Australian Journal of Crop Science AJCS
AJCS 6(3):470-474 (2012) ISSN:1835-2707

Sequence analysis of a specific fragment associated with Ogura CMS in Kale (*Brassica oleracea* var. *acephala*)

Pengfang Zhu[1*],Xing Wang[1],Jian Zhang[1],Ming Xu[2],Yutang Wei[2]

[1]College of Forestry, Shenyang Agricultural University, Shenyang 110866, Liaoning, China;
[2]College of Horticulture, Shenyang Agricultural University, Shenyang 110866, Liaoning, China

***Corresponding author: pengfangzhu@yahoo.com.cn**

Abstract

In our previous works, we bred stable cytoplasmic male sterile (CMS) lines and their corresponding maintainer lines by inter- and intra-specific crosses and subsequent backcrosses in kale (*Brassica oleracea* var. *acephala*). In this study, we cloned a gene related to CMS based on *orf138*, a chimeric gene believed to closely associate with Ogura cytoplasmic male sterility in radish. Polymorphisms were detected among the kale CMS lines and their maintainer lines with a specific 474-bp full-length nucleotide sequence. Among the specific sequence, we identified an entire coding region contained 420-bp open reading frame (Accession No. HQ191478). Compared with the original *orf138* coding sequence, four insertions of nucleotide in 5´terminus and one deletion at the 21st site were found in coding region. The ORF encodes 139 amino acids polypeptide, which had five substitutions with ORF138, *orf138* coding sequence. Sequence analysis of the *orf138* under kale nuclear background contributes a better understanding to nucleocytoplasmic interaction mechanism in cruciferous crops.

Keywords: *Brassica oleracea* var. *acephala*; cytoplasmic male sterility; homology; morphology; *orf138.*
Abbreviations：CMS - cytoplasmic male sterile; NILs - near isogenic lines; ORF - open reading frame; PCR - Polymerase chain reaction.

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait characterized by the inability to produce or release functional pollens without affecting female fertility. Molecular studies on CMS systems have either led to the characterization of new genes or specific to the male sterile cytoplasm. So far, CMS is believed to be associated with specific nuclear-mitochondrial interactions. A theoretical framework has been provided by the concept of nucleocytoplasmic conflict or incompatibility between the nucleus and the cytoplasm (Giancola et al., 2007). Molecular findings of chimeric genes, resulting from duplications, rearrangements, and recombination of mitochondrial DNA sequences, were involved in the control of CMS expression (Saumitou-Laprade et al., 1994; Schnable and Wise, 1998; Hanson and Bentolila, 2004). CMS has been successfully used for production of hybrid seeds in cruciferous species, where *Ogu*, *Pol*, *Nap*, *Cam*, *Nsa*, *Nca*, *Shan 2A* and *Kos* cytoplasm are main types (Wei et al., 2009). Ogura-type CMS, which is derived from wild radish (*Raphanus sativus*) (Ogura, 1968), is one of the most analyzed CMS cytoplasm. It is caused by an aberrant mitochondrial gene, *orf138*, which works in CMS *Raphanus* and *Brassica* plants carrying Ogura cytoplasm (Bonhomme et al., 1992). According to existed molecular mechanism in Ogura CMS, the coding sequence in *orf138* gene was probably inherited steadily from same cytoplasm of radish. In Ogura mitochondrial genome, *orf138* is linked to, and co-transcribed with, another mitochondrial gene, *orf158*, while *orf158* did not relate to CMS for its transcriptions in both sterile and fertile plants (Bonhomme et al., 1992). To date, numbers of *orf138-related* nucleotide variations have been revealed in cruciferous crops. A deletion of 39 nucleotides which consist of one of three repeats in the 3´ part of the coding region was demonstrated in a Japanese radish cultivar and Japanese wild radish (Yamagishi and Terachi, 1996). Ogura *orf138* sequences were classified into nine types on the basis of coding genes in wild radish, cultivated radish and *Raphanus raphanisturum* (Yamagishi and Terachi, 2001). A European wild Ogura-related cytoplasm did not cause sterility while crossed with maintainer radish lines (Giancola et al., 2007). However, what is unclear is how the mitochondrial DNA sequences rearrange under kale nuclei backgrounds. Kale, a popular ornamental and edible plant in the world, plays an important role both on landscapes and vegetables. Kale CMS breeding programmes have been implemented in China recently (Li and Yu, 2006). In our previous study, new and stable CMS lines of kale were bred by inter- or intra-specific hybridization and subsequent backcrosses (Zhu and Wei, 2006). Apart from the practical interest, cytoplasmic male sterility provides us with an invaluable opportunity to study the interaction between mitochondrial and nuclear genes. There were two adverse traits, seedlings chlorosis and nectary degenerating in our original CMS donor parents *Brassica rapa*, but they were both overcome by inter-specific hybridization in F_1. In order to gain a better understanding of the evolution and differentiation of Ogura male-sterile cytoplasm, we conducted the research to analyze gene sequences associated with CMS in kales. To our knowledge, it is the first report on kale CMS molecular characterization. In this study, results of coding nucleotide and deduced polypeptide of the specific Ogura-associated *orf138* could provide unique information to nucleocytoplasmic interaction, reveal the possible mechanism

in CMS kale, and be helpful in understanding the evolution of Ogura male-sterile cytoplasm in *Cruciferae*. Furthermore, the comparison between targeted genes could guide kale CMS breeding programs by means of using relevant information from the same family.

Results

Polymorphism of primers

All candidate primers were amplified under kale genomic DNA. But K_1, K_2, K_3 and K_4 did not show any polymorphism between CMS lines "A1", "A8" and their mutual maintainer line "B1". Polymorphisms were detected under primer K_5 between the above CMS lines and maintainer line with one band at 500bp or so. In order to get more evidences, we amplified two more pairs of CMS lines and corresponding maintainer lines with K_5. Similar polymorphisms were obtained in four male sterile lines "A16", "A18", "A12" and "A20" and their respective maintainer lines "B16" and "B21" (Fig. 2). The results indicated that the polymorphic bands were specific to male sterility.

Sequencing and homology analysis

The nucleotide sequences of PCR products and an entire coding region were examined. Sequencing results showed that all the detected CMS-related fragments were 474 bp full-length nucleotide sequences (Fig. 3), which contained an entire coding region 420-bp open reading frame (Accession No. HQ191478). Compared with the original *orf138* coding sequence (Accession no. Z12626, Bonhomme et al. 1992), four insertions of nucleotide in 5´terminus and one deletion at 21st site were found in the ORF (Table 3). An imperfect 39-bp nucleotide by deletion of "GG" in the 3´ terminus in the last one in the ORF was identical with the original *orf138* (Fig. 3). The specific 420-bp open reading frame coding 139 amino acids polypeptide was designated as ORF139 in current study. Compared to ORF138, five substitutions totally were deduced in ORF139 (Fig. 4). Therefore, differences in nucleotides and coded amino acids indicated specificity of the CMS kale we focused. The homologies between the specific 474-bp nucleotides and *orf138*-related sequences in *Brassica* were high. For instance, we found that there were only two deleted "A" in our sequence (HQ191478) compared with CMS *B. oleracea* mitochondrial Ogura CMS-related protein gene (GQ464371) and *B. napus* cybrids mitochondrial atpA gene (Z12627). All the analysis above indicated that the specific 420-bp nucleotide was homologous to Ogura CMS-related gene, *orf138*.

Discussion

In this study, PCR analysis revealed that *orf138* was present in the kale male sterile lines within a specific 474-bp full-length sequence, which was absent in their corresponding maintainer lines. A total of five mutations (four nucleotide insertion and one deletion) were identified in the *orf138* of Ogura-type cytoplasm. This is the first report exploring Ogura CMS-related nucleotide-sequence variations in kale. The results benefit evolutionary genetics at intra- and inter-specific or intergenetic levels in cruciferous plants. Sequencing of the specific *orf138*-related gene under kale nuclear background contributes a better understanding of nucleocytoplasmic interaction mechanism. Previous researches have indicated that CMS trait was closely associated with mitochondrial DNA

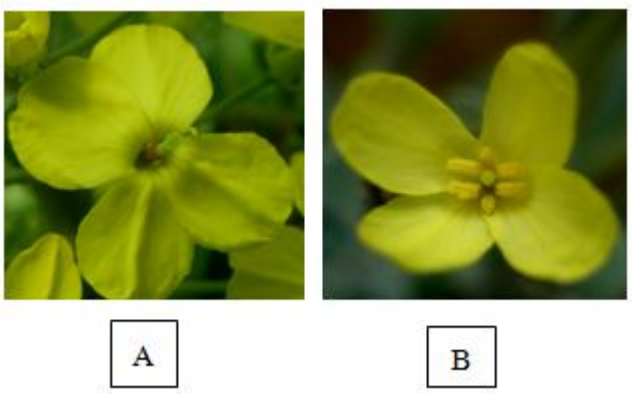

Fig 1. Comparison of floret between a cytoplasmic male sterile line and its fertile maintainer line. *A* male sterile line, indicates short and undeveloped stamens. *B* maintainer line, indicates six long and developed stamens.

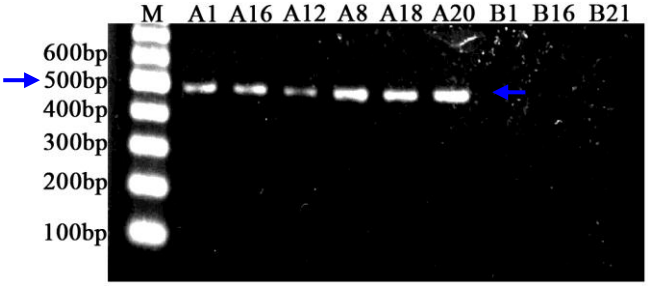

Fig 2. Amplifications and polymorphisms found between CMS lines and their respective maintainer lines under primer K_5. *M*: DNA ladder. *A lanes*: CMS lines A1, A16, A12, A8, A18, and A20, the six accessions had been detected targeted CMS-related bands in 500bp or so. *B lanes*: Maintainer lines B1, B16, and B21, the targeted bands were all absent in the three accessions.

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ACGGGAAGTG ACAATACCGC TTTTCTTCAG CATATAAATG CATGATTACC TTTTTCGAAA
ATTGTCCACT TTTTGTCATA ATCTCACTCC TACTGAATGT AAAGTTAGTG TAATAAGTTT
CTTTCTTTTA GCTTTTTTAC TAATGGCCCA TATTTGGCTA AGCTGGTTTT CTAACAACCA
ACATTGTTTA CGAACCATGA GACATCTAGA GAAGTTAAAA ATTCCATATG AATTTCAGTA
TGGGTGGCTA GGTGTCAAAA TTACAATAAA ATCAAATGTA CCTAACGATG AAGTGACGAA
AAAAGTCTCA CCTATCATTA AAGGGGAAAT AGAGGGGAAA GAGGAAAAAA AAGAGGGGAA
AGGGGAAATA GAGGGGAAAG AGGAAAAAAA AGAGGGGAAA GGGGAAATAG AGGGGAAAGA
GGAAAAAAAA GAGGTGGAAA ATGGACCGAG AAAATAATGC TTTGTGAACC CAAT
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Fig 3. A 474-bp, full-length, *orf138*-related nucleotide sequence in kale. The box indicates the 420-bp open reading frame. The underlines with arrows indicate three 39-bp repeats, the last one an imperfect in 3´ terminus.

rearrangements, which resulted in formation of novel chimeric genes. In *Cruciferae*, molecular data are in agreement with theoretical models that consider CMS as a stage in the co-evolution between nucleus and mitochondria, and not simply as a deleterious mitochondrial mutation (Bonhomme et al., 1992). In our study, differences in open reading frame and polypeptides deduced were both present. Similar evidence about *orf138* polymorphisms has been reported (Yamagishi and Terachi, 2001). In the processing of the kale CMS lines breeding, several kinds of genomes such as Chinese cabbage (*Brassica rapa*) and cabbage (*B. oleracea*) have been involved

Table 1. Accessions of cytoplasmic male sterile lines and their corresponding maintainer lines used in this study.

Name /type	Accession number	Type of leaves	Color of inner leaf	Male fertility	Origin
B1/M[a]	sykB1	wavy	pink	MF[c]	selfing
A1/CMS[b]	sykA1	wavy	pink	MS[d]	interspecific cross[e] and backcross with B1
A8/CMS[b]	sykA8	wavy	pink	MS[d]	intraspecific cross[f] and backcross with B1
B16/M[a]	sykB16	crinkled	white	MF[c]	selfing
A16/CMS[b]	sykA16	crinkled	white	MS[d]	interspecific cross[e] and backcross with B16
A18/CMS[b]	sykA18	crinkled	white	MS[d]	intraspecific cross[f] and backcross with B16
B21/M[a]	sykB21	crinkled	purple red	MF[c]	selfing
A12/CMS[b]	sykA12	crinkled	purple red	MS[d]	interspecific cross[e] and backcross with B21
A20/CMS[b]	sykA20	crinkled	purple red	MS[d]	intraspecific cross[f] and backcross with B21

Notes: [a] maintainer line, [b] cytoplasmic male sterile line, [c] male fertile, [d] male sterile, [e] interspecific cross between improved radish cytoplasmic CMS *Brassica rapa* and *B. oleracea* var. *acephala*, [f] intraspecific cross between improved radish cytoplasmic CMS *B. oleracea* var. *capitata* and var. *acephala*

Table 2. Primer sequences and general information.

Primer name		Sequence (5′ to 3′)	References
K_1	K_1^+	TGGTCAACTCATCAGGCTC	Chen et al. (2009)
	K_1^-	GCCTCTAGGAGTAGTGAAGAAC	
K_2	K_2^+	CCATATTTGGCTAAGCTGGTTTTCT	Wang et al. (2006)
	K_2^-	TATCATCTCGGTCCATTGTCCAC	
K_3	K_3^+	GAATTTCAGTATGGGTGGC	Zhao et al. (2008)
	K_3^-	AGCAGTTGGTTCCGTAGTT	
K_4	K_4^+	GCAATGATTACCTTTTTCGA	Li et al. (2009)
	K_4^-	GCATTATTTTCTCGGTCCAT	
K_5	K_5^+	ACGGGAAGTGACAATACC	Chen et al. (2009)
	K_5^-	ATTGGGTTCACAAAGCAT	

Notes: "+": forward primer, "–": reverse primer

Table 3. Comparison of nucleotide changes between the CMS-related ORF in kale and the original *orf138* coding sequences. *a* single-base deletion.

Accession no.	Nucleotide site in the coding region of *orf138*				
	-4	-3	-2	-1	21
HQ191478	A	T	G	C	-[a]
Z12626					A

Notes: *a* single-base deletion.

in it. Compared to the original Chinese cabbage what we used for CMS donor parent, kale cytoplasmic male sterility lines overcome seedlings chlorosis and nectariy degenerating. So interaction between nuclear genes in kale and *orf138*, or variation of *orf138* sequences under kale nucleus background may be hypothesized reasons of it. Further studies on the relationship of molecular mechanism and phenotypes of kale CMS need to be continued in more details. CMS is not only an important characteristic for the analysis of nuclear and mitochondrial DNA interactions, but also for plant breeding in the production of F_1 hybrids. CMS is often induced by interspecific or intergeneric sexual crosses or somatic protoplast fusion in cruciferous plants. Kale, an old-fashioned vegetable in Europe, has become more and more popular in China recently. Apart from abundantly nutritious as vegetable, kale displays its beauty as an ornamental plant in winters by bright colors and beautiful flat, wavy or crinkled leaves during stage of rosette. Kale cytoplasmic male sterility lines have been developed in our previous work. The achieving of the male sterility lines and their respective maintainer lines plays an important role on heterosis breeding programme in kales. Restoration of fertility is another concern in crops such as rapeseed (*Brassica napus*), rice (*Oryza sativa*), maize (*Zea mays)* and petunia (*Petunia hybrida*), where seeds are always needed. Optimistically, restorer lines are unnecessary in kale hybrid seed production because foliage are normally specific economic parts, so traits related to leaves or vegetative parts are normally concerned instead of seeds.

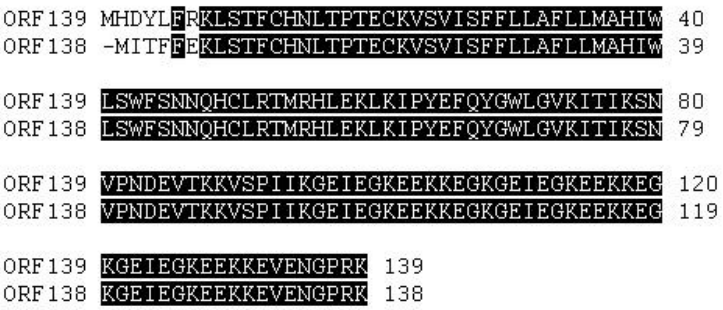

Fig 4. Sequence comparison of ORF139 and the original ORF138. The letters did not blackened indicate five substitutions deduced in ORF139 except M.

Materials and methods

Plant materials

Six male sterile lines and corresponding maintainer lines were listed in Table 1. Male sterile lines were continuously bred by intra- and inter-specific hybridization and backcrosses with the recurrent parents. Simultaneously, maintainer lines, which were used as the recurrent parent, were self-crossed. All the accessions were grown in experimental field for vegetative growth and greenhouse for reproductive growth. Fertility of the floral was assessed by identification of stamens morphology and observation of anthers under microscope. The CMS lines and their maintainer lines could be nearly treated as near isogenic lines (NILs). Except for stamens morphology, differences between NILs were not clear, take main ornamental traits in rosette for instance (Table 1). CMS lines ("A" lines) could be classified with maintainer lines ("B" lines) for undeveloped stamens in "A" than "B" lines (Fig. 1).

DNA isolation

Ten individual plants from each accession were chosen randomly for genomic DNA isolation. Genomic DNA was extracted from fresh young leaves during rosette under protection of liquid nitrogen using a Plant DNA Kit provided by Tiangen Biotech (Beijing) Co. LTD, P.R. China (http://www.tiangen.com). DNA quality and quantity were evaluated on 1.0% agarose gel and micro-spectrophotometer, respectively.

Primer screening and PCR amplification

Primers specific to Ogura CMS were selected (Table 2). All these primers were synthesized by GenScript Corporation (Shanghai, P.R. China). Polymerase chain reaction (PCR) amplification was performed using 50 ng of genomic DNA in a final volume of 20 μL, containing 10 × PCR buffer (containing Mg^{2+}), 1.6 μM of each primer, 4 μM of each dNTP and 0.75 unit r*Taq* DNA polymerase (Takara, Dalian, China). The following amplification protocol was carried out in a thermal cycler. An initial denaturing step was performed at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min and extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min. The amplification products were analyze by electrophoresis on 2.0 % (w/v) agarose gels. The gels were stained with ethidium bromide and visualized under UV light.

Nucleotide sequencing and sequence analysis

Target PCR products were excised under UV light, and then purified by using gel extraction and purification kit (Tiangen Biotech Co. LTD, Beijing, P.R. China). Sequencing was preformed by Takara Biotechnology (Dalian, China) Co., Ltd.

Conclusion

Cloning of an entire coding region contained 420-bp open reading frame (Accession No. HQ191478) in kale contributes to a specific understanding of nucleocytoplasmic interaction mechanism in cruciferous plants. Compared with the original *orf138* coding sequence, four insertions of nucleotide in 5´terminus and one deletion at the 21st site were found in coding region. The ORF encoded 139 amino acids polypeptide, which had five substitutions compared to *orf138* coding sequence.

Acknowledgments

This work was supported by scientific project Foundation of Liaoning Province Office of Education, China (Grant No. 2008664). Thanks to Prof. Fang from Chinese Academy of Agricultural Science for providing CMS cabbage and Prof. Wei from Shenyang Agricultural University for providing CMS Chinese cabbage.

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