPR592 (Petunia) (Bentolila et al., 2002), Rfk1 (radish, Brassica) (Koizuka et al., 2003), Rf5 (rice) (Hu et al., 2012), Rf1b (rice) (Wang et al., 2006), Rf2 (rice) (Itabashi et al., 2011), Rf17 (rice) (Fujji and Toriyama, 2009), Rf1 (sorghum) (Klein et al., 2005), and Rf1 (bvORF20) (sugar beet) (Matsuhira et al., 2012). Rf-PPR592, Rf0, Rf1a, Rf1b and sorghum Rf1 encode the PPR protein. PPRs are a group of RNA-binding proteins and most of them play a role in organellar posttranscriptional mRNA processing such as editing, splicing, cleavage, degradation and translation (Delannoy et al., 2007; Schmitz-Linneweber and Small, 2008). In maize CMS-T, the first isolated plant restorer gene, Rf2 encodes an aldehyde dehydrogenase (Cui et al., 1996). Rf17 in CMS-CW encodes a mitochondrial sorting protein containing the 178 amino acids of the acyl carrier
protein synthase-like domain (Fujii and Toriyama, 2009). \textit{Rf2} in CMS-LD encodes mitochondrial glycine-rich protein (Itabashi et al., 2011). Recently, \textit{bvORF20}, which encodes a putative peptidase of the M48 family, has been shown to be a strong candidate for \textit{Rf1} in sugar beet CMS-Owen (Hagihara et al., 2005; Matsuhira et al., 2012). Therefore, in addition to the conserved PPR \textit{Rf} gene, the \textit{Rf} gene is highly diverse.

\section*{Cytoplasmic-genic male sterility (CGMS) in pepper}

In pepper, only a CMS cytoplasm from the Indian germplasm (USDA accession PI 164835) has been characterized (Peterson 1958) and used for hybrid seed production. Isolation of candidate genes for CMS related genes in pepper. Candidate genes for CMS-associated gene were isolated in pepper. A chimeric mitochondrial gene, \textit{orf507}, was identified in the mitochondrial genome of CMS peppers (Gulyas et al., 2006; Kim et al., 2007). The CMS-related protein, ORF507, shows interaction with ATP synthase 6kDa subunit protein (Li et al., 2012). Since decrease in the ATP synthase activity was detected in CMS pepper lines, it was suggested that the binding of ORF507 with ATP synthase activity as a possible mechanism for CMS in pepper (Li et al., 2012). Another candidate gene, named as \textit{Ψatp6-2} gene, is generated by novel rearrangement on 3' region of normal \textit{atp6-2}. Transcriptional pattern of \textit{Ψatp6-2} was different between male sterile and restorer lines, indicating a possible association of this gene with CMS (Kim and Kim, 2006).

For \textit{Rf}, several \textit{Rf}-linked markers, OPP13-CAPS, AFRF8-CAPS, PR-CAPS and CRF-SCAR (Lee et al., 2008; Kim et al., 2006; Gulyas et al., 2006) have been
developed respectively. But these markers have limited applications in broad pepper lines. Recently, a genomic region co-segregating with the restoration-of-fertility phenotype was identified and candidates for the $R_f$ gene were showed. Among the candidates, one PPR type gene, *CaPPR6* was assumed as a strong candidate for $R_f$ gene, and several molecular markers related to *CaPPR6* were developed (Jo et al., 2016). But direct evidence that *CaPPR6* function as a $R_f$ gene in pepper is still lacking and markers related to *CaPPR6* also showed limitation in some pepper breeding lines.
REFERENCES


CHAPTER 1

Genetic and expression analysis of candidate genes for the pepper \( R_f \) gene

ABSTRACT

*Restorer-of-fertility* (\( R_f \)) is a nucleus-encoded gene which suppresses the induction of cytoplasmic male sterility (CMS) caused by CMS-associated genes located on mitochondrial genome. The CMS/\( R_f \) systems are useful agricultural traits for production of hybrid varieties. These traits enable seed production without manual emasculation or contamination of seeds caused by self-pollination. In pepper, a genomic region co-segregating with the restoration-of-fertility phenotype was identified and four candidates for the \( R_f \) gene were proposed. Among the candidates, one PPR type gene, \( CaPPR6 \) was assumed to be a strong candidate for the \( R_f \) gene, and several molecular markers derived from \( CaPPR6 \) were developed. However, application of \( CaPPR6 \) derived markers to several pepper breeding lines showed that genotypes of these markers did not match with perfectly the \( R_f \) phenotype indicating \( CaPPR6 \) is not the \( R_f \) gene or there are other \( R_f \) loci. To address these questions, the other 3 \( R_f \) candidate genes were analyzed by sequence and RT-PCR. We used CMS lines and restorer lines of Bukang and Chungyang for chili pepper, and sweet pepper CMS line, MSGR-A and sweet pepper unstable maintainer line, SPR03, which has \( R_f^r R_f^r \) genotype. All 4 candidate genes showed no expression in Bukang A comparing in Bukang C. But only CDS3 was expressed
in SPR03 but not in MSGR-A as same as Bukang lines, while CDS1 and CaPPR6 were expressed in MSGR-A, but not in SPR03 and CDS2 showed no expression in both MSGR-A and SPR03. Sequence analysis revealed that CDS1 and CDS3 encoded a NTM1-like 9 protein and a TIR1 (transport inhibition response 1)-like protein, respectively, while CDS2 encoded an uncharacterized protein. Only CDS3 was predicted to have mitochondrial transit peptides at its N-termini, while CDS1 and 2 were predicted to have no signal peptide. Based on detected SNPs in each candidate genes between Bukang and Chungyang, we developed markers for each candidate and applied to F₃BC₄ population between MSGR-A and SPR03, and panel of breeding lines provided by Enza Zaden. MSGR-A follows the haplotypes of the Bukang and Chungyang CMS lines in CDS1 but follows the haplotypes of restorer lines in CDS2 and CDS3, whereas SPR03 follows the haplotypes of CMS lines in CDS1, CDS2 and CDS3. In panels of breeding lines, the marker developed based on the promoter region of CDS3 showed the highest accuracy among the newly developed markers and it was comparable accuracies with previously marker related to CaPPR6. In conclusion, reverse haplotype of Rf candidates compared to chili pepper lines suggests that MSGR-A and SPR03 have different type of Rf co-segregated region, and specific expression of CDS3 in restorer lines and marker accuracy in breeding lines strengthened the hypothesis that this gene is a strong candidate for pepper Rf and we expected that it will be possible to develop restorer pepper lines using the CDS3 related marker.
INTRODUCTION

Restorer-of-fertility (Rf) is a nucleus-encoded gene which suppresses the induction of cytoplasmic male sterility (CMS) caused by CMS-associated genes located on mitochondrial genome. The CMS/Rf systems are useful agricultural traits for production of hybrid varieties. These traits enable seed production without manual emasculation or contamination of seeds caused by self-pollination. The obtained hybrid varieties exhibit increased harvest yields through heterosis. All Rf gene products identified so far are mitochondrial proteins. Furthermore, many Rf members are known to encode pentatricopeptide repeat (PPR) proteins, such as Rf–PPR592 in Petunia hybrida, Rfo/Rfk in Raphanus sativus, and Rf1 in Sorghum bicolor (Bentolila et al., 2002; Brown et al., 2003; Koizuka et al., 2003; Klein et al., 2005). The Rf gene in BT–CMS rice, Rf1, is also known to encode a PPR protein (Kazama and Toriyama, 2003; Akagi et al., 2004; Komori et al., 2004). PPR proteins are sequence-specific RNA-binding proteins that are involved in various post-transcriptional processes (RNA editing, RNA splicing, RNA cleavage and translation) for organellar RNAs (Schmitz-Linneweber and Small, 2008). Several PPR-type RF proteins have been shown to suppress the expression of CMS-associated genes in the mitochondria of lines with restored fertility (Gillman et al., 2007; Kazama et al., 2008; Uyttewaal et al., 2008).

PPR genes encode proteins which gave a repeated motif composed of array of amino acids motif or slightly different length of motifs which can bind RNA through its superhelix structure. They were seems to be involved in RNA editing, splicing, processing, cleavage and degradation. Recent study showed that each 35
amino acid array of PPR genes determined the specificity of the protein to one nucleotide of target RNA. Combination of the first and sixth amino acids in PPR protein was crucial for the determination of specificity implying direct interaction of these amino acids with RNA. Altogether, these characteristics of PPR type $R_f$ genes supports the hypothesis that $R_f$ genes has been evolved by birth-and-death process with the appearance of new CMS genes.

Although the molecular mechanisms underlying fertility restoration by PPR-type $R_f$ genes have been extensively investigated, the function of non-PPR type $R_f$ genes is poorly understood. The first identified $R_f$ was $R_f^{2a}$ from maize (Zea mays), which encodes aldehyde dehydrogenase (Cui et al., 1996). $R_f^{2a}$ is required for normal anther development, but does not reduce the accumulation of the CMS-associated gene product URF13 (Liu and Schnable, 2002). Rice $R_f^{17}$ encodes an acyl carrier protein synthesis like domain-containing protein (Fujii and Toriyama, 2009). The presence of $R_f^{17}$ does not change the RNA profile of the putative CMS-associated gene CW-orf307 (Fujii et al., 2010). Reduction of $R_f^{17}$ expression was shown to restore the fertility of the CW-CMS line, suggesting that $R_f^{17}$ may be involved in mitochondria-to-nucleus signaling in the CMS line (Fujii and Toriyama, 2009).

In pepper, a genomic region co-segregating with the restoration-of-fertility phenotype was identified and candidates for the $R_f$ gene were proposed. Among the candidates, one PPR type gene, $CaPPR6$ was assumed as a strong candidate for $R_f$ gene, and several molecular markers related to $CaPPR6$ were developed (Jo et al., 2016). However, application of $CaPPR6$ derived markers to sweet pepper breeding lines showed that genotypes of these markers did not match perfectly the $R_f$ phenotype. Especially, in sweet pepper CMS line MSGR-A containing $rfrf$
genotype and unstable maintainer line SPR03 containing $Rf^aRf^u$ genotype, CaPPR6 linked marker showed reverse genotype compared to phenotype. To overcome these limitations, sequence and expression study of the other 3 $Rf$ candidate genes is required.

In this study, we considered the possibility that the other 3 $Rf$ candidate genes, previously identified, are pepper $Rf$ rather than CaPPR6. To confirm their fertility restoration function and facilitate the reliable and efficient molecular breeding for $Rf$ gene, sequence analysis, protein function prediction and expression check of the candidates for $Rf$ gene were performed. Also, molecular markers were newly developed from the SNPs between CMS and restorer lines in pepper and applied to $Rf^u$ segregation population between MSGR-A and SPR03 and panel of breeding lines provided by Enza Zaden.
MATERIALS AND METHODS

Plant materials

Five hot pepper breeding lines and two sweet pepper breeding lines including CMS, maintainer, and restorer lines were used for sequence and expression analysis. Table 1 lists the plant materials and their phenotypes. All plants were grown under 16 h light and 8 h dark condition at constant 23°C. A population consists of 29 F3BC4 plants between MSGR-A and SPR03 were used to check co-segregation of marker genotype and phenotype. DNA samples of a total of 50 lines from Enza Zaden (Enkhuizen, The Netherlands) were used to test marker applicability.

Genomic DNA extraction

Young leaves from plants of each accession were used for DNA extraction. The concentration and purity of DNA samples was measured using a Take3 (BioTek, Winooski, USA) after DNA was extracted using the cetyl trimethylammonium bromide (CTAB) method (Park et al., 2009). DNA samples showing absorbance ratios at 260/280 nm above 1.8 were used for marker analysis.

DNA sequence analysis

Sequence analysis was performed using amplicons obtained from each breeding lines to detect SNPs in each Rf candidate gene. Amplified PCR products from genomic DNA of each breeding lines were sequenced directly. A Labopass Gel and
Table I-1. List of hot pepper and sweet pepper CGMS lines used in this study

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Pungency</th>
<th>Fruit shape</th>
<th>Rf phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bukang A</td>
<td>pungent</td>
<td>elongated</td>
<td>rfif</td>
</tr>
<tr>
<td>2</td>
<td>Bukang C</td>
<td>pungent</td>
<td>elongated</td>
<td>RfRf</td>
</tr>
<tr>
<td>3</td>
<td>Chungyang A</td>
<td>pungent</td>
<td>elongated</td>
<td>rfif</td>
</tr>
<tr>
<td>4</td>
<td>Chungyang B</td>
<td>pungent</td>
<td>elongated</td>
<td>rfif</td>
</tr>
<tr>
<td>5</td>
<td>Chungyang C</td>
<td>pungent</td>
<td>elongated</td>
<td>RfRf</td>
</tr>
<tr>
<td>6</td>
<td>MSGR-A</td>
<td>sweet</td>
<td>small blocky</td>
<td>rfif</td>
</tr>
<tr>
<td>7</td>
<td>SPR-03</td>
<td>sweet</td>
<td>large blocky</td>
<td>Rf°Rf&quot;</td>
</tr>
</tbody>
</table>
PCR Clean-up Kit (Cosmogenetech, Seoul, Korea) was used to purify PCR products according to the protocol supplied by the manufacturer, prior to sequencing with an ABI3730 sequencer (NICEM, Seoul, Korea). The sequences of PCR products amplified from accession in each breeding lines were analyzed. All obtained sequences were aligned using a MEGA6 software.

**HRM analysis conditions**

A Rotor-Gene 6000 real-time PCR thermocycler (Corbett Research, Sydney, Australia) was used to detect to SNP markers via HRM analysis. The real-time PCR amplification conditions were 95°C for 10 min, then 55 cycles of 94°C for 20 s, 56°C for 20 s and 72°C for 30 s. For HRM analysis, temperature were increased 0.1°C every minute from 65 to 90°C.

**RT-PCR analysis**

Total RNA was isolated from anthers (obtained from floral buds which are 3-5mm in size) from using MG Total RNA extraction kit (MGMED, Seoul, Republic of Korea) according to manufacturer’s description. cDNA was synthesized from 2 μg of total RNA using EasyScript cDNA synthesis kit (Transgene biotech, Beiging, China). The reverse-transcriptase PCR (RT-PCR) was performed using gene specific primer set in 25 μl with 10 mM Tris-HCl (pH8.3), 50mM KCl, 1.5 mM MgCl2, 0.2mM of each dNTP, 0.2μM of each primer, 1 μl of reverse transcription products, and 1 unit of taq polymerase.
Table I-2. SNP-based primers designed from direct sequencing of candidate genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Tm (°C)</th>
<th>Amplicon (bp)</th>
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<td>201</td>
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<tr>
<td></td>
<td>R: CATAGTCTCTACGGTCATCATC</td>
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</table>
RESULTS

Expression analysis of $Rf$ candidate genes

In the previous study, 4 candidate genes, CDS 1, 2, 3 and $CaPPR6$, were predicted to be downregulated in Bukang A comparing in Bukang C. To confirm the expression of candidate genes, primer sets for RT-PCR analysis were specifically designed to amplify a portion of each candidate genes. RT-PCR was carried out to reveal differential expression pattern between sterile and fertile breeding lines. All 4 candidate genes expressed in Bukang C whereas there was no expression in Bukang A (Fig. 2). For one more checking, we used sweet pepper sterile line, MSGR-A and sweet pepper fertile line, SPR03. When $CaPPR6$ derived marker was applied to these sweet pepper lines, the marker showed reverse results that MSGR-A had $Rf$ genotype and SPR03 had $rf$ genotype. Interestingly, CDS1 and $CaPPR6$ were expressed in MSGR-A, but not in SPR03. CDS2 showed no expression in both MSGR-A and SPR03. Only CDS3 was expressed in SPR03 but not in MSGR-A as same with hot pepper breeding lines.

To check whether CDS3 is only expressed in fertile plants in $Rf$ gene segregation population, RT-PCR analysis was also performed to BC$_4$F$_2$ population derived from F2 of MSGR-A X SPR03 using primer sets for CDS3. We choose 6 sterile plants and 1 fertile plant in population. Amplification products of CDS3 were found in anther tissues of the fertile individuals, but not in the anther tissues of the sterile individuals (Fig. 3).
Figure I-1. Expression of Rf candidates in the mature anthers of sterile line (Bukang A and MSGR-A) and fertile line (Bukang C and SPR03)
Figure I-2. mRNA expression of $Rf$ candidates in paprika $Rf$ segregation population.
Sequence variations of \textit{Rf} candidate genes

To investigate sequences of 4 candidate genes, using primers designed from the 5’ and 3’ end of each \textit{Rf} candidate genes, full sequence of each candidate genes were amplified from the Bukang and Chungyang restorer and CMS line. It turned out CDS 1, 3 and PPR6 encode a NTM1-like 9 protein, a TIR1 (transport inhibition response 1)-like protein and a PPR protein, respectively, while CDS2 encoded an uncharacterized protein. Putative SNP sites were identified by comparing the coding sequences from Bukang and Chungyang restorer and CMS line. TargetP (http://www.cbs.dtu.dk/services/TargetP/) program was used to predict the cellular localization of each candidate genes. Only CDS 3 was predicted to have mitochondrial transit peptides at its N-termini, whereas CDS 1 and 2 were predicted to have no signal peptide and PPR6 was predicted to have chloroplast transit peptide. The full-length CDS3 consist of a 537, 345 and 516 bp ORF encoding 178, 114 and 171 amino acids with a predicted molecular mass of 20.67, 13.52 and 19.2 kDa respectively. Analysis of the predicted amino acid sequence of each candidate showed that CDS3 has a mitochondrial targeting sequence at the N-terminus (predicted by TargetP) and one leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily (predicted by NCBI; https://www.ncbi.nlm.nih.gov/Structure/cdd/), while no targeting sequence or conserved domain was identified in CDS1 and CDS2 (Table 3).

To investigate sequence variations between the restorer and CMS, we sequenced the candidate genes of Bukang and Chungyang lines. In CDS1 gene of Bukang lines, a total of 4 amino acid substitutions were detected; Val^{102}-to-Leu, Leu^{119}-to-His, Arg^{125}-to-His and His^{142}-to-Gln caused by G^{304}-to-C, T^{356}-to-H, G^{374}-to-A, and
T^{423}\text{-to-G}. These mutations were same at CDS1 of Chungyang lines. In CDS2 gene of Bukang lines, a total of 6 amino acid substitutions were detected; Arg^{11}\text{-to-Leu, Thr^{17}\text{-to-Met, Val^{30}\text{-to-Leu, Ser^{51}\text{-to-Ile, Lys^{60}\text{-to-Gln and Cys^{85}\text{-to-Ser caused by G^{32}\text{-to-T, C^{50}\text{-to-T, G^{115}\text{-to-C, G^{152}\text{-to-T, A^{178}\text{-to-C, and T^{253}\text{-to-A. These mutations were the same at CDS2 of Chungyang lines. A total of 15 amino acid substitutions were detected in CaPPR6 gene; Val^{71}\text{-to-Phe, Asn^{108}\text{-to-Ser, Lys^{168}\text{-to-Glu, Asn^{244}\text{-to-Asp, Gly^{299}\text{-to-Ala, Ile^{311}\text{-to-Val, Arg^{424}\text{-to-His, Gly^{456}\text{-to-Glu, Lys^{473}\text{-to-Glu, His^{475}\text{-to-Gln, Ala^{476}\text{-to-Ser, Ile^{477}\text{-to-Val, Lys^{480}\text{-to-Asn, Leu^{487}\text{-to-Pro, and Pro^{488}\text{-to-Leu caused by G^{211}\text{-to-T, A^{323}\text{-to-G, A^{503}\text{-to-G, A^{730}\text{-to-G, G^{896}\text{-to-C, A^{931}\text{-to-G, G^{1271}\text{-to-A, G^{1367}\text{-to-A, A^{1417}\text{-to-G, T^{1425}\text{-to-G, G^{1426}\text{-to-T, A^{1429}\text{-to-G, G^{1440}\text{-to-T, T^{1460}\text{-to-C, and C^{1463}\text{-to-T. These mutations were the same at CaPPR6 of Chungyang lines except Arg^{424}\text{-to-Ser caused by G^{1271}\text{-to-C instead of Arg^{424}\text{-to-His (Table 4). No amino acid substitution was in CDS3, but 14 bp of indel was detected in promoter region (Fig. 4). In the case of sweet pepper line, both MSGR-A and SPR03 follow the haplotype of CDS1 in CMS line. But MSGR-A follows the haplotype of CDS2 and CDS3 in restorer line, whereas SPR03 follows CMS line. Two amino acid substitutions were detected in CaPPR6 of MSGR-A compared to CaPPR6 in restorer line; Asn^{108}\text{-to-Ser and Lys^{168}\text{-to-Glu caused by A^{323}\text{-to-G, and A^{502}\text{-to-G. These mutations are also existed in Bukang and Chungyang CMS lines. CaPPR6 of SPR03 follows the haplotype of Bukang CMS line.}
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<tr>
<th>Name of candidate gene</th>
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<th>Information on protein sequence in GenBank showing the highest similarity with ORF</th>
<th>GenBank ID</th>
<th>Characteristics</th>
<th>Length of CDS</th>
<th>Localization</th>
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Nucleotide and amino acid variations among \textit{Rf-CDS1} (A), \textit{CDS2} (B) and \textit{CaPPR6} (C) between restorer and CMS lines

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<td>Chungyang C</td>
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<td>SPR03</td>
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<td>c/L a/H a/H g/Q</td>
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<td>t/L t/M c/L t/I c/Q a/S</td>
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<td>MSGR-A</td>
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<tr>
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<td>rf-PPR6</td>
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</tr>
<tr>
<td>SPR03</td>
<td>Rf\textsuperscript{a}-PPR6</td>
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Figure 1-3. SNP positions of *Rf* candidates in *C. annuum* ‘Bukang A’ and ‘Bukang C’, CDS1 (A), CDS2 (B) and CDS3 (C), respectively. Red triangles indicate the positions of SNPs. The open triangle indicates the position of InDel. Black triangles indicate the position of marker primers used in this study.
**Marker development and application in breeding lines**

Based on detected SNPs in each candidate genes between Bukang and Chungyang, one HRM marker for CDS1 and three HRM markers for CDS2 were designed based on SNPs in the coding sequences. In the case of CDS3 which has no SNP in the CDS region, five markers were designed based on SNPs in the 3’UTR region, 5’UTR region and 14 bp indel of promoter region. Each marker was tested to Bukang parental lines and polymorphisms between sterile and fertile lines were confirmed. Among these markers, CDS1_HRM for CDS1, CDS2_HRM2 for CDS2 and CDS3_pro_HRM1 for CDS3 showed polymorphism and co-segregated with phenotype (Fig. 5).

Newly developed and previously developed CaPPR6 related (Co1Mod1) markers were applied to panels of breeding lines provided by Enza Zaden. In the application to 50 lines provided by Enza Zaden, Co1Mod1 showed 80% of accuracy (80%), although only the marker developed from promoter region of CDS3 (88%) exhibited better accuracy with Co1Mod1 marker. Other markers developed from CDS1 and CDS2 coding sequence showed comparatively low success rate (60% and 52% respectively) (Table 5).
Figure I-4. HRM results for Rf-candidates associated markers. Normalized HRM curves of SNP based markers, CDS1_HRM (A), CDS2_HRM2 (B) and CDS3_pro_HRM1 (C).
Table I-5. Application of Rf related markers to a panel of pepper breeding lines from Enza Zaden

<table>
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<tr>
<th>Markers</th>
<th>Marker haplotypes</th>
<th>Number of lines classified as RfRf (total 24 lines)</th>
<th>Number of lines classified as Rff (total 12 lines)</th>
<th>Number of lines classified as rff (total 14 lines)</th>
<th>Ratio of successful genotyping (%)</th>
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<td>rff</td>
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<td>7</td>
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<tr>
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DISCUSSION

The RT-PCR of $Rf$ candidate genes using primer sets for each candidate was done in Bukang A, Bukang C, Chungyang A, Chungyang C, MSGR-A and SPR03. It has been reported that all of candidates were known to be expressed in Bukang C but not in Bukang A based on transcriptome analysis. In RT-PCR analysis, all of candidates showed expression in chili pepper restorer line, but not in chili pepper CMS line. But in SPR03, which is known to be carrying $Rf^u/Rf^u$ genotype, only CDS3 was expressed, whereas CDS1, CDS2 and CaPPR6 were not expressed. In MSGR-A, CDS1 and CaPPR6 were expressed, whereas CDS2 and CDS3 were not. This result indicates that CaPPR6 in MSGR-A does not appear to restore the male sterility, which is not matched with the results of the previous study that revealed CaPPR6 is the strongest candidate gene for pepper $Rf$. Comparison of genomic sequence of CaPPR6 between MSGR-A and Bukang C showed that there are two amino acid substitutions, Asn-to-Ser and Lys-to-Glu. These substitutions occurred at the 6th amino acid in 2nd PPR motif and the 31st amino acid in 3rd PPR motif, which are also found in existed in other CaPPR6 in Bukang A and Chungyang A. It can be inferred that this amino acid substitution is involved in the fertility restoration function of CaPPR6. Analysis of Genomic sequence of CaPPR6 of SPR03 showed nonsense mutation that results in a premature stop codon. This demonstrates that fertility restoration mechanism in sweet pepper is not controlled by CaPPR6 in MSGR-A or SPR03, and sweet peppers may have other $Rf$ controlled genes rather than CaPPR6. Among four candidate genes, CDS3 expressed in fertile lines but not in sterile lines. We also confirmed whether the expression of CDS3 follows fertility restoration phenotype or not using RT-PCR.
analysis in a $Rf$ segregating population. The result showed that CDS3 is only expressed in fertility restored plants. This implies that CDS3 can be regarded as the candidate for $Rf$ among genes in the $Rf$ co-segregating region.

NTM1, which is the CDS1 encoding protein, belongs to the NAC transcription factor family that are unique to plants. The NAC proteins contain a highly conserved NAC DNA-binding domain (DBD) that consists of approximately 160 residues in their N-terminal regions. The transcriptional activities reside in their C-terminal regions, although the C-terminal sequences are quite diverse, which is thought to impart their distinct and varied individual functions (Kim et al., 2007a). The NAC family is one of the largest transcription factor families in plant genomes. There are approximately a hundred of NAC transcription factors in each of the Arabidopsis and rice genomes. Several NAC members have been functionally studied in floral development, apical meristem formation, growth hormone signaling, ER stress responses and cell-cycle control. However, most NAC transcription factors have not yet been functionally characterized, and the protein structures of the NACs have not been thoroughly examined (Kim et al., 2007b).

Until now, a number of NAC proteins have been shown to bind fragments of this promoter. A number of genes are known to be upregulated by overexpression of Arabidopsis NAC genes. So the protein encoded by CDS1 has a possibility that can regulate transcription of factors related to fertility restoration.

TIR1 protein, which is the CDS3 encoding protein, is the substrate receptor subunits of the complex and function as auxin receptors. Auxin acts as a ‘molecular glue’ to stabilize the interaction between TIR1 and AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins, a transcriptional repressor, thus promoting their ubiquitination and degradation to induce auxin-regulated responses (Dharmasiri et
TIR1 is a member of a small gene family that contains five additional AFB proteins (AFB1–AFB5), which collectively mediate auxin response and are essential for the growth and development of plants. Some aspects of auxin-regulated transcription are understood. Two families of transcription factors, the auxin response factor (ARF) and Aux/IAA proteins, have been involved in this process. The ARF proteins bind DNA directly and either activate or repress transcription depending on the ARF. The Aux/IAA proteins exert their effects by binding to the ARF proteins through a conserved dimerization domain. At least in the case of the activating ARFs, the effect of Aux/IAA binding is to repress transcription (Terrile et al., 2012). If an ARF affects transcription of Rf related factor, the protein encoded by CDS3 can interacts with Aux/IAA proteins. It can reduce the transcription repression of the ARF, and finally regulates fertility restoration mechanisms.

Analysis of genomic sequence of Rf candidates between CMS and restorer pepper lines revealed that four SNPs in CDS1, six SNPs in CDS2 and no SNP in CDS3 that commonly existed both in Bukang and Chungyang lines and resulted in amino acid changes. It is interesting that CDS3 showed no SNPs between CMS and restorer lines although CDS3 can be candidate gene for sweet pepper Rf. The lack of divergence in the gene sequence implicated that differences in the promoter region are the likely cause of differences in CDS3 expression. Cellular localization prediction program showed only CDS3 has mitochondrial transit peptides among four candidate genes. Since the most Rf genes previously identified in other crops encode mitochondrion-targeted proteins that interact with CMS related genes or are involved in metabolism or signaling in mitochondria, based on previous observations and this presented here, we suggest that CDS3 is the most likely
candidate for the pepper $Rf$. Further function analysis of CDS3 will help to reveal the molecular mechanism of fertility restoration in pepper. MSGR-A follows the haplotype of Bukang and Chungyang CMS lines in CDS1, but follows the haplotype of the restorer lines in CDS2 and CDS3. SPR03 follows the haplotype of the CMS lines in CDS1, CDS2 and CDS3. So, MSGR-A and SPR03 seems to have different type of $Rf$ co-segregated region compared to chili pepper.

For efficient molecular breeding of CGMS in pepper, development of broadly applicable markers that are tightly linked to $Rf$ has been attempted. But still now, The CGMS system is not currently used for several pepper breeding lines, especially for sweet pepper. The marker developed from the promoter region of CDS3 performs better than previously developed markers in terms of their applicability to a broad range of breeding lines. This marker is expected to be useful in practical molecular breeding of pepper CGMS lines. Although this marker was not able to correctly detect the genotype in some breeding lines, different haplotype can exist even for an allele of the CDS3. Also, there is possibility that there are other $Rf$ sources. In case of rice, two $Rf$ genes ($Rf_5$, $Rf_6$) are involved in fertility restoration of HL type CMS rice (Hu et al., 2012). Further studies on the classification of haplotypes for CDS3 and genetic analysis of $Rf$ for lines in which marker was not applicable is needed to investigate these possibilities.
REFERENCES


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protein is part of a large mitochondrial complex that interacts with transcripts of the CMS-associated locus. *Plant J* 49:217-227


Raphanus sativus Pentatricopeptide Repeat Proteins Encoded by the Fertility Restorer Locus for Ogura Cytoplasmic Male Sterility. Plant Cell 20:3331-3345
CHAPTER 2

Transgenic Expression of the Pepper CMS Associated Gene orf507 in *Nicotiana benthamiana*

ABSTRACT

Cytoplasmic male sterility (CMS) is an inability to produce functional pollen which is caused by mutation on mitochondrial genome. In pepper, CMS is known to be associated with the orf507 gene but direct evidence that this gene directly causes male sterility is still lacking. To test the hypothesis that ORF507 is sufficient to induce male sterility, a set of chimeric constructs were developed. The cDNA of orf507 was cloned in frame with yeast coxIV pr-sequence, and was expressed under tapetum-specific promoter TA29 (construct designated as TCON). For developing control constructs, orf507 was cloned with mitochondrial transit sequence under constitutive CaMV35S promoter (SCON) and without mitochondrial transit sequence under the TA29 promoter (TON) or constitutive CaMV35S promoter (SON). Among several independent transformants obtained with each of the gene cassettes, none of the transgenic plants were male sterile. Furthermore, no differences in floral morphology, pollen viability and germination rate were observed between transgenic and non-transgenic plants. To confirm cellular localization of ORF507 protein, GFP signal of each transgenic construct was
checked and no GFP signal was detected in all of transgenic lines. This may be due to the post-translational process of transgene in tobacco cells.
INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternal genetic attribute that prevents plants from producing functional pollen grains. CMS has been widely used to produce F₁ hybrids to increase fruit yield and reduce labor (Budar and Pelletier, 2001). Economically feasible hybrid seed production requires a good pollination system to prevent self-pollination of the female line. CMS lines, which cannot produce functional pollen grains, are often used as female lines in hybrid seed production to enhance seed purity (Schnable and Wise, 1998). In most cases, CMS is associated with premature degradation of the tapetal cells that nurtures pollen mother cells (Hernould et al., 1998). In sunflower, the ORF (orfH522) related to CMS induced male sterility after introduced into tobacco plants and expressed under the tapetum specific promoter TA29. Bright-field microscopy demonstrated ablation of the tapetal cell layer in transgenic sterile plants containing the orfH522 gene. Premature DNA fragmentation and programmed cell death (PCD) were observed at meiosis stage in the anther of sterile plants (Nizampatnam et al., 2009). In general, CMS is believed to be due to rearrangement of the mitochondrial genomes, which is attributed to the production of new orfs (Schnable and Wise, 1998; Budar and Pelletier, 2001; Hanson and Bentolila, 2004; Yang and Zhang, 2007). Some experimental evidence has confirmed the correlation between CMS-related orfs and occurrence of CMS (Hanson and Bentolila, 2004). In Brassica juncea, transgenic plants containing the orf220 gene, which is associated with CMS, and the mitochondrial target sequence of the β subunit of F1-ATPase were male sterile. In addition, transgenic stem mustard plants exhibited the same
aberrant flower development as the CMS stem mustard phenotype (Yang et al., 2010). As described above, targeted expression in mitochondria of novel orfs has been shown to induce male sterility or semi-sterility in some cases (He et al., 1996; Wang et al., 2006; Kim et al., 2007; Yamamoto et al., 2006). Most of the orfs in the CMS line are associated with mitochondrial energy metabolism complexes. For example, orf522 of sunflower PET1-CMS encodes a protein that shares 18 amino acids with ORFB that is homologous to ATP8 of Reclinomonas (Balk and Leaver, 2001), and orf79 of rice is co-transcribed with the B-atp6 gene and encodes a cytotoxic peptides (Wang et al., 2006). All novel orfs have been shown to be associated with CMS, and mutations in genes encoding mitochondrial enzyme subunits also lead to male sterility (Li et al., 2010).

In pepper (Capsicum annuum L.), CMS was first isolated from an Indian C. annuum entrustment (PI164835) (Peterson, 1958). Molecular studies indicate that male sterility in many CMS lines is associated with the expression of co-transcribed orf456 located downstream with the mitochondrial CoxII gene (Kim et al., 2007), and the pseudogene ψatp6-2, 3'- truncated form of atp6-2 in a maintainer line (Kim and Kim, 2006). Later orf456 was mistakenly annotated due to a sequencing error at the 3'end and reannotated as orf507 (Gulyas et al., 2010).

Expression of the mitochondrion-targeted orf456 gene under the tapetum specific promoter TA29 in the transgenic Arabidopsis has shown that 45% of the T1 transgenic plants were male sterile and seedless, and pollen grains of semi-sterile T1 plants had exine layer defects and vacoulated pollen (Kim et al., 2007). Expression of the orf507 gene in the flowers and leaves encodes a toxin protein that inhibits cell growth and specifically interacts with the subunit of ATP synthase 6. The activity and content of ATP synthase in CMS plants is reduced by more than
half compared with normal plants. Based on these results, the researchers speculated that ORF507 could make impaired MtATP6 (Li et al., 2013). However, there is no experimental evidence that ORF507 is sufficient to induce male sterility.

Based on these reports, we postulated that if ORF507 is sufficient to induce male sterility in pepper and the protein would be able to induce male sterility in a heterologous system. When the CMS related factor ORFH522, in sunflower was targeted into mitochondria, full or partial male sterile was induced in tobacco plants, but no pollen disruption was observed when the protein lacked the mitochondrial targeting sequence. Therefore, we hypothesized that if orf507 is cloned under the tapetum-specific promoter TA29 from tobacco and the protein is targeted into mitochondria using the transit peptide (coxIV pre-sequence), ORF507 might induce male sterility in tobacco. Towards this end, I transformed orf507 under TA29 promoter and the mitochondria transit sequence into tobacco and evaluated fertility of transgenic plants.
MATERIALS AND METHODS

Plant materials

The pepper (*Capsicum annuum L.*) CMS line ‘Bukang A’ carrying the male sterile cytoplasm was used in the study for cloning the CMS related gene, *orf507*. *Nicotiana benthamiana* was used for transformation.

DNA extraction and analysis

Total genomic DNA was extracted from young leaf tissue of plants in a 1.5ml micro tube by the CTAB method (Doyle and Doyle 1987). DNA quality and quantity were measured using a Take3 spectrophotometer (COMPANY, CITY, COUNTRY). Polymerase chain reaction (PCR) analysis was done with different sets of primers to confirm the gene cassette in the transgenic plants. The PCR reaction mixture consist of 100 ng of genomic DNA, 200 μM of each dNTP, 0.2 μM of each forward and reverse primer, 1x concentration of Taq DNA polymerase buffer (10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, 0.01% gelatin), 1 unit of Taq DNA polymerase and triple distilled water to make up the volume to 25 μl. The PCR was programmed for a 5 min denaturation at 95°C, followed by 35 cycles of 95°C for 30 s, annealing temperature for 30 s 72°C for 1min, and a final extension at 72°C for 10 min.

Vector Construction

To construct the vector, the tapetum-specific promoter (TA29) from tobacco and a
mitochondrial transit sequence, and the first 54 amino acids of COXIV from yeast were used. The orf507 gene was taken from the Bukang A, CMS line of *C. annuum*. The PCR amplified sequences were first cloned in the T-Blunt vector using T-Blunt PCR cloning system (Solgent, Daejeon, South Korea). The ligated vectors were sequenced (NICEM, Seoul, Korea) and excised with specific enzymes that were introduced into the primers. The TA29 promoter, coxIV pre-sequence and orf507 were cloned sequentially into T-Blunt vector and transferred into the pCR®8/GW/TOPO® Vector using TOPO TA cloning system (Invitrogen, California, USA). Then TA29-coxIV pre-sequence-orf507 sequence was cloned into pMDC107 vector by Gateway LR Clonase II Enzyme Mix (Invitrogen, California, USA). Plasmids were isolated from cultured cells using AccuPrep® Plasmid Mini Extraction Kit (Bioneer, Daejeon, South Korea). The resultant clone TCON was used for plant transformation. The control was constructed without a mitochondrial transit sequence, TON (pMDC107: TA29-orf507-nos terminator). The SCON (pMDC83: CaMV35S-coxIV presequence-orf507-nos terminator) and SON (pMDC83: CaMV35S-orf507-nos terminator) vector were also constructed for the comparison with the TCON transgenic plants. The vectors were confirmed by sequencing. The confirmed vectors were then transformed into *Agrobacterium tumefaciens* strain GV3101 following the freeze-thaw method of transformation.

**Plant Transformation**

The constructed vectors were used to transform the *N. benthamiana* via leaf disk transformation procedure (Horsch et al. 1985). Explants were infected with the *A. tumefaciens* strain GV3101 containing transformation vector for 1 min. The
infected explants were co-cultured in a solid medium (MS + 1.5 ml/L BAP) for 3 d and then transferred into selection medium (MS + 10 mg/L Hyg + 200 mg/L Cef + 1.5 mg/L BAP). The explants were transferred into fresh medium every 2 week. When shoots began to grow, the shoots were cut and transferred into rooting medium (1/2 MS + 5 mg/L Hyg + 100 mg/L Cef + 0.1 mg/L NAA) for root production. When roots were elongated, the transformants were transferred and acclimated to soil.

**RT-PCR analysis**

Total RNA was isolated from anthers (obtained from floral buds which are 3-5 mm in size) using MG Total RNA extraction kit (MGMED, Seoul, Republic of Korea) according to manufacturer’s description. cDNA was synthesized from 2 μg of total RNA using EasyScript cDNA synthesis kit (Transgene biotech, Beiging, China). The reverse-transcriptase PCR (RT-PCR) was performed using gene specific primer set in 25 μl with 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.2 μM of each primer, 1 μl of reverse transcription products, and 1 unit of Taq polymerase.

**Pollen analysis**

After flowering, transgenic and control plants were scored for pollen production and pollen viability. Plant that failed to produce pollen grains was determined to be male sterile. If pollen dust was noticed, it was stained with fluorescein diacetate (FDA) to check pollen viability. After dust down pollens on a slide glass, FDA acetone stock solution (2 mg/ml) diluted in 0.5 M sucrose solution (final
concentration of 0.5 ug FDA/0.5 M sucrose) was dropped on the pollens and checked viability using Axiophot microscope (Zeiss, Germany). To study the germination rate, pollen grains were cultured on sucrose and boric acid medium (Hernould et al. 1993) and the percentage of germination was recorded at regular intervals.

Based on pollen stainability and germination results, plants were classified as sterile and fertile. If the pollen grains stained negative with FDA and failed to germinate then such plants were classified as male sterile while the plants that produced normal pollen grains were designated as fertile.
RESULTS

Development of gene constructs

Four gene constructs viz, TON (TA29 promoter - orf507 - GFP tag - nos terminator), TCON (TA29 promoter - coxIV pre sequence - orf507 - GFP tag - nos terminator), SON (CaMV35S promoter – orf507 - GFP tag - nos terminator) and SCON (CaMV35S promoter - coxIV pre-sequence-orf507 - GFP tag - nos terminator) were developed using the CMS candidate mitochondrial gene orf507. This gene was cloned either under tapetum-specific (TA29) promoter or under no promoter and with or without the mitochondrial target sequence coding for the first 54 amino acids of COXIV peptide from yeast. The chimeric genes cloned under no promoter were ligated into the vector pMDC83 which contain CaMV35S promoter in front of multiple cloning sites (MCS) and the other chimeric genes were ligated into the vector pMDC107 which contain no promoter in front of MCS (Fig. 1). Both vectors contain the hptII gene driven by 35S promoter for facilitating selection of the putative transformants using hygromycin in vitro. The confirmed vectors were transferred into A. tumefaciens strain GV3101.
Figure II-1. Schematic illustration of gene constructs developed and used. Open arrows indicate promoters. The mitochondrial transit sequence was indicated as dotted boxes.
Transformation of tobacco with the constructs

Using a genetic transformation system, transformed plants were obtained through the shoot differentiation, elongation, and rooting stages (Fig. 2). There were no phenotypic abnormalities between the transformants and the controls that had been infected with the empty vectors. The transformants were checked for the transgenicity using GFP tag specific PCR primers with the non-transgenic plant as the negative control. For each construct at least 6 independent transgenic plants showed the presence of the gene cassette, while no PCR products were observed in the non-transgenic plants (WT) were obtained (data?). Further, we performed qRT-PCR analysis using GFP tag specific PCR primer to check the expression of gene cassettes in the flower buds. The qRT-PCR results showed that expression of the gene cassettes under CaMV35S promoter and TA29 promoter was detected in flower buds. No qRT-PCR products were observed in flower buds in the WT plants.
Figure II-2. Steps showing transformation of *N. benthamiana* with agrobacterium. (A) Seed germination. (B) Blot drying of dissected leaf explants on co-cultivation medium. (C) Callus formation after 4 weeks on selection medium. (D) Shoot elongation in selection medium for 2-4 weeks. Rooting and plant development in rooting medium. (F) Transfer and acclimation of rooted shoots to soil.
Figure II-3. Molecular analyses of $T_0$ transgenic plants. (A) PCR confirmation of transgenic tobacco plants using gfp specific primers. (B) qRT-PCR confirmation of transgenic tobacco plants using gfp specific primers.
Targeting ORF507 into mitochondria in tapetal cell layer doesn’t leads to male sterility

All 8 transgenic plants containing TCON construct showed pollen grains on the anther. The plants carrying either TON, SCON or SON constructs were also all fertile (Table 1). The floral morphology, length of filaments, size and color of anthers were similar between the transgenic male sterile and control plants. Fluorescein diacetate (FDA) staining of pollen grains, which can distinguish viable and non-viable pollen grains, showed that the pollen grains produced on transgenic plants containing TCON construct were all viable compared to transgenic plants containing other construct and WT. The germination frequency of pollen grains from untransformed plants which ranged between 50 and 60% was taken as 100% for calculating the frequency of germination (%) in transgenic plants. Pollen grains produced on TCON transformants showed similar germination frequency indicating normal development of pollen grains (Fig. 4). To check whether orf507 successfully targeted to mitochondria in TCON transgenic plants, we observed GFP signal on leaves compared to wild type and other control plants. There was no GFP signal on leaves of wild type and all of transgenic plants.
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<th>Construct</th>
<th>No of shoots transported into NRM</th>
<th>No of root differentiated transformants</th>
<th>No of soil acclimated transformants</th>
<th>No of fertile plants</th>
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**Table II-1.** Number of transformants realized with each of the chimeric constructs.
**Figure 4.** Comparison of the floral morphology and pollen viability in transformants and wild type control plants. Fully open flower and normal anther filaments in non-transgenic control plant (A) and transgenic plant TCON 2 (D). FDA staining of pollen grains in non-transgenic control plant (B) and transgenic plant TCON 2 (E). Pollen dust from both non-transgenic control plant © and transgenic plant TCON2 (F) germinated normally.
DISCUSSION

CMS is known to be associated with novel orfs resulting from rearrangement of the mitochondrial genome. In pepper, CMS is associated with the expression of a novel mitochondrial gene, orf507, which is expressed as a mitochondrial polypeptide in all the tissues of male-sterile plants. The CMS related orfs regulate sterility by affecting the expression of the co-transcription gene, or by interacting with the protein and changing its biological function, such as the interaction of orfH79 with the atp6 gene in rice. In pepper, orf507 is co-transcribed with the coxII gene, and it was observed that the dysfunction of mitochondrial cytochrome c oxidase in the CMS line. Although the dysfunction of cytochrome c oxidase in the CMS line has been confirmed, it is largely unknown the relationship of the CMS-specific orf507 gene with cytochrome c oxidase and male sterility.

The four chimeric constructs developed with orf507 allowed testing of the effect of expressing this gene either constitutively or only in tapetal cell layer with the option of targeting ORF507 into mitochondria or allowing it to be cytosolic. Transgenic plants were made by agrobacterium-mediated plant transformation method for each orf507 construct. All of transgenic plants were analyzed by PCR and RT-PCR to check gene insertion and expression.

Transgenic tobacco plants carrying orf507 cloned under tapetum specific promoter with mitochondrial transit sequence were not male sterile. GFP tag was used to show intercellular localization of fusion protein but GFP signal was not detected in all of transgenic plants and wild type plants. Therefore we could not confirm whether transformed genes normally translated the proteins or not. There is similar
example that stable transgenic plants of GFP didn’t detected in *Arabidopsis* (Haseloff and Amos. 1995). It seems that no signal detection of GFP sometimes occurs in transgenic plants. Although the exact reason of no GFP was not revealed, there is possibility of post translational regulation mechanism of ectopic proteins in plants. If it is the case, ORF507 protein could be also degraded and it would not be able to see the effect of ORF507 in inducing the male fertility transgenic tobacco. Another possibility is that tag cleavage from a GFP tagged fusion protein. In this case, ORF507 could function its own role without GFP tag but GFP signal could not be detected. To check accumulation of ORF507 in mitochondria, immunoblot analysis of ORF507 is needed.

Even if ORF507 was accumulated in the mitochondria of the tapetal cells, ORF507 could not induce male sterility in the transgenic plants. This lack of male sterility after nuclear expression of *orf507* can be explained by several CMS systems. Inappropriate temporal and spatial control of transgene expression is often presented as the primary explanation for this failure of induction of male sterility. The tapetum has been shown to be a target tissue for a sterility because the tapetal development is initially impaired in male sterile plants (Gourret et al., 1992). The induction of male sterility by the nuclear *orf507* gene will require specific expression of this gene in tapetal cells. Thus, we generated a construct comprising the tapetal-specific promoter TA29 with an ideal spatial and temporal expression profile for this purpose. However, the TA29-*coxIV-orf507* construct did not induce male sterility in transgenic plants. Thus, the lack of *orf507* expression in the correct tissue and at the right time may not be a not the main reason for the failure of induction male sterility in these transgenic plants.

Other possibility of failure of induction male sterility may be to too little
accumulation of the Orf507 protein which must be accumulated a certain threshold levels. Alternatively, the submitochondrial localization of the CMS protein may be responsible (Wintz et al., 1995). If CMS-related genes are expressed in plant species that are different from the plant species from which they originated, the lack of a specific partner for the CMS protein necessary for male sterility may also be a problem. This seems unlikely in this study, but this possibility cannot be ruled out. The orf507 originates from Capsicum annuum and has been successfully transferred to Nicotiana species. As Capsicum belongs to the same family as Nicotiana, partners of CMS in Capsicum are probably also present in Nicotiana. It is also possible that ORF507 alone may not be sufficient for inducing male sterility. To address this question, further study is required using other CMS-related genes such as Ψatp6-2 and orf300 (Jo et al., 2014).
REFERENCES


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세포질 유전자적 웅성불임(CGMS)은 식물 수확량을 향상시키고 병이나 스트레스에 대한 저항성 및 내성을 향상시키기 위해 잡종 종자를 생산하는데 널리 사용되어왔다. 식물 CGMS 는 미토콘드리아와 핵 사이의 유전자 비 양립성(genetic incompatibility)의 결과이며, 일반적으로 미토콘드리아의 비정상적인 키메라 유전자와 관련된다. 고추에서 세포질적 웅성불임(CMS)과 관련된 미토콘드리아 유전자와 임성회복(Rf) 후보유전자가 밝혀졌고, 임성회복 유전자와 연관된 분자 마커가 개발되었다. 하지만 아직까지 분자 육종 시스템에서의 임성회복 유전자 연관 분자 마커의 활용은 제한적이었으며 CMS 관련 미토콘드리아 유전자로 인해 웅성불임이 야기된다는 직접적인 증거는 여전히 부족하다. 그래서 본 연구에서는 임성회복 후보 유전자에 대해 발현량과 염기서열을 분석하였고, 광범위하게 적용 가능한 Rf 마커를 개발하였다. 또한, 고추의 CMS 관련 유전자인의 orf507 을 담배에 형질전환하여 발현시켰다. 첫 번째 장에서는 Rf 후보 유전자인 CDS1, CDS2, CDS3 및 CaPPR6 의 발현량과 염기서열 분석을 수행하였다. 그 결과, Rf 후보 유전자들 중 CDS3 만이 희복전에서 특이적인 발현을 보였다. 분자 마커는 각 후보 유전자의 SNP 와 InDel 을 바탕으로 개발되었으며 Enza zaden 사에서 제공받는 육종 계통에 적용하였다. 그
결과, CDS3 및 CaPPR6 에 기초한 분자 마커가 가장 높은 정확도를 나타냈다.

두 번째 장에서, ORF507 이 식물에서 웅성불임을 유도한다는 가설을 확인하기 위해 orf507 의 cDNA 를 미토콘드리아 전이 서열인 coxIV 와 함께 클로닝하고 담배 식물에서 융단조직 특이적 프로모터인 TA29 하에 발현시켰다. 그러나 예상과는 달리 orf507 이형질 전환된 담배 식물은 웅성불임 표현형을 나타내지 않았다.

주요어: 고추(Capsicum annuum), 세포질적 웅성불임, 웅성불임 회복 유전자, 식물 형질전환

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