Plant Breeding

doi:10.1111/pbr.12416

Cytological observation of anther structure and genetic investigation of a new type of cytoplasmic male sterile 0A193-CMS in *Brassica rapa* L.

Qianxin Huang[1], Xiaoyang Xin[1], Yuan Guo[1,2] and Shengwu Hu[1,2]

[1]State Key Laboratory of Crop Stress Biology in Arid Areas and College of Agronomy, Northwest A&F University, Yangling, Shaanxi, 712100, China; [2]Corresponding author, E-mails: guoyuan2109@163.com; hushengwu@hotmail.com

With 5 figures and 4 tables

Received March 30, 2016 / Accepted August 22, 2016
Communicated by A.-M. Chevre

Abstract

Cytoplasmic male sterility (CMS), a maternally transmitted failure in pollen formation, is an effective pollination control system in hybrid rapeseed (*Brassica napus*) breeding. However, CMS is not widely used in the related oilseed species *Brassica rapa.* In the past years, several male sterile plants have been isolated from the *B. rapa* landrace '0A193', collected in Shaanxi, China, in 2011. It is noteworthy that the fertility expression of 0A193-CMS was affected by temperature. In contrast to *pol* CMS, fertility tests with 18 *B. rapa* and 9 *B. napus* accessions suggest that a different system of maintaining and restoring is responsible for the observed phenotype. Further on, genetic investigation evidenced that fertility of 0A193-CMS is controlled by both cytoplasmic and one pair of nuclear recessive genes. Interestingly, plants of the 0A193-CMS type possess a highly specific fragment of the mitochondrial gene *orf222*, a crucial regulator of male sterility in *nap* CMS. Our study broadens the CMS resources in *B. rapa* and provides a highly applicable alternative to *pol* CMS and *ogu* CMS for hybrid breeding production.

Key words: *Brassica rapa* L. — cytoplasmic male sterility — anther abortion — inheritance — maintaining and restoring relationship

Brassica rapa L. (AA, 2n = 20) is an important oilseed crop worldwide and is also a major vegetable and fodder plant (Prakash and Hinata 1980). *Brassica rapa* has a broader geographic distribution than its related hybrid species *B. napus* (AACC, 2n = 38). Plants of this species are widely cultivated in Europe, East Asia and South Asia. Up to date, *Brassica* species have been very successful taking advantage of the heterosis phenomenon in hybrid cultivars. This fact is due to very effective pollination control systems, such as cytoplasmic male sterility (CMS), genic male sterility (GMS), ecological male sterility (EMS), self-incompatibility (SI) and chemical hybridization agent (CHA) (Fu and Tu 2002). Among these pollination control systems, CMS is the most widely distributed, providing an expedient mechanism for the large-scale production of *Brassica* hybrid seeds (Havey 2004). In addition, CMS exemplifies the importance of mitochondrial function in pollen development.

Cytoplasmic male sterility is a maternal genetic trait encoded in the mitochondrial genome that produces the offspring with no viable pollen in higher plants (Chen and Liu 2014). According to the anther developmental stages and mechanism of anther abortion, CMS can be classified into three major classes: (i) no-pollen-sac abortion, (ii) microspore mother cell abortion and (iii) microspore abortion (Yu and Fu 1990). Among the no-pollen-sac abortion class, *pol* CMS and *nap* CMS, in which anther development is inhibited at sporogonium differentiation stage, are representative types (Bartkowiak-Broda et al. 1979, Yu and Fu 1990). The anthers of SaNa-1A CMS type are aborted at the late pollen mother cell stage with the tapetum showing prior vacuolization, suggesting that this CMS type belongs to the microspore mother cell abortion class (Wang et al. 2014). Further on, anther abortion in *Nsa* CMS is initiated from the first meiotic division to tetrad stage (Liu et al. 2015) and that in *ogu* CMS, *NCa* CMS from tetrad stage to uninucleate stage (Ogura 1968, Gonzalez-Melendi et al. 2008, Wei and Wang 2009), so indicating that these CMS types belong to the microspore abortion class.

Concerning regulatory genes, the male sterile phenotype of CMS lines arises as a result of alterations in chimeric open reading frames (ORFs) in the mitochondrial genome (Yamagishi and Bhat 2014). It has been proven that *ogu*-, *nap*- and *pol* CMS are associated with the mitochondrial genes *orf138*, *orf222/nad5c/orf139* and *orf224/atp6*, respectively (L'Homme and Brown 1993, L'Homme et al. 1997, Duroc et al. 2005, An et al. 2014).

In agriculture, several CMS systems have been used in rapeseed (*B. napus*) F_1 hybrid production. Among them, *pol* CMS is regarded as the first practical valuable CMS system with widely distributed maintainers and restorers. However, this system is sensitive to environment factors in some nuclear backgrounds, leading to undesired self-fertilization during the process of hybrid seed production (Fu 1981, Fan and Stefansson 1986, Verma et al. 2000). On the other hand, the *nap* CMS type has many restorers in European and Japanese materials, however, its sensitiveness to temperature limits the widespread of its use for hybrid production (Fan and Stefansson 1986). Further on, the *ogu* CMS system is stable in several genetic backgrounds, but its implementation is hampered by the patent protection of restorers and difficulties in finding the *Rf* gene. Particularly, the genetic drag associated with the introgression of radish genomic fragments in most existing restorers makes it hard to get double low hybrids based on this CMS (Engelke et al. 2011).

The dominance of a few types of cytoplasm in crop heterosis breeding has a great risk on the production. For example, due to the large use of T-cytoplasmic maize hybrids, *Helmintosporium maydis*, a major disease was spread having devastating consequences for the maize production in America (Laughnan and Gabay-Laughnan 1983). At the present time, only the *pol* CMS is extensively used in commercial rapeseed hybrid production in China, not excluding a high risk for disease epidemics, and may decrease the yield in rapeseed hybrids due to its single cytoplasm (Liu et al. 2012). Therefore, the discovery and characterization of new male sterility source is one of the most important milestones for heterosis utilization in rapeseed (including *B. napus* and *B. rapa*).

Within the last six years, several male sterile plants were found in one *B. rapa* accession '0A193' collected from Yimen Town, Binxian, Shaanxi Province, China, in April 2011. In the

present study, these male sterile plants were characterized by morphological observation of floral organs, cytological observation of the anther development and genetic investigation. Our results suggested that fertility of this male sterile accession was controlled by both cytoplasmic and one pair of nuclear recessive genes. Gene-specific PCR results indicate that 0A193-CMS plants belong to the *nap* CMS type. Our study provided useful information for broadening the CMS resources in *B. rapa*, overcoming the negative effects of using single cytoplasm heterosis breeding.

Materials and Methods

Plant materials: In April 2011, our group found several male sterile plants in *B. rapa* materials collected from Yimen Town, Binxian, Shaanxi Province, China. This material received the accession code '0A193'. These plants were characterized by crossing with several rapeseed accessions and F_1 plants were sterile, and no restorers were found in 2012. Moreover, their testcross offspring were male sterile at low temperature in early florescence, while fertility was regained when temperature increased in later florescence. Two male sterile lines named 2A345 and 2A397 derived from *B. rapa* accession '0A193', along with other 27 tested accessions consisting of 18 *B. rapa* and 9 *B. napus*, were used as plant materials in this study. The origins of materials are listed in Table 1.

Observation of floral organ morphological characteristics: Morphological characterization of the plant materials was carried out along March 2013. Sampling of the plants was carried out at the same time window (between 8:00 and 9:00). The sterile flower buds of male sterile lines 2A345 and 2A397 were sampled at early flowering stage (19 March) marked as 2A345(x) and 2A397(x), and the fertile flower buds were taken at fully flowering stage when fertility stamens appeared (27 March) and marked as 2A345(y) and 2A397(y), respectively. The flower buds of fertile material Baiyu (Table 1), named as 2A347, were used as control.

Cytological observation of anther development: The floral buds of this male sterile material before and after fertility changeover were collected and fixed in Carnoy's fluid for 2 h, sequentially followed by steps of dehydration, finally transferred to 70% ethanol and stored at 4 °C until use. For paraffin sectioning, the samples were dehydrated and cleared with xylene, sequentially embedded and transversely sectioned (7 μm) with YD-202A slicing machine (Yi Di Medical Facility Factory, China). Finally, the anthers were observed under an OLYMPUS BX51 microscope (Olympus, Japan) after dewaxing, rehydration and dyeing. The procedure of whole clearing technique followed the methodology reported by Yu et al. (2009).

Genetic research: The male sterile line 2A345 was used as female parent to be crossed with 18 *B. rapa* and 9 *B. napus* accessions (Table 1). The fertility of their offspring indicated that two *B. rapa* accessions Beiji Youcai and Yimen Youcai can restore the fertility of 2A345. Therefore, F_1, BC_1 and F_2 populations derived from the crosses between Beiji Youcai, Yimen Youcai and 2A345 were used for genetic study.

DNA isolation and mitochondrial gene-specific primer design: Young leaves were collected for total DNA extraction using a modified CTAB method (Doyle 1990). The DNA samples were then diluted with RNase-free water to about 50 ng/μl. Specific primer pairs for the genes *Orf138*, *Orf222* and *Orf224,* which were reported by Wei et al. (2005) were synthesized (Table 2).

PCR amplification: The PCR mixture (20 μl) contained 50 ng template DNA, 150 μM dNTPs, 1 × PCR buffer, 0.15 μM each primer and 0.25U *Taq* DNA polymerase (TianGen, China). PCR amplification was carried out with predenaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 56 °C for 50 s, 72 °C for 70 s and a final extension at 72 °C for 10 min.

Cloning and sequencing: After the detection of DNA on agarose gel electrophoresis, target DNA fragments were purified with Gel Extraction

Table 1: The tested accessions and fertility of their testcross F_1 populations with the *B. rapa* male sterile accession

No.	Name	Origin	Type	Fertile plants	Sterile plants
1	Baiyu	Zhejiang, China	*B. rapa*	0	61
2	Xinming Youcai	Binxian, Shaanxi, China	*B. rapa*	0	150
3	Winter Rape No.1	Gansu, China	*B. rapa*	0	90
4	Fenyang Youcai	Shanxi, China	*B. rapa*	0	15
5	Gaokeyinzhong	Gansu, China	*B. rapa*	0	75
6	Yellow sarson	India	*B. rapa*	0	30
7	Jingninghongheizi	Zhejiang, China	*B. rapa*	0	30
8	Linyi Youcai	Shanxi, China	*B. rapa*	0	30
9	Cuimu Youcai	Linyou, Shaanxi, China	*B. rapa*	0	90
10	Longquanhei Youcai	Zhejiang, China	*B. rapa*	0	165
11	Tianyou No.8	Gansu, China	*B. rapa*	0	75
12	Tianyouxinxuan	Gansu, China	*B. rapa*	0	75
13	Xinjiangxian Youcai	Shanxi, China	*B. rapa*	0	30
14	Yongshou 147	Yongshou, Shaanxi, China	*B. rapa*	0	90
15	Huaipinglinchang Youcai	Yongshou, Shaanxi, China	*B. rapa*	0	90
16	Tongshuwan Youcai	Yongshou, Shaanxi, China	*B. rapa*	0	90
17	Beiji Youcai	Binxian, Shaanxi, China	*B. rapa*	60	0
18	Yimen Youcai	Binxian, Shaanxi, China	*B. rapa*	60	0
19	HYZ-01	Pol CMS restorer, Shanghai, China	*B. napus*	60	0
20	Westar	Nap CMS restorer, Canada	*B. napus*	90	0
21	Zhongshuang No.7	Pol CMS maintainer, Hubei, China	*B. napus*	165	0
22	Zhongshuang No.2	Pol CMS maintainer, Hubei, China	*B. napus*	40	0
23	Zheshuang 72	Pol CMS maintainer, Hubei, China	*B. napus*	40	0
24	Shaan 2B	Pol CMS maintainer, Shaanxi, China	*B. napus*	0	40
25	Pol B	Pol CMS restorer, Hunan, China	*B. napus*	0	40
26	9722	Pol CMS restorer, Shaanxi, China	*B. napus*	40	0
27	8C	Pol CMS restorer, Shaanxi, China	*B. napus*	0	40

Table 2: PCR primer used in this study for amplifying specific mitochondrial gene fragments

Primer name	Orientation	Sequence (5'-3')	Target Gene	Expected size in the target gene
P11-F	Forward	GAAACGGGAAGTGACAAT	*Orf138*	465 bp
P12-R	Reverse	GCATTATTTTCTCGGTCCAT	*Orf138*	465 bp
P21-F	Forward	AGCTGTCTGGAGGGAATC	*Orf222*	1102 bp
P22-R	Reverse	GCGGTCTCACGCACTAATC	*Orf222*	1102 bp
P31-F	Forward	AGCTGTCTGGAGGGAATC	*Orf224*	747 bp
P32-R	Reverse	ACGACATCAAGGAGGAAC	*Orf224*	747 bp

Kit (TianGen, China), later ligated to the pMD19-T Vector (Takara, China) and transformed into DH5α (TianGen, China). Five positive clones were selected of each PCR product for sequencing.

Effects of temperature on male fertility materials: The experimental materials were sown at 25 September 2013 in Yangling, Shaanxi, China (latitude 34°N; longitude 108°E). After vernalization, the seedlings of 2A397 were transplanted into greenhouse for one week and then transplanted into growth chambers. According to previous field work, it is most likely that the critical stage for fertility was around 10 days before flowering. '0A193' exhibits male sterility at average air temperature below 11.83 °C and became male fertile over 16.17 °C (data not shown). Therefore, three different temperature regime treatments were applied in growth chamber: 15 °C/10 °C (mean = 12.5 °C), 20 °C/8 °C (15 °C) and 22 °C/17 °C (19.5 °C) (day/night, 14 h/10 h, light intensity: 40000 Lux). The fertility of newly opened flowers was observed everyday according to the grading standard of fertility by Yang and Fu (1991).

Results

Morphological characteristics of floral organ of the *B. rapa* male sterile line

Flowers of male fertile line 2A347 were well developed (Fig. 1, F). Compared with the control plants, the flowers of male sterile lines 2A345 and 2A397 displayed obvious morphological differences before and after the fertility changeover. Before the sterility changeover, various floral organ abnormalities were observed in 2A345(x) and 2A397(x), including crimpy petals and significantly degraded, dehydrated and dried triangular stamens, thinner and shorter filaments (Fig. 1a–S). Additionally, irregular numbers of stamens and pistil were observed and the sterile anthers produced little or no pollen grains (Fig. 1a–S, Fig. 2S). After the sterility changeover, the flowers of 2A345(y) and 2A397(y) looked normal as the control plants (Fig. 1b–S), and anthers of 2A397(y) could produce enough pollen grains (Fig. 2T).

Cytological observations of anther development

To determine the sterile stage, fertile and sterile anthers were cytologically observed in different development stages. The transverse sections of the anther at engorged pollen stage showed marked differences between the male sterile accessions and the control. The anther was normal in the fertile material 2A347, and the wall of pollen sac was composed of three layers in immature anther: epidermis, middle lamella and tapetum, which is closely arranged at the innermost of anther chamber (Fig. 3F). After the maturation of anther, only epidermal cells remained in the wall of pollen sac, and the rest layers disappeared. Various abnormalities were observed in 2A345(x), which is male sterile during the anther development. Most pollen sacs stopped to develop even before the differentiation of archesporial. Thus, only parenchymal cells instead of pollen sac were produced, and the epidermis, fibrous layer and tapetum were not able to be formed, impairing the differentiation of the pollen mother cells (Figs 3, S2). Only a few individual corners were differentiated into pollen sacs, however, abnormal epidermis and vacuolization of middle lamella were observed in certain degree (Figs 3, S3). Tapetum and microspore degraded earlier at engorged pollen stage (Figs 3, S2). Anther locules were not able to open (Figs 3, S4), and adhesive pollen (Figs 3, S3) was observed in mature anther. In addition, zero or multiple anther connectives (Figs 3, S1, S2) and concrescent anthers (Figs 3, S1) were also observed in sterile materials. After the sterility changeover, the progress of anther development in 2A345(y) became normal as the fertile line 2A347, except that a few pollen sacs showed a retarded growth (Fig. 3T).

Genetic research

Twenty-seven accessions (18 *B. rapa* and 9 *B. napus*) were selected to testcross with the aforementioned male sterile

Fig. 1: Flower organs of the male sterile plants before and after sterile-to-fertile transition. (a): Before fertility transition, (b): After fertility transition. S: Male sterility, F: Male fertility

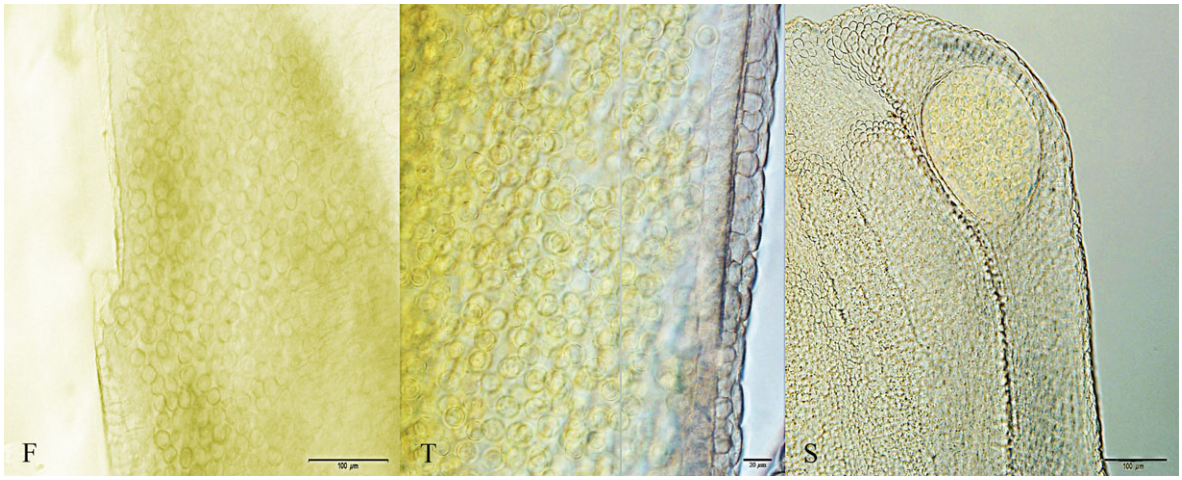

Fig. 2: Comparison of pollen grains number between the fertile accession with male sterile accession before and after fertility transition by whole transparent observation. F: Fertile material, T: Fertility-transformed sterile material, S: Male sterile material

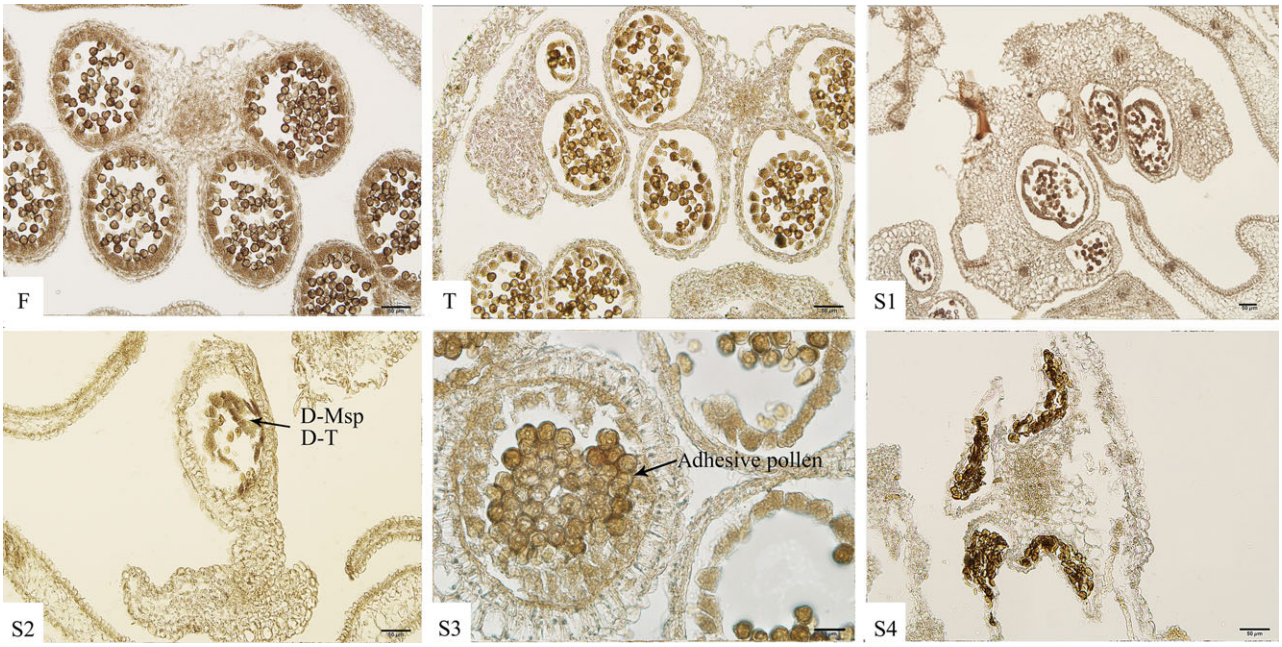

Fig. 3: Comparison of anther features of sterile materials and normal fertile materials in *B. rapa*. S1-4: Sterile anthers before fertility transition, T: Sterile anthers after fertility transition, F: Fertile anther. D–T: degenerated tapetum; D-Msp: degenerated microspores. Scale bars = 50 μm

accession. The testcross F_1 of two *B. rapa* accessions (Beiji Youcai and Yimen Youcai) and six *B. napus* accessions (HYZ-01, Westar, Zhongshuang 7, Zhongshuang 2, Zheshuang 72 and 9722) was fertile, however, that of other 19 accessions was sterile. Interestingly, fertility results of F_1 indicated that the *B. rapa* male sterile line used in this study has different maintaining and restoring relationship with *pol* CMS. 8C is the restorer of *pol* CMS, but here is the maintainer of this male sterile accession, Zhongshuang 7 and Zhongshuang 2 is the maintainer of *pol* CMS, but is the restorer in the present study (Table 1). The inheritance of this male sterile accession was investigated by observing the fertility results of the testcrossed F_1, F_2 and BC_1 populations with its restorers (Beiji Youcai and Yimen Youcai). The F_1 populations between this male sterile accession and its restorers were all male fertile, the F_2 population segregated in a 3 : 1 ratio for fertility to sterility, and the BC_1 population segregated in a 1 : 1 ratio for fertility to sterility (Table 3). Consequently, it was proposed that fertility of this male sterile accession was controlled by both cytoplasmic gene and a pair of genic recessive genes.

Identification of cytoplasm type by the multiple PCR

The associated genes for CMS were reported in *Brassica* include *orf138* for *ogu* CMS (Duroc et al. 2005), *orf222*/*nad5c*/*orf139* for *nap* CMS (L'Homme and Brown 1993) and *orf224*/*atp6* for *pol* CMS (L'Homme et al. 1997). Three pairs of primers that were specifically designed for *orf138*, *orf222* and *orf224* were used to amplify male sterile accessions 2A345 and 2A397 together with RF04(R) (*ogu* CMS type), Westar (*nap* CMS type) and Zhong 9A (*pol* CMS type) through the multiple PCR (Wei et al. 2005, Zhao et al. 2010) in the present study. As expected,

Table 3: Inheritance of the male sterile accession in *B. rapa*

Code	Pattern of crossing	Name of male parent	Fertile plants	Sterile plants	χ^2_C (3:1)	χ^2_C (1:1)
1A160	F_1	Beiji Youcai	45	0	–	–
1A161	F_1	Yimen Youcai	45	0	–	–
2A174	F_2	Beiji Youcai	43	19	0.77	–
2A155	F_2	Yimen Youcai	47	16	0.01	–
2A170	BC_1	Beiji Youcai	48	58	–	0.76
2A152	BC_1	Yimen Youcai	65	60	–	0.13

$\chi^2_{0.05,1} = 3.84$, $\chi^2_{0.01,1} = 6.63$.

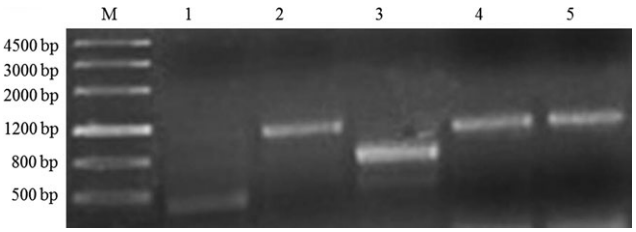

Fig. 4: Analysis on the male sterile accessions by a multiplex PCR. M: Marker, Lane 1: RF04(R) (*ogu* CMS type), Lane 2: Westar (*nap* CMS type), Lane 3: Zhong 9A (*pol* CMS type), Lane 4: 2A345, Lane 5: 2A397

a specific amplification product of about 465 bp was yielded in RF04(R), which was specific for *orf138* (*ogu* CMS type), 1102 bp in Westar, which was specific for *orf222* (*nap* CMS type), and 747 bp in Zhong 9A, which was specific for *orf224* (*pol* CMS type). A specific amplification product of approximately 1102 bp was amplified in both male sterile materials 2A345 and 2A397 (Fig. 4) and was cloned and sequenced. Sequence alignment with DNAman software showed that these products shared 100% nucleotide and protein (669 amino acid) identity with the sterility gene at the *orf222* (gi: 114148846) locus of *nap* CMS, indicating that this male sterile accession had the same cytoplasm type as *nap* CMS (Fig. 5).

Effect of temperature on fertility

In the field experiments, we found that the fertility of this material changes over gradually from sterile to fertile during florescence about 1 week to 10 days. The later result of growth chamber experiment revealed that this male sterile accession is temperature sensitive and it tends to be in sterile condition under low temperature (15 °C/10 °C), and it turns into fertile when temperature increases to a certain high. When day/night temperature was 22 °C/8 °C and 22 °C/17 °C, there were no obvious differences in male sterility (Table 4). However, the lowest (15 °C/10 °C) treatment made the fertility-level rise from 2.68 to 5.84 after flowering of 10–12 days (Table 4). Thus, based on the fertility of newly opened flowers, it could be concluded that the temperature-responding stage was 10–12 days after flowering in growth chamber (Table 4), and the critical temperature of fertility sensitivity of '0A193' accession is under 15 °C/10 °C (day/night) (Table 4). Moreover, the fertility of this male sterile accession is not only affected by a range of certain temperatures but also did by the cumulative effect of temperature.

```
2A345     ATGCCTCAACTGGATAAATTCACTTATTTTTCACAATTCTTCTGGTTATGCCTTTTCTTC
2A397     ATGCCTCAACTGGATAAATTCACTTATTTTTCACAATTCTTCTGGTTATGCCTTTTCTTC
orf222    ATGCCTCAACTGGATAAATTCACTTATTTTTCACAATTCTTCTGGTTATGCCTTTTCTTC
          ************************************************************
protein    M  P  Q  L  D  K  F  T  Y  F  S  Q  F  F  W  L  C  L  F  F

2A345     TTTACTTTCTATATTTTCATATGCAATGATGGAGATGGAGTACTTGGGATCAGCAGAATT
2A397     TTTACTTTCTATATTTTCATATGCAATGATGGAGATGGAGTACTTGGGATCAGCAGAATT
orf222    TTTACTTTCTATATTTTCATATGCAATGATGGAGATGGAGTACTTGGGATCAGCAGAATT
          ************************************************************
protein    F  T  F  Y  I  F  I  C  N  D  G  D  G  V  L  G  I  S  R  I

2A345     CTAAAACTACGGAACCAACTGCTTTCACACTGGGGTAAGACCATCCAGAGCAAGCTAAAG
2A397     CTAAAACTACGGAACCAACTGCTTTCACACTGGGGTAAGACCATCCAGAGCAAGCTAAAG
orf222    CTAAAACTACGGAACCAACTGCTTTCACACTGGGGTAAGACCATCCAGAGCAAGCTAAAG
          ************************************************************
protein    L  K  L  R  N  Q  L  L  S  H  W  G  K  T  I  Q  S  K  L  K

2A345     CTTGGTGGAAAAGATCGTACAAGTAAGTTCGGGGTCTTAGCGTTCGCCACGCGCTATTTC
2A397     CTTGGTGGAAAAGATCGTACAAGTAAGTTCGGGGTCTTAGCGTTCGCCACGCGCTATTTC
orf222    CTTGGTGGAAAAGATCGTACAAGTAAGTTCGGGGTCTTAGCGTTCGCCACGCGCTATTTC
          ************************************************************
protein    L  G  G  K  D  R  T  S  K  F  G  V  L  A  F  A  T  R  Y  F

2A345     CTCATGTTCGTGGTCCCAAAAATGCGGCTAGCTATATATCTAATATATGGTTTGAATTTT
2A397     CTCATGTTCGTGGTCCCAAAAATGCGGCTAGCTATATATCTAATATATGGTTTGAATTTT
orf222    CTCATGTTCGTGGTCCCAAAAATGCGGCTAGCTATATATCTAATATATGGTTTGAATTTT
          ************************************************************
protein    L  M  F  V  V  P  K  M  R  L  A  I  Y  L  I  Y  G  L  N  F

2A345     ATTTTTGGGATTAAATGGGGGTTGCTAGGAAATGAGATATTTCAGTTCGGCGTCGGACCA
2A397     ATTTTTGGGATTAAATGGGGGTTGCTAGGAAATGAGATATTTCAGTTCGGCGTCGGACCA
orf222    ATTTTTGGGATTAAATGGGGGTTGCTAGGAAATGAGATATTTCAGTTCGGCGTCGGACCA
          ************************************************************
protein    I  F  G  I  K  W  G  L  L  G  N  E  I  F  Q  F  G  V  G  P

2A345     GATGGCGTCGCGCCCCCAGCTCTAGATCTCAACGAGCGCCCGCCACTGCATCTTTTGTAC
2A397     GATGGCGTCGCGCCCCCAGCTCTAGATCTCAACGAGCGCCCGCCACTGCATCTTTTGTAC
orf222    GATGGCGTCGCGCCCCCAGCTCTAGATCTCAACGAGCGCCCGCCACTGCATCTTTTGTAC
          ************************************************************
protein    D  G  V  A  P  P  A  L  D  L  N  E  R  P  P  L  H  L  L  Y

2A345     GCGGATGTTGAGAGTTCCGACTCTCAACAAGCGCGGAATGCTGATATGCTAGCGCATATT
2A397     GCGGATGTTGAGAGTTCCGACTCTCAACAAGCGCGGAATGCTGATATGCTAGCGCATATT
orf222    GCGGATGTTGAGAGTTCCGACTCTCAACAAGCGCGGAATGCTGATATGCTAGCGCATATT
          ************************************************************
protein    A  D  V  E  S  S  D  S  Q  Q  A  R  N  A  D  M  L  A  H  I

2A345     AGCCGAGTGCAAGAGATAACCCGTGACCTAGAGGGTGAGCATGATATCGCGCGGCGTCAA
2A397     AGCCGAGTGCAAGAGATAACCCGTGACCTAGAGGGTGAGCATGATATCGCGCGGCGTCAA
orf222    AGCCGAGTGCAAGAGATAACCCGTGACCTAGAGGGTGAGCATGATATCGCGCGGCGTCAA
          ************************************************************
protein    S  R  V  Q  E  I  T  R  D  L  E  G  E  H  D  I  A  R  R  Q

2A345     GCCCTCGTCGATATCATGAAGTGGGAGGTCAGGAGCTTGGATCACCACTTCCGGGTCTTT
2A397     GCCCTCGTCGATATCATGAAGTGGGAGGTCAGGAGCTTGGATCACCACTTCCGGGTCTTT
orf222    GCCCTCGTCGATATCATGAAGTGGGAGGTCAGGAGCTTGGATCACCACTTCCGGGTCTTT
          ************************************************************
protein    A  L  V  D  I  M  K  W  E  V  R  S  L  D  H  H  F  R  V  F

2A345     CGGTACCTAGACCGTCTGCGAGATTCGAAGAGAGCCAAGGTGAACGAAATACTCGATCTA
2A397     CGGTACCTAGACCGTCTGCGAGATTCGAAGAGAGCCAAGGTGAACGAAATACTCGATCTA
orf222    CGGTACCTAGACCGTCTGCGAGATTCGAAGAGAGCCAAGGTGAACGAAATACTCGATCTA
          ************************************************************
protein    R  Y  L  D  R  L  R  D  S  K  R  A  K  V  N  E  I  L  D  L

2A345     TTTCGATGA
2A397     TTTCGATGA
orf222    TTTCGATGA
          *********
protein    F  R  *
```

Fig. 5: Alignment of DNA and protein sequences of male sterile accessions 2A397 and 2A345 with *orf222* in *B. rapa*

Table 4: The influence of different temperature treatment on male fertility of '0A193'

Day/night temperature	Degree of fertility					
	1–3d	4–6d	7–9d	10–12d	13–15d	16–18d
15 °C/10 °C	2.68D	3.92C	4.07C	5.84A	5.57AB	5.24B
22 °C/8 °C	5.24B	5.64A	5.16B	5.23B	5.20B	–
22 °C/17 °C	5.88A	5.41B	5.67AB	5.95A	5.83A	5.80A

Data means grading standard of fertility: 0 (complete male sterility), 1–4 (partly male sterility) and 5–6 (male fertility); data followed by different capital letters in the same row indicated a significant difference at 0.01 level.

Discussion

Identification and classification of CMS types are important because it helps to understand the inheritance patterns and to study the genetic mechanisms, thus providing a theoretical basis for the development of maintainer lines and the efficient use of male sterile lines (Chen et al. 2006). In the present study, the aforementioned *B. rapa* male sterile 0A193-CMS has different maintaining and restoring relationship with *pol* CMS. The result of multiplex PCR (Zhao et al. 2010) and molecular cloning and sequencing of the specific PCR bands revealed that the cytoplasm of this male sterile accession was identical to that of *nap* CMS. Together, this male sterile accession belonged to the *nap* CMS type, and its fertility was controlled by a pair of genic recessive genes. It is the second report in *B. rapa* after Yang et al. (1998), who discovered male sterile plants in a southern *B. rapa* landrace 'Xishuibai' and revealed that their cytoplasm was identical to that of *nap* CMS by using restriction fragment length polymorphism (RFLP) technique. However, they could not find restorers in the tested oilseed *B. rapa* accessions. In the present study, we found maintainers and restorers for the male sterile accession 0A193-CMS in both *B. rapa* and *B. napus*, which will lay a foundation for its utilization in *B. rapa* heterosis breeding.

Cytological observations reported that anther development in the *nap* CMS aborted at the sporogenous cell stage, even a few pollen sacs could form, which still could not release viable pollens because of delayed pollen development, indehiscent anthers or adhesive pollens (Bartkowiak-Broda et al. 1979, Hu et al. 2012). In our study, it was found that most pollen sacs stopped developing at archesporial cell differentiation stage, which were similar to those of *nap* CMS reported previously. Intriguingly, many abnormalities, not reported before, were observed including most abnormally dehiscence pollen sacs (Figs 3, S4), no connective (Figs 3, S2), concrescent anthers (Figs 3, S1), multiple connectives (Figs 3, S1) in '2A345(x)'. Our cytological study provides some new and interesting characterizations of pollen abortion in *nap* CMS materials.

The effects of environmental conditions on cytoplasmic male sterility have been investigated in rapeseed (Fan and Stefansson 1986, Dong et al. 2008). Fan and Stefansson (1986) found that temperature interacts strongly with rapeseed fertility. *B. napus nap* and *pol* CMS systems were expressed sterile or partially sterile consistently at lower temperature (22 °C), while it displayed a distinct pattern of fertility expression when temperature becomes high (26 °C and 30 °C). In the present study, field observation and growth chamber experiment revealed that this male sterile accession is temperature sensitive. The critical temperature is close to 15 °C/10 °C (day/night) (Table 4). More interesting is that although a treatment of this male sterile material is under a relatively low temperature 15 °C/10 °C (day/night) during the whole flowering period, anthers of flowers at early flowering stage showed higher degree of sterility than those of final flowering stage, which suggested the male fertility of this male sterile accession is also affected by the cumulative effect of temperature. This *B. rapa* male sterile accession can find application in the heterosis breeding by the selection of maintainers, which would produce more stable male sterile lines (Dong et al. 2008), or in combination with the temperature-sensitive genetic male sterile (TGMS) accessions such as TE5A (Zeng et al. 2014) and SP2S (Yu et al. 2015). In addition, it could be also used in 'two-line hybrid system', one male sterile line which can be multiplied in summer rape areas such as Qinghai, Gansu in China, and one male parent.

In conclusion, we have identified a new cytoplasmic male sterile source 0A193-CMS in *B. rapa*. Genetic investigation and molecular verification revealed that this male sterile accession belonged to the *nap* CMS type, and its fertility was controlled by a pair of genic recessive genes. Maintainers and restorers for this male sterile accession were found in both *B. rapa* and *B. napus* accessions. The result will enrich the male sterile resources. The present study provided useful information for broadening the CMS resources in *B. rapa* and lay the foundation for heterosis breeding of *B. rapa* crops.

Acknowledgements: This research was supported by the earmarked fund for Modern Agro-industry Technology Research System (CARS-13) and Key Projects in the National Science & Technology Pillar Program during the Eleventh 5-year Plan Period (2010BAD01B02). The authors are grateful to anonymous reviewers for critical reading of the manuscript and their constructive comments for revisions.

References

An, H., Z. H. Yang, B. Yi, J. Wen, J. X. Shen, J. X. Tu, C. Z. Ma, and T. D. Fu, 2014: Comparative transcript profiling of the fertile and sterile flower buds of *pol* CMS in *B. napus*. BMC Genom. **15**, 1—10.

Bartkowiak-Broda, I., P. Rousselle, and M. Renard, 1979: Investigations of two kinds of cytoplasmic male sterility in rapeseed (*Brassica napus* L.). Genetica Polonica **20**, 487—497.

Chen, L. T., and Y. G. Liu, 2014: Male sterility and fertility restoration in crops. Annu. Rev. Plant Biol. **65**, 579—606.

Chen, Q. B., X. L. Hou, J. J. Wang, Y. Q. Leng, F. L. Jiang, and P. Xue, 2006: Molecular identification of cytoplasmic male sterility line 101A of Onion. Acta Botanica Boreali-Occidentalia Sinica. **26**, 2430—2433.

Dong, J. G., Z. S. Dong, C. S. Liu, X. X. Liu, and F. N. Xie, 2008: Selection and identification of thermo-sensitive male sterile line 417S in *Brassica napus* L. Chinese Journal of Oil Crop Sciences **30**, 306—311.

Doyle, J., 1990: Isolation of plant DNA from fresh tissue. Focus **12**, 13—15.

Duroc, Y., C. Gaillard, S. Hiard, M. Defrance, G. Pelletier, and F. Budar, 2005: Biochemical and functional characterization of ORF138, a mitochondrial protein responsible for Ogura cytoplasmic male sterility in *Brassiceae*. Biochimie **87**, 1089—1100.

Engelke, T., J. Hirsche, and T. Roitsch, 2011: Metabolically engineered male sterility in rapeseed (*Brassica napus* L.). Theor. Appl. Genet. **122**, 163—174.

Fan, Z., and B. R. Stefansson, 1986: Influence of temperature on sterility of two cytoplasmic male sterility systems in rape (*Brassica napus* L.). Can. J. Plant Sci. **66**, 221—227.

Fu, T.D., 1981: Production and research of rapeseed in the People' Republic of China. Eucarpia Cruciferae Newsletter. **6**, 6—7.

Fu, T.D. and J.X. Tu, 2002: Present situation and prospects on the research and utilization of hybrid rapeseed. In: Liu H.L.(ed), Analects of Crop Breeding. China Agricultural University Press, Beijing. 235—250.

Gonzalez-Melendi, P., J. R. H. Mora, S. Fajardo, F. Budar, and M. M. Lucas, 2008: A light and electron microscopy analysis of the events leading to male sterility in Ogu-INRA CMS of rapeseed (*Brassica napus* L.). J. Exp. Bot. **59**, 827—838.

Havey, M. J., 2004: The use of Cytoplasmic Male Sterility for Hybrid Seed Production, 623—634. Springer, Netherlands.

Hu, Y. M., J. G. Dong, Q. Meng, Y. F. Guo, and Z. S. Dong, 2012: Cytological observation on anther development of five CMS lines in *Brassica napus*. Acta Agriculturae Boreali-occidentalis Sinica. **21**, 95—99.

Laughnan, J. R., and S. Gabay-Laughnan, 1983: Cytoplasmic male sterility in maize. Annu. Rev. Genet. **17**, 27—48.

L'Homme, Y., and G. G. Brown, 1993: Organizational differences between cytoplasmic male sterile and male fertile *Brassica*

mitochondrial genomes are confined to a single transposed locus. Nucleic Acids Res. **21**, 1903—1909.

L'Homme, Y., R. J. Stahl, X. Q. Li, A. Hameed, and G. G. Brown, 1997: *Brassica nap* cytoplasmic male sterility is associated with expression of a mtDNA region containing a chimeric gene similar to the *pol* CMS-associated *orf224* gene. Curr. Genet. **31**, 325—335.

Liu, Z., P. W. Liu, F. R. Long, D. F. Hong, Q. B. He, and G. S. Yang, 2012: Fine mapping and candidate gene analysis of the nuclear restorer gene *Rfp* for *pol* CMS in rapeseed (*Brassica napus* L.). Theor. Appl. Genet. **125**, 773—779.

Liu, J., R. Xiang, W. Wang, D. Mei, Y. Li, A. S. Mason, L. Fu, and Q. Hu, 2015: Cytological and molecular analysis of *Nsa* CMS in *Brassica napus* L. Euphytica **206**, 279—286.

Ogura, H., 1968: Studies on the new male-sterility in Japanese Radish, with special reference to the utilization of this sterility towards the practical raising of hybrid seeds. Mem Fac Agric Kagoshima Univ. **6**, 39—78.

Prakash, S., and K. Hinata, 1980: Taxonomy, cytogenetics and origin of crop brassicas, a review. Opera Botanica. **55**, 3—57.

Verma, J. K., Y. S. Sodhi, A. Mukhopadhyay, N. Arumugam, V. Gupta, D. Pental, and A. K. Pradhan, 2000: Identification of stable maintainer and fertility restorer lines for 'Polima' CMS in *Brassica campestris*. Plant Breeding **119**, 90—92.

Wang, J., Y. N. Gao, Y. Q. Kong, J. J. Jiang, L. I. Ai-Min, Y. T. Zhang, and Y. P. Wang, 2014: Abortive process of a novel rapeseed cytoplasmic male sterility line derived from somatic hybrids between *Brassica napus* and *Sinapis alba*. J Integr Agric. **13**, 741—748.

Wei, W. L., and H. Z. Wang, 2009: Cytological and molecular characterization of a new cytoplasmic male sterility in rapeseed. Plant Breeding **128**, 426—428.

Wei, W. L., H. Z. Wang, and G. H. Liu, 2005: Molecular identification of the sterile cytoplasm of *NCa* of a cytoplasmic male sterile line in rapeseed (*Brassica napus* L.). Sci. Agric. Sin. **38**, 1965—1972.

Yamagishi, H., and S. R. Bhat, 2014: Cytoplasmic male sterility in *Brassicaceae* crops. Breed. Sci. **64**, 38—47.

Yang, G. S., and T. D. Fu, 1991: A preliminary study on the restoring-maintaining relationship in rapeseed (*Brassica napus* and *Brassica campestris*). Acta Agronomica Sinica. **17**, 151—156.

Yang, G. S., T. D. Fu, and G. G. Brown, 1998: Genetic investigation on a cytoplasmic male sterility system in oilseed *Brassica campestris* L. Acta Agronomica Sinica. **24**, 705—710.

Yu, F. Q., and T. D. Fu, 1990: Cytomorphological research on anther development of several male sterile lines in *Brassica napus* L. J of Bota Res. **8**, 209—216.

Yu, C. Y., J. G. Dong, S. W. Hu, and P. R. He, 2009: Efficiency of a novel gametocide amidosulfuron on rapeseed (*Brassica napus*). Plant Breeding **128**, 538—540.

Yu, C. Y., Y. F. Guo, J. Ge, Y. M. Hu, J. G. Dong, and Z. S. Dong, 2015: Characterization of a new temperature-sensitive male sterile line SP2S in rapeseed (*Brassica napus* L.). Euphytica **206**, 473—485.

Zeng, X. H., W. P. Li, Y. H. Wu, F. Liu, J. L. Luo, Y. L. Cao, L. Zhu, Y. J. Li, J. Li, Q. B. You, and G. Wu, 2014: Fine mapping of a dominant thermo-sensitive genic male sterility gene (*BntsMs*) in rapeseed (*Brassica napus*) with AFLP- and *Brassica rapa*-derived PCR markers. Theor. Appl. Genet. **127**, 1733—1740.

Zhao, H. X., Z. J. Li, S. W. Hu, G. L. Sun, J. J. Chang, and Z. H. Zhang, 2010: Identification of cytoplasm types in rapeseed (*Brassica napus* L.) accessions by a multiplex PCR assay. Theor. Appl. Genet. **121**, 643—650.