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ORIGINAL PAPER

Genetic characterization of a new cytoplasmic male sterility system (*hau*) in *Brassica juncea* and its transfer to *B. napus*

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Abstract A novel cytoplasmic male sterility (CMS) was identified in *Brassica juncea*, named as *hau* CMS (00-6-102A). Subsequently, the male sterility was transferred to *B. napus* by interspecific hybridization. The *hau* CMS has stable male sterility. Flowers on the A line are absolutely male sterile, and seeds harvested from the line following pollinations with the maintainer gave rise to 100% sterile progeny. The anthers in CMS plants are replaced by thickened petal-like structures and pollen grains were not detected. In contrast, in other CMS systems viz. *pol*, *nap*, *tour*, and *ogu*, anthers are formed but do not produce viable pollen. The sterility of *hau* CMS initiates at the stage of stamen primordium polarization, which is much earlier compared with the other four CMS systems. We have successfully transferred *hau* CMS from *B. juncea* to *B. napus*. Restorer lines for *pol*, *ogu*, *nap*, and *tour* CMS systems were found to be ineffective to restore fertility in *hau* CMS. Sixteen out of 40 combinations of mitochondrial probe/enzyme used for RFLP analysis distinguished the *hau* CMS system from the other four systems. Among these sixteen combinations, five ones alone could distinguish the five CMS systems from each other. The evidence from genetic, morphological, cytological and molecular studies confirmed that the *hau* CMS system is a novel CMS system.

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Introduction

Rapeseed (*Brassica napus* L.) is one of the major oilseed crops grown widely in European countries, Canada, China and Australia. Cytoplasmic male sterility (CMS) is an important approach to exploit heterosis in this crop (Fu 1995). CMS is a common trait in angiosperms (Kaul 1988) and is maternally inherited resulting in failure to produce functional pollen. CMS occurs in a variety of plant species and is often associated with novel mitochondrial open reading frames, which interfere with the proper functioning of mitochondria and pollen development (Hanson 1991; Hanson and Folkerts 1992; Bonen and Brown 1993). Several CMS systems have been reported in rapeseed which include *Raphanus*/*ogu* (Ogura 1968), *tour* (Mathias 1985; Stiewe and Röbbelen 1994), *polima or pol* (Fu 1981), and *nap* (Shiga and Baba 1971, 1973; Thompson 1972). *Pol* CMS was discovered in 1972 (Fu 1981), and became the first CMS system to be extensively utilized for hybrid seed production (Fan et al. 1986; Röbbelen 1991). The *pol* CMS is sensitive to environment in certain nuclear backgrounds leading to breakdown of sterility, which reduces hybridity levels in F_1 hybrids.

A number of studies have been carried out for the characterization and identification of CMS systems in many crops including rice, wheat, sugar beet, sunflower, common bean, onion, *Arabidopsis*, pearl millet and radish based on mitochondrial DNA analyses (Delorme et al. 1997; Schnable and Wise 1998; Horn and Friedt 1999; Nahm et al. 2005). According to restorer and maintainer relationships, rice cytoplasm in sterile lines was classified into WA type, HL type and Dian-type (Li 1980). Analyses of mitochondria DNA sequence can also distinguish the cytoplasm types in CMS rice and maize (Kadowaki et al. 1988; Wei et al. 1997). In maize, mtDNA of the T, S and C group had

unique electrophoresis bands, which could be used to classify the CMS types (Kemble et al. 1980). So far, nine CMS lines in *B. napus* have been characterized into four types as listed above (*pol*, *ogu*, *nap*, *tour*) by analysis of restorer and maintainer relationships and mtDNA RFLPs (Yang et al. 1998). The recent reports of CMS in *Brassica* include CMS 681A of spontaneous origin in a mutant line of a *B. napus* cv. Xiangyu 13. It has the same restorer and maintainer relationship as *pol* CMS but possessed different mitochondrial DNA sequences (Liu et al. 2005). Another CMS system named "126-1" was identified in a population of doubled haploids of synthetic *B. napus* ISN 706 and subsequently transferred to *B. juncea* (Sodhi et al. 2006). Several CMS systems of alloplasmic origin have also been reported in *B. juncea* containing cytoplasm of several wild species viz. *B. oxyrrhina* (Prakash and Chopra 1990), *B. tournefortii* (Pradhan et al. 1991; Arumugam et al. 1996), *Diplotaxis siifolia* (Rao et al. 1994), *Moricandia arvensis* (Prakash et al. 1998), *Erucastrum canariense* (Prakash et al. 2001), *Diplotaxis erucoides* (Bhat et al. 2006) and *D. berthautii* (Bhat et al. 2007).

We have identified a new cytoplasmic male sterility system in mustard (*B. juncea*), and we report here its characterizations based on genetic, morphological, cytological and molecular studies, and its transfer to *B. napus* by interspecific hybridization.

Materials and methods

Plant materials

All materials used in this study belong to Huazhong Agricultural University (HAU) and are listed in Table 1. The *hau* CMS (00-6-102A) was originally found as a spontaneous male sterile mutant in *B. juncea* in the experimental field of HAU in 1999 (T.D. Fu, unpublished). The *hau* CMS (00-6-102A) lines were examined for 10 consecutive generations during a 5-year period in Lanzhou in summer (altitude 2,200 m, latitude 37°N, average temperature 20.4°C) and in Wuhan in spring (altitude 50 m, latitude 30°N, average temperature 17°C) in China. The maintainer was another *B. juncea* line 00-6-102B. Lines 6w-301 and 6w-307 of *B. napus* were used as recurrent parents in crosses with *hau* CMS for ten generations (BC_{10}) to establish this CMS system in *B. napus*.

The assessment of sterility

To assess "sterility degree" in the 11th generation plants of backcross, 3,000 flowers from 300 plants (ten flowers per inflorescence per plant) were covered with pollination bags to prevent pollinations. The number of flowers inside the pollination bags resulting in pods were recorded at maturity. To assess "sterility rate", 1,000 plants of *B. juncea hau* CMS were planted, and the fertility of individual plants (the number of plants with stamens in flowers) was recorded at flowering.

Cytological studies

Immature buds (about 1.8 mm in length) of 00-6-102A and 00-6-102B were randomly collected for cytological study. They were fixed in carnoy I solution for 24 h and subsequently transferred to 70% ethanol for long-term storage. The buds were embedded in wax, sectioned and observed under Leica DMLB microscope (wetzlar, Gemany) equipped with CCD (LEICA DL73300F) (Yu and Fu 1988).

Analysis of restorer and maintainer relationship

Fourteen breeding lines (restorers and maintainers of different CMS systems) were tested for their ability to act as restorers and maintainers across several CMS lines (Table 1). Pollination was carried out by hand and F_1 seeds were harvested and F_1 plants were assessed for the degree of male sterility. The following categories could be recognized according to the method of Pahwa et al. (2004): (1) Male fertile: flowers with normal sized petals and anthers containing fertile pollen, and seed set on selfing to a level similar to that in a fertile euplasmic check. (2) Male sterile: flowers with narrow petals and rudimentary anthers with little or no fertile pollen and no seed set on selfing. (3) Partially fertile: flowers having medium sized petals and small mal-formed anthers positioned significantly below the stigma with reduced pollen load and poor seed set on selfing.

DNA extraction and mitochondrial probes

Buds collected from five different CMS and maintainer lines in the field were used for extraction of total genomic DNA. Total DNA from six lines, one maintainer line and five different CMS, were extracted using a modified CTAB method (Doyle and Doyle 1990). Young and healthy buds were ground into a fine powder with liquid nitrogen. About 10 g of frozen powder was transferred into pre-labelled 50 ml polypropylene tubes, which were chilled with liquid nitrogen. A preheated (65°C) 20 ml extraction buffer (0.5 M NaCl, 100 mM Tris–HCl (pH 8.0), 50 mM EDTA (pH 8.0), 2% CTAB) was adjusted to pH 7.5 and 0.5 ml β-mercaptoethanol was then added to each tube. Each tube was mixed thoroughly with gentle agitation and incubated for 60 min at 65°C. Chloroform: isoamyl alcohol (24:1) was then added to the

Table 1 The origins of major experimental materials

Name of accessions	Origin	Species	Fertility
hau CMS and its maintainer line			
00-6-102A[a]	*hau* CMS	*B. juncea*	Sterile
00-6-102B[a]	Maintainer line of *hau* CMS	*B. juncea*	Fertile
The materials used for analysis of restorer and maintainer relationships			
6W-301A	*hau* CMS	*B. napus*	Sterile
1141A[a]	*pol* CMS	*B. napus*	Sterile
6-300A[a]	*ogu* CMS	*B. napus*	Sterile
6-350A[a]	*tour* CMS	*B. napus*	Sterile
6-360A[a]	*nap* CMS	*B. napus*	Sterile
6-270B	Maintainer line of *tour* CMS	*B. napus*	Fertile
6-260R	Restorer line of *tour* CMS	*B. napus*	Fertile
6-301B	Maintainer line of *ogu* CMS	*B. napus*	Fertile
6-301R	Restorer line of *ogu* CMS	*B. napus*	Fertile
02-102	Maintainer line of *nap* CMS	*B. napus*	Fertile
5021C	Restorer line of *nap* CMS	*B. napus*	Fertile
3706	Restorer line of *pol* CMS	*B. napus*	Fertile
3721	Restorer line of *pol* CMS	*B. napus*	Fertile
5148	Restorer line of *pol* CMS	*B. napus*	Fertile
5200	Restorer line of *pol* CMS	*B. napus*	Fertile
5900	Restorer line of *pol* CMS	*B. napus*	Fertile
71-1	Restorer line of *pol* CMS	*B. napus*	Fertile
Hui10	Restorer line of *pol* CMS	*B. napus*	Fertile
6-300R	Restorer line of *pol* CMS	*B. napus*	Fertile
05A-409	Synthetic *B. napus*	*B. napus*	Fertile
The materials used as receptors for the transfer of hau CMS from *B. juncea* to *B. napus*			
6W-301	Maintainer line	*B. napus*	Fertile
6W-307	Maintainer line	*B. napus*	Fertile

[a] Six materials marked were also used in RFLP analysis.

Table 2 Mitochondrial probes used for RFLP analysis

Probe	Size of fragment (kb)	Digestion site (PCRed)	Source	Reference
atp6	2.7	*Hin*dIII	Maize	Dewey et al. (1985a)
atp9	2.2	*Xba*I	Maize	Dewey et al. (1985b)
atpα	4.2	*Hin*dIII	Maize	Braun and Levings (1985)
coxI	4.5	*Bam*HI	Wheat	Bonen et al. (1987)
coxII	2.8	*Kpn*I/*Bam*HI	Maize	Fox and Leaver (1981)
Orf222	0.66	PCR amplified	Rapeseed	L'Homme et al. (1997)
orf263-atp6	1.2	PCR amplified	Rapeseed	Landgren et al. (1996)
atp1	1.47	PCR amplified	*Arabidopsis thaliana*	NC-001284.2, GI:26556996
cob	1.06	PCR amplified	*Arabidopsis thaliana*	NC-001284.2, GI:26556996
Orf222-nad5-orf139	1.04	PCR amplified	Rapeseed	L'Homme et al. (1997)

tubes and mixed thoroughly with gentle agitation. The tubes were then centrifuged for 15 min at 6,500 rpm. Supernatant DNA was precipitated by the addition of an equal volume of ice-cold isopropanol. The precipitated DNA was rinsed twice with 70% ethanol, and transferred to a sterile 1.5 ml Eppendorf tube. The precipitated DNA was then dissolved in sterile water and quantified using agarose gel electrophoresis. DNA concentration and purity were measured by a Beckman spectrophotometer at a wavelength of 260 and 280 nm.

Mitochondrial probes were either cloned and PCR amplified based on published information of *Arabidopsis thaliana* (NC_001284.2, GI:26556996), wheat, maize or *B. napus* (Table 2).

Fig. 1 Flower morphology of *B. juncea hau* CMS line 00-6-102A (A, stamens degenerated into small petals) and its maintainer line *B. juncea* 00-6-102B (B, normal stamens)

RFLP analysis of mitochondrial DNA

RFLP analysis was performed as previously described (Kang et al. 2001), with some minor modifications. Total DNA was digested with five different restriction enzymes—*Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI, *Pst*I. Restriction digestion was carried out with 2 U restriction enzymes per microgram of DNA. Approximately 20 µg DNA was digested for 12 h with each enzyme, with the total volume of 50 µl. Genome DNA was separated on 0.8% agar in 1× TAE buffer, and then transferred to nylon membrane, after which the membrane was conserved in 20× SSC. Mitochondrial specific probes were used for RFLP analysis (Landgren et al. 1996; Delorme et al 1997; Nahm et al. 2005). The probes were labelled with α-[^{32}P]dCTP. The labelled probes were denatured by alkaline treatment with a final concentration of 0.4 N NaOH, and were added to filters in 30 ml of hybridization buffer (5× SSC, 0.4% SDS, 5× Denhardt's reagent). Hybridization was conducted at 65°C for 16 h. Filters were washed twice (cold 5 min and hot 15 min at 65°C) with 1× SSC, 0.1% SDS, and then again with 0.5× SSC, 0.1% SDS (hot 15 min at 65°C). The filters were then placed under Agfa CP-BU film for several days, depending on the strength of the signal.

Results

Development of *hau* CMS in *B. juncea* and in *B. napus*

The male sterility found in the spontaneous mutant in *B. juncea* could be maintained in this species After ten generations of backcrossings with *B. juncea* 00-6-102B, a stable sterile *B. juncea* line (*hau* CMS) was established. The "sterility degree" of 3,000 flowers was 100% and the "sterility rate" of 1,000 plants was also 100% in Lanzhou and Wuhan, indicating the stability of *hau* CMS in two different environments. This CMS was transferred to *B. napus* through interspecific hybridization. The resultant *B. napus* CMS was also absolute and stable when 6w-301 and 6w-307 were used as maintainers.

Cytological observation of *hau* CMS anther development

Anthers of *hau* CMS line 00-6-102A transformed into thickened petal-like structures, which had no anther and filament (Fig. 1). These structures were similar in shape and colour to normal petals. However, the transverse section showed that they were thicker with increased cellular layers than normal petals and had no pollen sacs (Fig. 2). The stamen primordium deviated from the normal polarization and formed petal primordium, which developed into petal-type structures at the stamen location. These characteristics differed from other CMS systems reported so far. The pollen abortion stage was earlier than other CMS types (Yu and Fu 1988; Grant et al. 1986). The anther development of maintainer 00-6-102B was normal, and each anther had four normal pollen sacs in papilionaceous shape (Fig. 2). The A-lines of *pol*, *nap*, *tour*, and *ogu* CMS systems formed anthers, but were devoid of functional pollen.

The restorer and maintainer relationship of *hau* CMS

When *pol* CMS restorer 6-300R was crossed with five different CMS systems, progenies of *pol* CMS were fertile but progenies of the other four CMS lines were completely male sterile. When *pol* CMS restorers such as 3706, 3721, 5148, 5200, 5900, 71-1, Hui10 were crossed with five CMS systems, progenies of *pol* CMS and *nap* CMS were all fertile but progenies of the other three CMS lines were completely male sterile (Table 3). Similarly, *tour* CMS restorer 6-260R restored the fertility of *tour* CMS but not the other four CMS lines. *Ogu* CMS restorer 6-301R was also specific only to *ogu* CMS, and *nap* CMS restorer 5021C was specific to *nap* CMS. When crossed to restorers of other CMS systems, the progenies of *hau* CMS, were all sterile. Therefore, none of the restorers of other CMS systems was effective in restoring *hau* CMS (Table 3). To identify restorers and maintainers of *hau* CMS in *B. napus*, more than 140 different lines of wide origin were used as pollinators in Lanzhou and Wuhan during a 5-year period (data not shown), but restorer lines could not be found. On being crossed to a synthetic *B. napus* 05A-409 (the progeny of

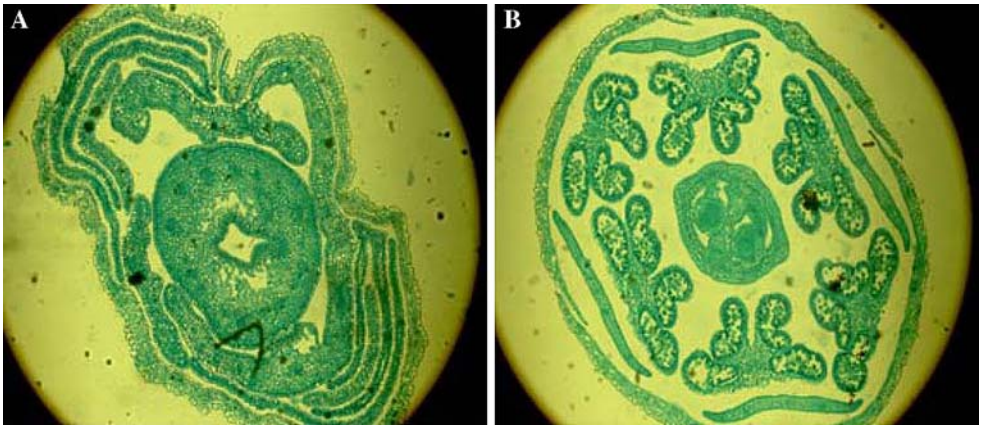

Fig. 2 Micrographs (×40) of microstructure of anther development. **a** *B. juncea hau* CMS line 00-6-102A: stamens completely degenerate into small and thick petals. Petals have many layers of cell, no polarization of archesporial cell. **b** *B. juncea hau* maintainer line 00-6-102B (normal cytoplasm): the outermost layer is calyx, which co-exists with petals, and there are six normal stamens with normal anther development

Table 3 Restorer and maintainer relationships between different CMS types

Name of *B. napus* accession	Function	*hau* CMS in *B. napus*	*tour* CMS	*pol* CMS	*ogu* CMS	*nap* CMS
3706	*pol* restorer	S	S	F	S	F
3721	*pol* restorer	S	S	F	S	F
5148	*pol* restorer	S	S	F	S	F
5200	*pol* restorer	S	S	F	S	F
5900	*pol* restorer	S	S	F	S	F
71-1	*pol* restorer	S	S	F	S	F
Hui10	*pol* restorer	S	S	F	S	F
6-300R	*pol* restorer	S	S	F	S	S
6-270B	*tour* maintainer	S	S	S	S	S
6-260R	*tour* restorer	S	F	S	S	S
02-102	*nap* maintainer	S	S	S	S	S
5021C	*nap* restorer	S	S	S	S	F
6-301R	*ogu* restorer	S	S	S	F	S
05A-409	Synthetic *B. napus*	PF	S	S	S	S

F fertile, *S* sterile, *PF* partially fertile

hybrids between *B. oleracea* and *B. rapa*), the progenies of *hau* CMS were partially fertile but no seed set was obtained on selfing.

RFLP analysis of mitochondrial DNA

The polymorphism of mitochondrial DNA was detected with mitochondrial probes *atp1*, *orf222*, *atp6*, *atp9*, *cob*, *coxII*, *Orf222-nad5-orf139*, *coxI*, *atpα* and *Orf263-atp6*. Out of 40 mitochondrial probe/enzyme combinations, 16 combinations exhibited polymorphism in Table S1 (Electronic supplementary material), such as *atp6*/*Hin*dIII, *atp6*/*Eco*RI, *atp6*/*Eco*RV, *atp9*/*Bam*HI, *atp9*/*Eco*RI and *atp1*/*Bam*HI (Fig. 3). *Hau* CMS 00-6-102A showed different banding patterns from the other four CMS lines and its maintainer line (00-6-102B). Out of 16 combinations, five probe/enzyme combinations alone distinguished the five CMS systems from each other (Table S1 of ESM; Fig. 3f). The *atp6* probe in combinations with three restriction endonucleases (*Hin*dIII, *Eco*RI and *Eco*RV) distinguished the *hau* cytoplasm *from pol*, *nap*, *ogu* and *tour* (Fig. 3a–c). In combination of *atp6/Hin*dIII, the main band (3.9 kb) of *pol*, *nap*, *ogu* and *tour* cytoplasms was the same as that of the maintainer line of *hau* (00-6-102B), but different from the main band (3.0 kb) of *hau* cytoplasms (Fig. 3a). In two other combinations (*atp6*/*Eco*RI and *atp6*/*Eco*RV), only one band was observed for each material, and the polymorphism of the bands could distinguish the *hau from pol*, *nap*, *ogu* and *tour* cytoplasms (Fig. 3b, c). The *atp9* probe in combinations with two restriction endonucleases (*Eco*RI and *Bam*HI) also allowed us to distinguish the *hau* from the other cytoplasms (Fig. 3d, e). The combination

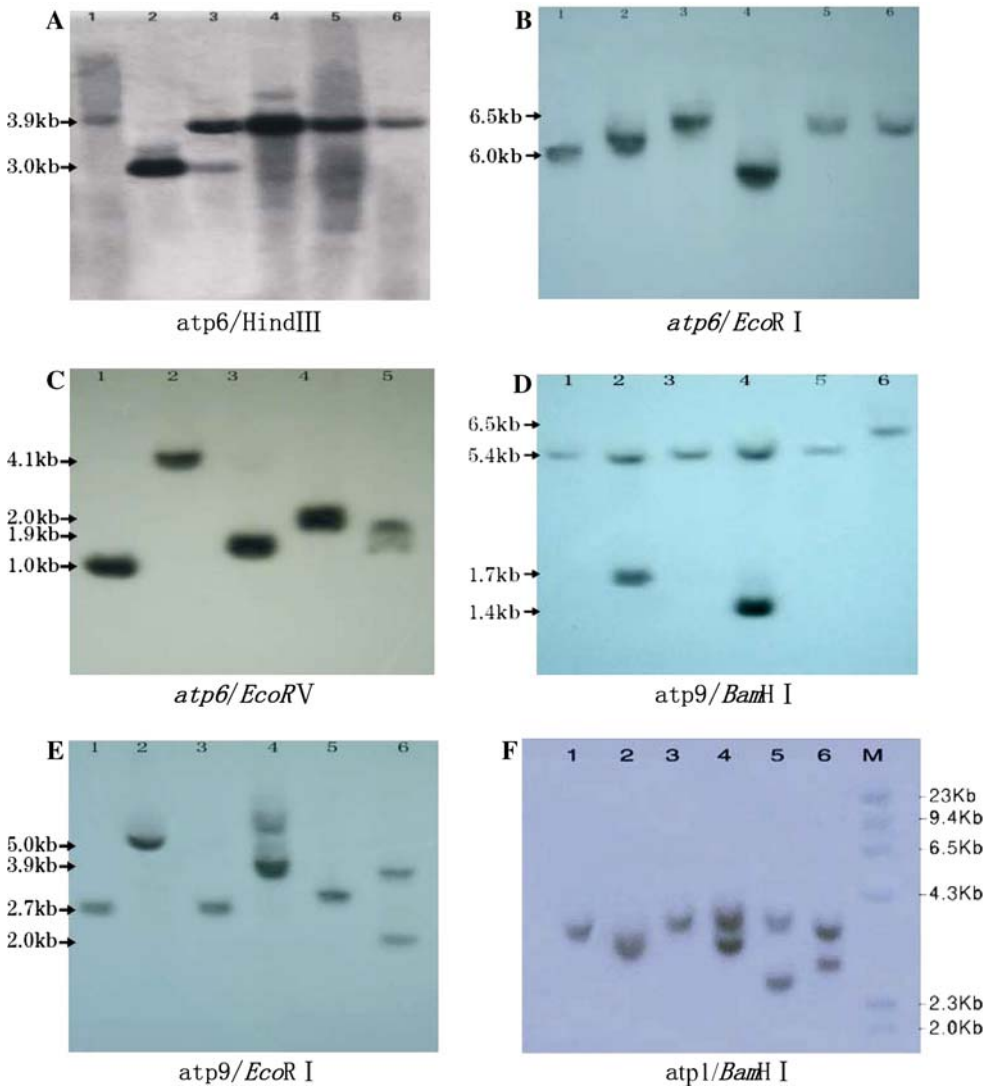

Fig. 3 Southern analysis between five CMS and one maintainer lines. Total DNAs were digested with *Eco*RV, *Eco*RI, *Hin*dIII and *Bam*HI, blotted onto a nylon membrane and hybridized with a gene-specific *atp9*, *atp1* and *atp6* mitochondrial probe. **a** *atp6*/*Hin*dIII, **b** *atp6*/*Eco*RI, **c** *atp6*/*Eco*RV, **d** *atp9*/*Bam*HI, **e** *atp9*/*Eco*RI, **f** *atp1*/*Bam*HI. *Lanes 1 pol* CMS, *2 hau* CMS 00-6-102A, *3 hau* maintainer 00-6-102B, *4 tour* CMS, *5 nap* CMS, *6 ogu* CMS, *M* markers

of *atp1*/*Bam*HI alone could distinguish the five CMS from each other (Fig. 3f). These results indicated that the mitochondrial DNA of *hau* CMS 00-6-102A was different from those of *pol*, *nap*, *ogu* and *tour*.

Discussion

Investigations on the *hau* CMS in *B. juncea* along with *pol*, *nap*, *ogu*, and *tour* CMS on cytology, general genetics and molecular genetics show that *hau* CMS is a novel type of cytoplasmic male sterility. Its sterility is stable and complete with the sterility degree and sterility rate both 100%. When the sterile cytoplasm of *hau* CMS was introduced into *B. napus* following interspecific hybridization, the sterility was very stable and absolute. Compared with other CMS systems, stable and complete sterility of *hau* system makes it very suitable for Cruciferous vegetable breeding where the potential of heterosis has already been reported (Ke et al. 1992). This sterility is probably due to its complete absence of anthers and their transformation to petals.

Several approaches have been used to characterize and classify various CMS systems by researchers, based on the original cytoplasm classification, restorer and maintainer relationships classification (Beckett 1971), anther abortion stage classification (Yu and Fu 1988), molecular genetic classification, and sporophytic and gametophytic sterility classification. In the present investigation, we adopted an integrated approach to classify the five types of CMS systems at morphological, cellular, genetic and molecular levels and confirmed that the *hau* CMS is different from others. The integrated approach to classify CMS systems, outlined in this paper, helps to avoid unnecessary duplicated researches and to correctly select CMS systems for different breeding purposes.

The main disadvantage of the *pol* CMS system is that male sterility is sensitive to environment in different nuclear backgrounds, leading to its breakdown and selfing

during the process of hybrid seed production (Fu 1995). *Nap* CMS is very sensitive to temperature, thus limiting its use in hybrid production. *Tour* CMS has poor "sterility degree" with pollen production and is not used in hybrid production (Fu 1995). At present, *pol* and *ogu* CMS are widely used over the world. The discovery of *hau* CMS helps to increase the genetic diversity of CMS. The *hau* CMS system is not affected by environment and also the sterility degree and rate were both 100%. This CMS was introgressed to *B. napus* and Cruciferous vegetables (such as *B. juncea* var. *capitata* Hort.ex Li, *B. juncea* var. *multiceps* Tsen et Lee, *B. juncea* Czern. et Coss. var. *tsatsai* Mao, *B. rapa* Linn. var. *purpurea* Bailey and *B. rapa* var. *pekinensis*) by hybridization. The sterility of obtained CMS lines was stable and complete, in contrast to other four CMS types.

The *hau* CMS system appears to result from a disruption of anther development at a very early stage of stamen primordium differentiation. In contrast, the anther development of *pol* CMS was inhibited at the polarization stage of the archespore, therefore no pollen sacs were formed (Yu and Fu 1988). The anther sterility of *nap* CMS results from delayed pollen development and indehiscent anthers (Grant et al. 1986). The anther development of *ogu* CMS was inhibited at the tetrad to single nucleus pollen formation stage. The development of sporule was similar to fertile lines, but tetrad release was very difficult and the duration was also long (Yu and Fu 1988). The results indicate that the *hau* CMS was unique and different from other CMS systems in rapeseed. Development disruption of the *pol*, *tour*, *ogu*, and *nap* CMS systems must occur at a later stage than the *hau* CMS system, as these systems form normal-looking anthers, but fail to develop normal archesporial or microspore cells.

The identification of the restorer and maintainer relationship is one of the most classical methods to differentiate the types of sterile cytoplasm. The *hau* CMS line 6W-301A has the same nucleus genetic background as its *B. napus* maintainer line 6W-301 after ten backcross generations (BC10). The results of test crossing with restorer lines indicated that fertility in the *hau* CMS was not restored by restorer lines from *pol*, *ogu*, *nap* and *tour* CMS systems. Therefore we conclude that *hau* CMS is a distinct type of cytoplasmic male sterility. A maintainer line for *hau* CMS has been developed but a restorer line has not been found so far. The absence of a restorer means that the *hau* CMS is not currently useful for rapeseed breeding or production. However, we observed that some progenies of *hau* CMS were partially fertile when crossed to a few testing materials, which indicates that the test materials carry some restorer genes for *hau* CMS.

RFLP technology was effective in identifying several polymorphisms in mitochondrial DNA, which clearly distinguished the five different CMS systems. The restoration and maintenance of *kos* CMS were the same as that of *ogu* CMS in radish. But RFLP and sequence analysis reveal that their mechanisms are different (Sakai and Imamura 1992). Similarly, 681A and *pol* CMS have the same restoring–maintaining relationship and belong to the same type of CMS revealed on the basis of restoring–maintaining relationship. However, their ORFs of sterility genes are probably different as well (Liu et al 2005).

In the present *hau* CMS, we need to study the fertility transition mechanism of *hau* CMS, to search for the restoring lines and to isolate and clone the *Rf* gene(s). It will be very interesting to study the expression of *Rf* gene and the interaction between *Rf* gene and sterility associated mtDNA location. We aim at discovering the mechanism of CMS fertility, maintenance and restoration. The molecular classification conducted in this research can be used as a foundation for the future cloning of the CMS genes, the study of transcription process and the expression of the genes at enzyme level.

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