Mitochondrial-targeted Expression of *orf456* Causes Male Sterility in Chinese Cabbage (*Brassica rapa* L.)

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ABSTRACT Cytoplasmic male sterility (CMS) is a phenomenon specific to plant reproduction that has been extensively exploited for hybrid seed production. *Orf456* was previously identified as a candidate gene mediating male sterility in pepper. To gain further insight of this candidate gene, we carried out experiments to transform Chinese cabbage (*Brassica rapa* L.). About 30 T₁ transgenic lines were obtained and approximately 50 % of T₁ transgenic Chinese cabbage lines showed male-sterility. To evaluate pollen viability, three different approaches including plasmolysis test, staining pollen and *in vitro* germination assay were used. Analysis of the CMS transgenic lines showed that transgenic Chinese cabbages produced aberrant pollen development while some were unable to produce pollen. In conclusion, the mitochondrial *orf456* gene could induce partial male sterility in transgenic Chinese cabbage. Nevertheless, how the *orf456* gene precisely functions to induce male sterility and its biochemical function remains to be discovered.

Keywords Chinese cabbage (*Brassica rapa* L.), Cytoplasmic male sterility, *orf456*, Molecular breeding

INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited condition in which a plant is unable to produce viable pollen (Gómez 1999). Since the first documentation of male sterility by Joseph Gottlieb Kölreuter, who observed anther abortion within species and specific hybrids, cytoplasmic male sterility has been identified in over 150 plant species (Schnabel and Wise 1998; Hanson and Bentolila 2004; Chowdhury and Ahmad 2010).

CMS obviously reflects a conflict between the maternally inherited mitochondrial genome, which induces male sterility without affecting the overall fitness of the plant. CMS can occur at different stages during reproductive developments (Linke and Börner 2005; Chase 2007). It is generally considered that CMS is caused by the rearrangement of mitochondrial genomes, which, in many cases, is attributed to the generation of novel Open Reading Frames (ORF) (Yang and Zhang 2007). In a number of cases, transcripts originating from these altered open reading

frames are translated into unique proteins that appear to interfere with mitochondrial function and pollen development (Yamamoto 2008). CMS anther degeneration is probably caused by dysfunction of tapetum or vacuolation of tapetum, PMCs, and microspores (Shi et al. 2010). Most studies have been performed on vegetative tissues that express the CMS- associated proteins; however, biochemical and physiological studies have provided some clues to the disruption in aborted pollen development (Shi et al. 2010). Nevertheless, the precise action of a mitochondrial locus that confers male sterility has not been determined definitively for any species.

The *Brassica* family contains about 3,500 species and 350 genera and is one of the economically most important plant families (Rich 1991; Quijada et al. 2007). *Brassica rapa* (2n=20, AA) is a diploid crop in this family, which is currently widely cultivated in China, Japan, Korea, India, Canada, and Europe to produce vegetable, oilseed and fodder (Hirai and Matsumoto 2007; Li et al. 2013). Since Ogura (1968) first reported the CMS line of radish, a

Received Jun 20, 2013; Revised Jun 24, 2013; Accepted Jun 27, 2013; Published June 30, 2013

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number of F₁ hybrid cultivars have been developed using the Ogura CMS system in many economically important *Brassica* family crops. Despite many studies on CMS, researchers and breeders have made efforts to search for new sources of CMS in breeding.

In the previous studies, a mitochondrial *atp6* gene was identified as a CMS associate gene (Kim et al. 2006a; Kim et al. 2001). Then the second CMS-associated gene *orf456* was identified through analysis DNA and RNA blot hybridization between male fertile and CMS lines of chili pepper (Kim et al. 2007). The gene was located at the 3'-end of the *coxII* gene in CMS pepper. Later, it was shown that overexpression of *orf456* severely induced male sterility in *Arabidopsis* (Kim et al. 2007). To investigate the

functional role of the ORF456 protein in *B. rapa*, we carried out transformation of *B. rapa* with *orf456* fused to a mitochondrion-targeted *coxIV* gene.

MATERIALS AND METHODS

Plant materials

The wild-type Seoul Baechu (*B. rapa*), were kindly provided by D.H. Kim (Seoul National University, Korea).

Plant chimeric gene construction and transformation

Orf456 and *CoxIV* were cloned into the plant transformation vector pMDC32, which contains 35S promoter. Purified

Fig. 1. *Orf456* chimeric gene construct and transformation in *B. rapa*. a) Schematic diagram illustrating the construction and ligation of *orf456* and *coxIV* into plant transformation vector, pMDC32, driven by the 35S promoter. b) Polymerase chain reaction (PCR) analysis of chimeric gene genetically modified situation in T₀ individual transgenic *B. rapa* plants. Actin was used as internal control to probe Chinese cabbage gDNA quality. c) Polymerase chain reaction (PCR) analysis of chimeric gene genetically modified situation in T₁ individual transgenic *B. rapa* plants. HPT was hygromycin B, an antibiotic produced by the bacterium *Streptomyces hygroscopicus*. d) RNA and RT-PCR analysis of T₁ individual transgenic *B. rapa* lines.

plasmid DNA of each pMDC32 construct (Fig. 1a) was electroporated into *Agrobacterium tumefaciens* LBA4404. The presence and stability of the transgenes was verified by PCR (Fig. 1b, c, d). Chinese cabbage cultivar 'Seoul' was transformed using leaf disk method at National Institute of Horticultural and Herbal Science. For the T₁ selection, a hygromycin (15mg mL⁻¹)-containing Murashige and Skoog (50%) medium (Sigma, USA) was used.

Growth conditions and sampling

Transgenic Chinese cabbage lines was sterilized by 70% ethyl alcohol and 50% Clorox and germinated on MS media (Sigma, USA) containing Hygromycin B (HPT) (Sigma, USA). After 2 weeks, the seedlings were transplanted to soil and were cultured in a growth chamber (2 or 3 weeks). When the fifth leaf was fully expanded, the seedlings were exposed to a 4°C low temperature in a period of 20 to 30 days to achieve a complete vernalization for bolting (National Institute of Horticultural and Herbal Science, Suwon, Korea).

Fresh buds and anther were collected and fixed in Carnoy's Fluid (a fixative composed of 60% ethanol, 30% chloroform and 10% glacial acetic acid) and samples were stored in a refrigerator at 4°C. Fresh pollen of the *orf456* transformants and wild-type line were collected by shaking a flower on a clean sheet of paper, pouring the pollen into a 1.5 mL microcentrifuge tube with 0.5 mL 70% (v/v) ethanol and stored at 4°C.

DNA and RNA extraction and RT-PCR

Genomic DNA was extracted from young leaves using hexadecyltrimethylammonium bromide (CTAB) method (Prince et al. 1997). Total RNA was extracted from young flowers buds of T₁ transgenic plants using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was then treated with RNase-free DNase I (Ambion, USA) to remove possible contamination of genomic DNA for RT-PCR. Total RNA (2 µg) was used for first-strand cDNA synthesis using M-MLV reverse transcriptase (Invitrogen, USA). For PCR analysis of *orf456*, the forward primer 5'-ATGCCAAAAGTCCCATGTA-3' and the reverse primer 5'-

TTACTCGGTTGCTCCATTGTTT-3' were used. For *coxIV*, the forward primer 5'-GGATCCATGTTGTCACTACGTCAATCTATAAGA-3' and the reverse primer 5'-CCCGGGACCTCTTTAGCACCAGGACC-3' were used. For HPT, the forward primer 5'-CCTGAACTCACCGCGACG-3' and the reverse primer 5'-AAGACCAATGCGGAGCATAT-3' were used. *Brassica* actin was used as control.

Pollen viability test

The plasmolysis test was performed to evaluate pollen viability. Fresh pollen of the *orf456* transformants and wild-type line were collected and added with 20 µL of hypertonic solution (25% glycerol) on glass slides. Pollen were stained with 1% aceto-carmine (Nassar et al. 2000) and iodine (Jeffrey 2004) to detect cytoplasmic content. After 10 min incubation at room temperature, stained pollen were observed under a light microscope. *In vitro* germination assays were performed to determine the germination ability of pollen. Growth medium contained 10% sucrose, 0.01% boric acid (pH 6.8). After incubation at 20-25°C for about 4 hrs, the solution has been covered with a glass cover slip and photographed.

RESULTS

Chimeric gene construction and transformation

In the previous study, a CMS-associated gene, *orf456* was found at the 3' -end of the *coxII* gene, which was identified as a strong candidate for determining the male-sterile phenotype in pepper (Kim et al. 2007). When this gene was ectopically expressed in Arabidopsis, transgenic plants showed abnormal pollen development and sterility. To test if transgenic expression of the *orf456* gene can induce male sterility in *Brassica*, transgenic plants were developed. To transform *B. rapa*, a chimeric gene was constructed by fusing *orf456* gene with a mitochondrial transit peptide sequence (54 codons) of the nuclear *coxIV* gene. The *orf456* gene was amplified from CMS lines of chili pepper and ligated after the mitochondrial-targeting sequence of *coxIV*. Subsequently the chimeric gene has been ligated to the plant transformation vector, pMDC32,

driven by the 35S promoter. The schematic structure of the chimeric *orf456* gene is shown in Fig. 1a. The constructs were introduced into *B. rapa* 'Seoul Baechu'. Putative transgenic plants were selected by PCR and RT-PCR (Fig. 1b, c, d) and 10 T₀ plants exhibiting male sterility were obtained (Fig. 1b). We used the *orf456* gene as a marker to prove that this gene was inherited and could be expressed in T₁ transgenic (Fig. 1c, d). The male-sterile phenotype was shown to be genetically transmitted to the T₀ and T₁ generation associated with *orf456* expression in *B. rapa* (Fig. 1b, c). All the 10 male-sterile T₁ plants expressed the *orf456* gene, and all the 30 male-fertile T₁ plants lacked *orf456* expression in *B. rapa*.

Phenotypes of *orf456* transformants

Morphological characteristics of transgenic plants harboring the chimeric *orf456* gene were evaluated. The vegetative growth of transgenic *B. rapa* was similar to that of wild-type plants, but their reproductive characteristics differed in numerous ways including delays in bolting and flowering time (Fig. 2a), aborted buds structures at the basal part of the raceme (Fig. 2b), aberrant floral organ

(Fig. 2c). Comparison of silique and pod among wild-type and transgenic *B. rapa* revealed the inability of transgenic plants to seed-set at an early developmental stage (Fig. 2d, e). The seed size and weight also differed between wild-type and transgenic *B. rapa*. The former showed spherical, brown and glossy seeds, while the latter showed shriveled shape of seeds, which implies embryo hypoplasia (Fig. 2f). When the transgenic plants were cross-pollinated with wild-type plants, seed-setting occurred indicating normal pistil fertility (Fig. 2e).

Additionally, four types of aberrant flowers were observed in transgenic plants; flowers with less or more than six normal stamens (Fig. 2c-1), flowers with degenerative stamens devoid of pollen (Fig. 2c-2), one flower with several styles and petal-like sepal (Fig. 2c-3), and two or more coalescing flowers (Fig. 2c-4, c-5). These phenotypes of aberrant floral organs in transgenic *B. rapa* are similar with others studies (Yang et al. 2010 and Kim et al. 2007).

Pollen viability of transgenic lines

Pollen of the *orf456* transformants and wild-type *B. rapa* at the anther dehiscent stage were fixed and observed under

Fig. 2. Phenotypes of wild-type and *orf456* transformants. The morphological characters were observed in transgenic plants in which the chimeric *orf456* gene was expressed. Results suggested that *orf456* expression may have functioned in the various reproductive growth performance of transgenic *B. rapa* lines. a) Transgenic T₁ *B. rapa* plants, b) buds, c) blossoms, d) siliques, e) pods, f) seeds.

a light microscope (LM). We used three different approaches to estimate pollen viability: plasmolysis test, staining pollen with dyes and *in vitro* germination assay.

The plasmolysis test was performed to check the intactness of pollen. When placed in hypertonic (25% glycerol) solution, pollen of *orf456*-transformed *B. rapa* burst within 10-15 min (Fig. 3b), while the wild-type pollen grains remained intact even after 24 h (Fig. 3a). This indicates that the pollen of the *orf456*-transformed *B. rapa* was undergoing ateliosis. Pollen staining was used to determine enzymatic activity and membrane integrity of pollen. The morphology of acetocarmine and iodine-stained pollen was altered in transgenic *B. rapa* (Fig. 3b). This indicates the different pollen activity taking place in transgenic plants. *In vitro* germination assays determine the actual germination ability of pollen. After 4 hrs incubation at 20-25°C, the pollen grain is slightly swollen, with one of the three pores (furrows) open and the pollen tube emerging. Pollens from wild-type *B. rapa* produced pollen tubes, whereas those of transgenic plant bursted hence no pollen tube was growing (Fig. 3c).

Statistical analyses of pollen abortion phenotypes in the CMS lines

To confirm the function of *orf456* in producing a male-sterile phenotype, two independent tests were conducted. We randomly selected 7 out of 10 transgenic T₀ lines that contained *coxIV-orf456*, and obtained T₁ transgenic plants. The malformed pollen ratio of 15 plants lower than 10%, which showed a male fertility (Table 1). We analyzed the aberrant flowers ratio and malformed pollen ratio in T₁ *B. rapa* lines. The result indicated that approximately 50% T₁ *B. rapa* exhibited the male-sterile malformed blossom and 33.3% T₁ *B. rapa* malformed blossom exhibited the high malformed pollen ratio (Table 1). Four T₁ lines 1, 3, 6 and 10, showed higher malformed pollen ratio compared with other lines. Normal blossoms were compared with aberrant blossoms and we found the aberrant blossoms had a higher malformed pollen ratio (Table 1). Three T₀ generation lines 1, 6 and 10, showed poor seed setting ability when compared to the wild-type (Fig. 4a). Moreover, we found four T₁ lines 1, 6, 7 and 10 having lower number of siliques and seeds compared with other lines (Fig. 4b).

Fig. 3. Pollen viability tests. a) The plasmolysis test was performed to probe the pollen. In hypertonic (25% glycerol) solution, pollen from *orf456* transformants *B. rapa* burst within 10-15 min, while the wild-type pollen grains remained intact even after 24 hr. b) Staining test to determine pollen enzymatic activity and membrane integrity. c) *In vitro* germination assays determine the actual germination ability of pollen under suitable conditions. After 4 hrs of *in vitro* germination, pollen from wild-type *B. rapa* were able to produce pollen tubes which did not occur in transgenic *B. rapa*.

DISCUSSION

Chimeric *orf* genes resulting from the rearrangement of a mitochondrial genome are generally thought to be a causal factor in the occurrence of CMS (He et al. 1996). Several transformants containing *orf* gene constructs, with or without a mitochondrial targeting sequence, exhibited a semi-sterile or male sterile phenotype (He et al. 1996; Wang et al. 2006; Kim et al. 2007; Yamamoto et al. 2008). The failure of mitochondrial expression of novel *orfs* to

induce male sterility is probably due to problems in disruption of mitochondrial membranes (Rhoads et al. 1995; He et al. 1996), effect on ATP synthase function (Bergman et al. 2000; Sabar et al. 2003) and pollen abortion by programmed cell death in transgenic plants. However, there is still little information about how the mitochondrial factor regulates the expression of the nuclear genes involved in male sterility. Previously, to provide definitive evidence that expression of an abnormal mitochondrial gene can interrupt pollen development, a CMS-associated

Table 1. Analysis of malformed pollens ratio of T₁ *B. rapa* plants.

<i>Brassica rapa</i>	Blossom			Pollen					
	Normal blossome	Malformed blossom	Malformed blossom ratio (%)	Normal blossom			Malformed blossom		
				Normal pollen	Malformed pollen	Malformed pollen ratio (%)	Normal pollen	Malformed pollen	Malformed pollen ratio (%)
WT	25	0	—	824	8	0.96	915	7	0.76
1-5	17	6	26.09	424	49	10.36	417	711	63.03
1-7	22	4	15.38	589	72	10.89	885	199	18.36
1-8	32	1	3.03	394	46	10.45	—	—	—
1-9	22	7	24.14	533	218	29.03	682	243	26.27
1-11	25	0	—	325	187	36.52	—	—	—
2-3	25	0	—	814	62	7.08	—	—	—
3-4	20	4	16.67	731	213	22.56	79	725	90.17
3-11	31	4	11.42	230	349	60.28	6	1291	99.54
3-12	6	1	14.28	229	152	39.89	—	—	—
3-15	12	4	25	574	78	11.96	328	517	61.18
3-16	79	4	4.82	417	57	12.03	5	500	99
4-1	25	0	—	661	46	6.51	—	—	—
4-4	25	0	—	703	57	7.5	—	—	—
4-8	25	0	—	718	49	6.39	—	—	—
4-9	25	0	—	897	42	4.47	—	—	—
6-1	25	0	—	719	34	4.52	—	—	—
6-7	25	0	—	974	29	2.89	—	—	—
6-8	25	0	—	485	40	7.61	—	—	—
6-9	12	9	42.86	636	212	25	355	335	48.55
6-10	25	0	—	712	12	1.66	—	—	—
7-1	12	1	7.69	921	55	5.64	—	—	—
7-2	13	1	7.14	894	210	19.02	—	—	—
7-3	25	0	—	702	27	3.7	—	—	—
7-4	47	13	21.67	528	265	33.42	246	87	26.13
7-6	25	0	—	582	192	24.81	—	—	—
10-5	25	0	—	592	45	7.06	—	—	—
10-6	25	0	—	482	72	12.99	—	—	—
10-7	25	0	—	633	48	7.05	—	—	—
10-8	9	3	25	732	108	12.86	312	270	46.39
10-9	23	1	4.17	505	23	4.36	—	—	—

Fig. 4. Analyses of pollen abortion phenotypes in the CMS lines. a) Three T₀ lines (1, 6, and 10) showed semi-sterile phenotype, and higher ratio than other lines. This classification was based on malformed pollen morphology. b) Analysis result of seeds and siliques of wild-type and transgenic T₁ *B. rapa* plants.

mitochondrial DNA sequence from chili pepper, *orf456*, was identified by comparison of cytoplasmic genomes in fertile and CMS lines (Kim et al. 2007). In this paper, *orf456* gene was introduced into the Chinese cabbage to test the candidate CMS-associated gene function.

Several attempts have been made to induce CMS by intergeneric hybrid or transformation of CMS-associated genes into *Brassica*. Ogura (1968) first reported the CMS line of radish, a number of hybrid cultivars have been developed using the Ogura CMS system in many economically important crops. The *atpA* and *orf220*, newly isolated from CMS stem mustard (*Brassica juncea*, var. *tumida* Tsen et Lee) (Yang et al. 2010). A chimeric gene *orf220-atp2-1* was transformed into in wild-type stem mustard. Kim et al (2007) showed that transgenic Arabidopsis plants carrying a chimeric *orf456* could induce male sterility phenotype which was similar to those observed in CMS chili pepper. In this study we also showed that chimeric *orf456* gene induce partial male sterility in *B. rapa*.

It is thought that the disruption in pollen development is a consequence of mitochondrial dysfunction (He 1996). Furthermore, many genes related to the mitochondrial respiratory complex and pollen development were observed to be down-regulated in transgenic plants and the CMS lines (Yang et al. 2010). To date, functional genes specifically related to pollen development have been well documented. Mutation of any of these genes causes failure of microsporogenesis or abortion of pollen (He et al. 1996; Kim et al. 2007; Shi et al. 2010; Yang et al. 2010). We used three different approaches to estimate pollen viability: plasmolysis test, staining pollen with dyes and *in vitro* germination assay (Tuinstra and Wedel 2000). The results of our study indicate that introduction of each of the *orf456* gene constructs resulted in aberrant pollen development and disturbance in function (Fig. 2-4). We compared normal and aberrant blossoms and found higher malformed pollen ratio in aberrant blossoms (Table 1).

In conclusion, the mitochondrial *orf456* gene may

represent a strong candidate gene involved in CMS in transgenic Chinese cabbage. Nevertheless, how the *orf456* gene functions precisely to induce male sterility and its biochemical function remain to be discovered, thus further research is required to explore the signaling pathway of mitochondrial-nuclear genome interactions.

ACKNOWLEDGEMENTS

This work was supported by a grant (Project No. 609002-5) from the Screening Center for Disease Resistance Vegetable Crops of the Technology Development Program and a grant (code: 710001-03) from the Vegetable Breeding Research Center through R D Convergence Center Support Program, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

REFERENCES

- Bergman P, Edqvist J, Farbos I and Glimelius K. 2000. Male-sterile tobacco is plays abnormal mitochondrial *atp1* transcript accumulation and reduced floral ATP/ADP ratio. *Plant Mol. Biol* 42:531-544.
- Chase CD. 2007. Cytoplasmic male sterility: a window to the world of plant mitochondrial-nuclear interactions. *Trends Genet.* 23:81-90.
- Chowdhury MJ, Ahmad S, Nazim Uddin M, Quamruzzaman AKM and Patwary MMA. 2010. Expression of Heterosis for Productive Traits in F1 Brinjal (*Solanum melongena* L.). *Hybrids the Agriculturists.* 8(2):8-13.
- Gómez CC. 1999. *Biology of Brassica Coenospecies.* Elsevier. 186-189.
- Hanson MR and Bentolila S. 2004. Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *Plant Cell.* 16:154-169.
- He S, Abad AR, Gelvin SB and Mackenzie SA. 1996. A cytoplasmic male sterility-associated mitochondrial protein causes pollen disruption in transgenic tobacco. *Proc Natl Acad Sci USA.* 93:11763-11768.
- Hirai M and Matsumoto S. 2007. *Brassica rapa.* *Genome Mapping and Molecular Breeding in Plants.* Springer-Verlag Berlin, Heidelberg. 5:185-190.
- Kim DH and Kim BD. 2006a. The organization of mitochondrial *atp6* gene region in male fertile and CMS lines of pepper (*Capsicum annuum* L.). *Curr. Genet.* 49:59-67.
- Kim DH, Kang JG and Kim BD. 2007. Isolation and characterization of the cytoplasmic male sterility-associated *orf456* gene of chili pepper (*Capsicum annuum* L.). *Plant Mol Biol.* 63:519-532.
- Kim DH, Kang JG, Kim SJ and Kim BD. 2001. Identification of *cox2* and *atp6* regions as associated to CMS in *Capsicum annuum* by using RFLP and long accurate PCR. *Hortic Sci.* 42:121-127.
- Linke B and Börner T. 2005. Mitochondrial effects on flower and pollen development. *Mitochondrion.* 5:389-402.
- Li XN, Nirala R, Vignesh D, Choi SR, Hur YK, Nou LS, Yoon MK and Lim YP. 2013. Quantitative trait loci mapping in *Brassica rapa* revealed the structural and functional conservation of genetic loci governing morphological and yield component traits in the A, B, and C subgenomes of Brassica species. *DNA Research.* 20:1-16.
- Nassar NMA, Santos ED and David S. 2000. The transference of apomixis genes from *Manihot. neusana* Nassar to Cassava, *M. eculenta* Crantz. *Hereditas.* 132:167-170.
- Ogura H. 1968. Studies on the new male sterility in Japanese radish with special reference to utilization of this sterility towards the practical raising of hybrid seeds. *Mem. Fac. Agr. Kagoshima Univ.* 6:39-78.
- Prince JP, Zhang W, Radwanski ER and Kyle MM. 1997. A versatile and high-yielding protocol for the preparation of genomic DNA from *Capsicum spp.* (pepper). *Hortiscience.* 32:937-939.
- Quijada P, Wang XW, Hirai JC and Kole C. 2007. Oil seeds. *Genome Mapping and Molecular Breeding in Plants.* Springer Berlin Heidelberg. 2:211-263.
- Rhoads DM, Levings CS and Siedow JN. 1995. URF13, a ligand-gated, pore-forming receptor for T-toxin in the inner membrane of cms-T mitochondria. *J. Bioenerg. Biomembr.* 27:437-445.
- Rich TCG. 1991. *Crucifers of Great Britain and Ireland.* Botanical Society of the British Isles, London. 36.
- Sabar M, Gagliardi D, Balk J and Leaver CJ. 2003. ORFB is a subunit of F1F0-ATP synthase: Insight into the basis of cytoplasmic male sterility in sunflower. *EMBO Rep.* 4:1-6.
- Schnabel PS and Wise RP. 1998. The molecular basis of cytoplasmic male-sterility and fertility restoration. *Trends Plant Sci.* 3:175-180.
- Shi SL, Ding D, Mei SY and Wang JB. 2010. A comparative light and electron microscopic analysis of microspore

- and tapetum development in fertile and cytoplasmic male sterile radish. *Protoplasma*. 241:37-49.
- Tuinstra MR and Wedel J. 2000. Estimation of pollen viability in grain sorghum. *Crop Sci*. 40:968-970.
- Wang ZH, Zou YJ, Li XY, Zhang QY, Chen L, Wu H, Su DH, Chen YL, Guo JX, Luo D, Long YM, Zhong Y and Liu YG. 2006. Cytoplasmic male sterility of rice with boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. *Plant Cell*. 18:676-687.
- Yang JH, Liu XY, Yang XD and Zhang MF. 2010. Mitochondrially-targeted expression of a cytoplasmic male sterility-associated orf220 gene causes male sterility in *Brassica juncea*. *BMC plant biology*. 10:231.
- Yang JH and Zhang MF. 2007. Molecular genetics of mitochondrial respiratory/ATP-related genes in relation to cytoplasmic male-sterility of higher plants. *Gene Genomes and Genomics*. 1:21-26.
- Yamamoto MP, Shinada H, Onodera Y, Komaki C, Mikami T and Kubo T. 2008. A male sterility-associated mitochondrial protein in wild beets causes pollen disruption in transgenic plants. *Plant J*. 54:1027-1036.