Genetic classification of a newly identified cytoplasmic male sterility *hau* CMS system in *Brassica napus* L.

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Abstract

In this study, we have investigated a novel cytoplasmic male sterility (CMS) system named *hau* CMS (6-102A). The genetic classification of this system was confirmed by comparison with classical systems such as *pol*, *nap*, *ogu*, and *tour* based on genetic, cytological and molecular genetic studies. Anthers of *hau* CMS were aborted during the stage of stamen anlage polarization and no archesporial cell polarization was found. Compared with the other four CMS systems, the abortive stage in *hau* CMS was earlier, when crossed with 140 different original accessions. The results of cross hybridization indicated that the offspring of *hau CMS* had different fertility when hybridized with *pol*, *ogu*, *nap* and *tour* CMS systems. The restorer-maintainer relationship of *hau* CMS was different compared with *pol*, *nap*, *ogu*, and *tour* CMS systems. Sixteen out of 40 combinations of mitochondrial probes/enzyme used for RFLP analysis could distinguish the *hau* CMS system from the remaining four CMS systems studied. Out of these 16 combinations, 5 probe/enzyme combinations could distinguish the five CMS systems with each other. These results showed that *hau* CMS is a novel system different from other known CMS systems.

Key words: cytoplasmic male sterility, *hau CMS*; RFLP; mitochondria; canola

Introduction

Brassica napus L. is one of the most important oilseed crops, and cytoplasmic male sterility (CMS) is an important approach to exploit heterosis in this crop (Fu 1995). CMS is a common trait in angiosperms (Laser and lersten 1972). It is a maternally inherited trait that results 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). In all cases to date, the mitochondrion has been the location of maternally-inherited traits that disrupt pollen development (Hanson et al 2004). Several CMS types have been reported in rapeseed, like *ogu* CMS (Ogura 1968), *tour* CMS (Rawat and Anand 1979), *Pol* CMS (Fu 1981), *nap* CMS (Thompson 1972; Shiga and Baba 1973; Shiga and Baba 1971). Nine CMS lines in *B. napus* were classified into four types listed above by analysis of restorer and maintainer relationships and mtDNA RFLP (Yang *et al* 1998). *Pol* CMS was discovered in 1972 (Fu, 1981), and is the first CMS system to be applied widely in hybrid seed production (Fan et al 1986; Downey 1989; Robbelen 1991). The main disadvantage of *pol* CMS is that male sterility is sensitive to environment in different nuclear backgrounds, and selfing occurs in some situations which reduces hybridity levels in F_1 hybrids.

The genetic classification of CMS systems is a very important aspect of hybrid breeding. In maize, mtDNA of the T, S and C group had unique electrophoresis bands which could be used to classify the type of CMS (Kemble 1980); other methods of classification include male fertility restoration testing, RFLP of mtDNA and Sl, S2 plasmid-like DNA analysis. Following this approach, the cytoplasm of WBMs in maize, which was selected from the WBM population developed by Huazhong agricultural University, was classified into S-type of CMS (Li et al 2004). According to restorer and maintainer relationship, rice cytoplasm in sterile lines was classified into wild abortive type, Honglian type and Dian type (Li 1980). Mitochondria DNA can distinguish the cytoplasm types in CMS rice and maize (Zheng 1997; Kadowakietal.1988). There are many reports of classification of CMS systems in sunflower, pearl millet and radish based on mitochondrial DNA (Horn et al. 1999; Seok-Hyen Nahm et al.2005; Delorme et al. 1997).

Materials and methods

Plant materials

Five CMS lines and fourteen fertile lines of *B. napus* used in the experiment are listed in Table 1, These materials were planted in farmland till blooming, used for mensuration restorer and maintainer relationship of *hau* CMS in *B. napus*, including wide origin breeds of restorer genes and maintainer genes in *B. napus CMS* from Canada, Sweden and China. These breeding lines were used for male parents to test the induction of male sterility by five CMS lines. These breeding lines were pollinated onto the stigma of several CMS and F_1 plants to obtain progeny and compare the degree of male sterility. Immature buds of 6-102A and 6-102B were chosen for cytological Study. Buds were collected from five different CMS and maintainer lines of 6-102B in the field for extraction of total genomic DNA. Different probe/enzyme combinations were used for southern hybridization, and the type of *hau* CMS was identified according to the hybridization patterns of RFLP markers.

Hau CMS (6-102A) was originally found in *B. juncea* by professor Fu Tingdong in the experimental field of Huazhong Agriculture University in 1999. It was a natural male sterile mutant in the population. The trait was maintained by crossing with another wild *B. juncea*. Seeds were planted at the University experimental station and the plants were coded as 00-6-102A. The wild male *B. juncea* parent was numbered 00-6-102B. All the F_1 plants (00-6-102A) were sterile and no normal anthers were observed. To perform directional selection, this sterile line was back-crossed with a maintainer 00-6-102B. A stable CMS line was established after 10 generations of back-cross. At the same time, 00-6-102A was also repeatedly back-crossed with 6w-301 and 6w-307 of *B. napus* for six generation to establish a B. napus CMS system.

Table 1. The origin of experimental materials

Name of accessions	Resource	Species	Fertility
6-102A	*Hau* CMS	*B. juncea*	sterile
6-102B	Maintainer line of *hau* CMS	*B. juncea*	fertile
1141A	*Pol* CMS	*B. napus*	sterile
5900R	restorer line of *pol* CMS	*B. napus*	fertile
6-350A	*tour* CMS	*B. napus*	sterile
6-260R	Restorer line of *tour* CMS	*B. napus*	fertile
6-270B	Maintainer line of *tour* CMS	*B. napus*	fertile
6-300A	*Ogu* CMS	*B. napus*	sterile
6-301B	Maintainer line of *ogu* CMS	*B. napus*	fertile
6-301R	restorer line of *ogu* CMS	*B. napus*	fertile
6-360A	*nap* CMS	*B. napus*	sterile
02-102	Maintainer line of *nap* CMS	*B. napus*	fertile
02-106	Maintainer line of *nap* CMS	*B. napus*	fertile
6-300R	restorer line of *pol* CMS	*B. napus*	fertile
5148	restorer line of *pol* CMS	*B. napus*	fertile
Hui10	restorer line of *pol* CMS	*B. napus*	fertile
5200	restorer line of *pol* CMS	*B. napus*	fertile
3707	restorer line of *pol* CMS	*B. napus*	fertile
3721	restorer line of *pol* CMS	*B. napus*	fertile
71-1	restorer line of *pol* CMS	*B. napus*	fertile
6W-301	Maintainer line	*B. napus*	fertile
6W-307	Maintainer line	*B. napus*	fertile

Note: All the materials were from the Rapeseed Lab of Huazhong Agricultural University in china.

Cytological studies

Immature buds of 6-102A and 6-102B (1 mm in length) were fixed in the carnoy I solution for 24 h and then transferred into 70% ethanol for long term storage. The buds were inbeded in wax, sectioned and observed under electron microscope.

Analysis of restorer and maintainer relationship

Thirteen breeding lines were used as male parents in order to test the induction of male sterility by several CMS lines (Table 2). These breeding lines were pollinated onto the stigma of several CMS and F_1 plants to obtain progeny and to compare the degree of male sterility occurring in their progenies. *Hau* CMS 6-102A has the same nuclear background as maintainer line 6-102B after eight backcross generations (BC8) with *B. napus*. The level of fertility was recorded using the classifying standards of Yang et al. (1991).

Total DNA extraction and mitochondrial probe

Total DNA from six lines was extracted using a modified CTAB method (Doyle et al 1990). Young and healthy leaves were ground into a fine powder with liquid nitrogen. About 10g of frozen powder was transferred into pre-labeled 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 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 nm and 280 nm.

Table 2 Seven mitochondrial probes used for RFLP analysis

Gene	Size of fragment	Digestion site (PCRed)	Resource	Antibiotic
atp6	2.7Kb	HindIII	Maize	Amp r
atp9	2.2 Kb	XbaI	Maize	Amp r
atpα	4.2 Kb	HindIII	Maize	Amp r
coxI	4.5 Kb	BamHI	Wheat	Amp r
coxII	1.7 Kb	KpnI/ BamHI	Wheat	Amp r
$OrF_2$22	0.66Kb	(PCR amplified)	Rapeseed	Amp r
$orF_2$63-atp6	1.2Kb	(PCR amplified)	Rapeseed	Amp r
atp1	1.47Kb	(PCR amplified)	Rapeseed	Amp r
cob	1.06Kb	(PCR amplified)	Rapeseed	Amp r
$OrF_2$22-nad5-$orF_1$39	1.04Kb	(PCR amplified)	Rapeseed	Amp r

Among six lines, five were different CMS types and one was a male RFLP analysis of mtDNA RFLP analysis was performed as previously described (Kang et al. 2001), with some minor fertile line. Total DNA was digested with five different restriction enzymes - *Eco*RI, *Eco*RV, *Hind*III, *BamH*I. Restriction digestion was carried out with 2 U restriction enzymes per microgram of DNA. Approximately 20 μg DNA was digested for 12 hour with each enzyme, with the total volume of 50 μl. Genome DNA was separated on 0.8% agar in 1X TAE buffer, and then transferred to nylon membrane, after which the membrane was conserved in 20xSSC. The probes were labeled with α- [^{32}P] dCTP. The labeled probes were denatured by alkali treatment with a final concentration of 0.4 N NaOH, and 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 15min 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*

After ten generations of backcrossing, a stable sterile line was obtained in *B. juncea*, which was not affected by environment. The sterile degree and sterile rate were both 100%. This CMS was also transferred into *B. napus* and cruciferous vegetables through interspecific hybridization. The obtained *B. napus* CMS was also entire and stable when 6w-301 and 6w-307 were used as maintainers.

Fertility of Hau CMS in B. juncea

In *B. juncea* 6-102A, the sterility was stable, complete, and not effected by environment. The CMS line was tested for 10 consecutive generations within a five year period in Lanzhou of Gansu province and Wuhan of Hubei province in china. The fertility of all the plants studied were recorded as 0 grade. The degree and rate of male sterility were both 100 % (Fig 1A)

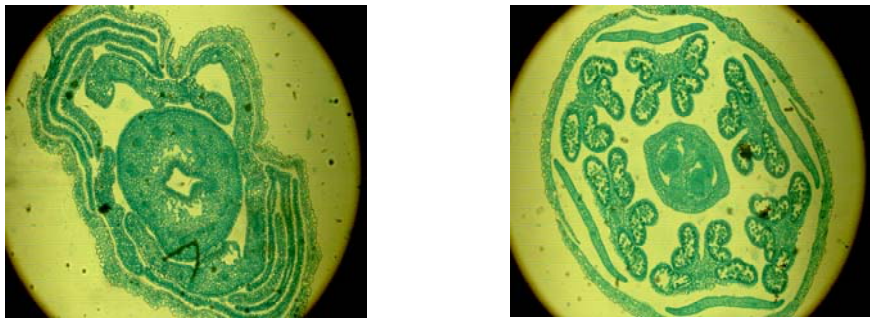
A

B

Fig. 1 A: Flower morphology of *B. Juncea hau* CMS line 6-102A. Its stamens were degenerated into small petals. B: Flower morphology of maintainer line *B.Juncea* 6-102B, showing normal stamens.

Cytological observation of hau *CMS anther development*

Observation of the intact flowers of 6-102A showed that all stamens were reverted to petals. The reverted petals were similar in shape and color to the normal petals, but from the transverse section they were thicker with increased cellular layers and no archesporial cell formation resulting in no anther formation (Fig 2A). Sterility of hau CMS happened at anlage differentiation stage was earlier than other CMS systems. The anther development of maintainer 6-102B was normal, and each anther had four normal pollen sacs in papilionaceous shape (Fig 2B).



A B

Fig. 2 A: Anther development microstructure of 6-102A: Stamens have completely degenerate into small petals, the degenerated petals are thicker than normal petals. The cell has many layers, no polarization of archesporial cell. B**:** Anther development microstructure of 6-102B (normal cytoplasm): The outtest layer is calyx, which co-exists with petals, there has six normal stamens, anthers develop normally.

Table 3: **Comparison of main stages for sterility in several CMS systems***

CMS in rapeseed	*Hau* CMS	*pol* CMS	*tour* CMS	nap CMS	*Ogu* CMS
Main abortion stage	Polarization of stamen anlage	Polarization of archespore	Polarization of archespore	Polarization of archespore	late stage of tetrad to single nucleus sporule stage

*Based on liu (2001)

The restorer and maintainer relationship of hau *CMS*

Elucidation of restorer and maintainer relationship is one of the classical methods to distinguish the types of cytoplasmic male sterility. Mensuration of restorer and maintainer relationship of *hau* CMS in *B. napus* used more than 140 kinds of wide origin materials, including breeds from Canada, swden etc. *Hau CMS* 6-102A had the same nuclear background of genetics with maintainer line 6-102B after eight backcross generations (BC8) with *B. napus*, Chose 13 tested breeding lines of *B. napus* to cross with *pol* CMS, *ogu* CMS, *our* CMS, nap CMS and *Hau* CMS. In order to compare the degrees of male sterility between five CMS lines, the five lines were used as female parents for cross with 13 male fertile lines breeding lines,The progenies of Breeding lines 5900,5148,5200,6-300R were found to be fertile when crossed with *pol CMS* but the progenies of other four CMS lines were completely male sterile. The progenies of Breeding lines 6-260R were found to be fertile when crossed with *tour CMS* but the progenies of other four CMS lines were completely male sterile, similarly The progenies of Breeding lines 6-301R were found to be fertile when crossed with *ogu CMS* but the progenies of other four CMS lines were completely male sterile. The fertility of the F_1 was assessed to indicate that the restorer and maintainer relations of 6-102A was different to other types of CMS (Table 4). The research of restorer and maintainer relation studied about *pol* CMS,*nap* CMS,*ogu* CMS and *cam* CMS (Yang et al. 1991). The result of cross hybridization indicates that the offspring of *Hau CMS* cross hybridizing with *pol* CMS, *ogu* CMS, *nap* CMS and *tour* CMS had different fertile reactions (Table 4), therefore we can conclude that *Hau CMS* is be a novel type of cytoplasmic male sterility.

Table 4: Mensuration of restorer and maintainer relationship between several kinds of sterilities

Name of accessions	*Hau* CMS	*tour* CMS	*Pol* CMS	*Ogu* CMS	*nap* CMS
5900	S	S	F	S	S
5148	S	S	F	S	S
5200	S	S	F	S	S
Hui 10	S	S	F	S	S
6-300R	S	S	F	S	S
6-270B	S	S	S	S	S
6-260R	S	F	S	S	S
02-102	S	S	S	S	S
02-106	S	S	S	S	S
6-301R	S	S	S	F	S
3706	S	S	F	S	S
3721	S	S	F	S	S
71-1	S	S	F	S	S

F=fertility and S= sterility

RFLP analysis of mitochondrial DNA

The polymorphism of mitochondrial DNA was detected with mitochondrial probes atp1, or$F_2$22, atp6, atp9 and Or$F_2$63-atp6. Out of 40 mitochondrial probe/enzyme combinations, 16 combinations exhibited polymorphisms. Out of 16 combinations, 5 probe/enzyme combinations distinguished the five CMS systems e,g, atp6/ EcoRI, atp6/BamhI, atp6/ EcoRⅤ,atp9/ EcoRI, atp1/ BamhI, or$F_2$22/ EcoRI (data not shown). Five probe/enzyme combinations identified the cytoplasm type of rapeseed CMS lines. 6-102A shows different bands from the other four CMS lines and maintainer line. The results indicates that 6-102A in *B. juncea* was quite different from *Pol* CMS, *nap* CMS, *ogu* CMS and *tour* CMS at the

mitochondrial DNA level. The mtDNA hybridization atlas of 6-102A was also entirely different from the other five types of cytoplasm's, which exhibited obvious polymorphisms (Figure3). At the same mtDNA strips of 6-102A and 6-102B were also detected obvious polymorphisms with 24 of 40 combinations. All bands showed that the amount and location of hybridization strips were different (Fig 3)

Discussion

Through studying a novel cytoplasmic male sterility system *hau* CMS in *B. juncea* along with *Pol* CMS, *nap* CMS, *ogu* CMS, *tour* CMS on cytology, general genetics and molecular genetics level, result showed that *hau* CMS was a novel type of cytoplasmic male sterility. Making use of its stable and complete sterility, moreover the sterility degree and sterility rate were all 100 percent, When the sterile cytoplasm of *hau* CMS was introduced into *B. napus* and Cruciferae vegetable by an interspecific cross of *B. napus* cv or Cruciferae vegetable with *B. juncea* CMS. There was significant application value in using of vegetable heterosis(ke,1992). The sterility was also very stable and absolute after transferring the sterile cytoplasm into *B. napus*. Now several restorer materials of *hau* CMS have been found, which makes the application prospect more wide in hybrid production.

There are many ways for CMS classification, such as, original cytoplasm classification, restorer and maintainer relationship classification, sterile stage of pollens classification. And according to the sterile ratio of F1 cross generation classification, it can aslo be divided into sporophytal sterile and gametal sterile lines, each method has its own importance. Here author started research and analysis from molecular, cellular, to individual level, and classified a new cytoplasmic male sterility system in Brassica, So, in practical application, on one hand choose the different classification methods according to the desire, and on the other hand, it's needed to know the classification status in the different classification ways before using the sterile lines. To serve the production, by using sterile materials it is needed to know the fertility behavior at first. Because of the integration of cytology, genetic and molecular biology, classification of CMS becomes more scientific and accurate.

The bud of 6-102A was used to make olefin slice (Yu et al 1998), and the microstructure observation shows that the sterility of 6-102A was initiated at the stage of stamen anlage polarization (Fig 2A). The stamen anlage deviated from the normal polarization and formed petal anlage, which could not form into anther, and developed into petal at the stamen growing location. These characteristics differ from the cytoplasmic male sterility in rapeseed as reported until now. In contrast, the anther development of *Pol* CMS was inhibited at the stage of polarization of archespore, therefore no anther were formed (Yu et al. 1988). The anther sterility of *nap* CMS was due to anther conglutination and resulted in delayed anther development, no polarization of sporogenous was found (Grant I 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 with normal fertile lines, but tetrad release is very difficult and duration was also long (Yu et al 1988).Microscopic observation of 6-102A in *B. juncea* indicated that *hau* CMS was different from the above sterile cytoplasts, sterile stamen exhibited as a small petal. This type of sterile mode had not yet been reported in rape,These results were showed (Table 3**.)**

The mensuration of restorer and maintainer relationship is one of the most classical methods to differentiate the types of sterile cytoplasm. Mensuration of restorer and maintainer relationship of *hau* CMS in *B. napus* used more than 140 kinds of wide origin materials, including breeds from Canada, sweden etc. *hau* CMS 6-102A have the same nucleolus background of genetics with maintainer line 6-102B after eight backcross generations(BC8) with *B. napus*. At present, The result of cross test indicates that the offspring of *hau* CMS cross hybridizing with *pol* CMS, *ogu* CMS, *nap* CMS and *tour* CMS had different fertile reactions. Therefore we can conclude that *hau* CMS is a novel type of cytoplasmic male sterility. Elucidation of restorer and maintainer relationship is one of the classical methods to distinguish the types of cytoplasmic male sterility. It was sure that this research could distinguish 6-102A CMS from *pol* CMS, *ogura* CMS, *nap* CMS and *tour* CMS. But mensuration of restorer and maintainer relationship needes more materials and more time, and the fertility is easy to be influenced by temperature, environmental factors, so the results easily appeared warp. Molecular methods applied to analyze the polymorphism of mitochondrial DNA give more accurate and credible results. It was easy to find a maintainer line for 6-102A in *B. juncea*,but its restorer line was hard to be sought. Through enlarging the cross scope three restorer materials had been found among domestic and Indian materials. This finding will enlarge the the using of heterosis in rapeseed.

RFLP technology was used to analyze six kinds of different cytoplasm. Out of 40 probe/enzyme combinations. In these 40 probe/enzyme combinations, 16 combinations could differ 6-102A from other CMS systems, five combinations could completely differ six types of different cytoplasms from each other. Another important thing is that, 6-102A could not be distinguished from other five different cytoplasm by PCR analysis of 12 pairs primer (data not shown), the reasons may be that the aberrance of mitochondrial genomes were mostly caused by the recombination in repeated sequences between molecules or within the molecules and the mutation of dots, but the differentiae caused by the permutation of alkali or the inserting and missing of lesser segment were difficult to be detected by PCR amplification. The area of primer covering the mitochondrial expression was limited in this study, so it was difficult to find differentiation.

Mechanisms of CMS in *B. napus* is very complex. As for molecular biology research of cytoplasmic male sterility in rapeseed is concerned, formers had made great progress in many aspects, such as the relative gene area of sterility, the effect of CMS on relative DNA location, expression of *Rf* gene, and the atlas locating and cloning of fertility restorer genes. As rapeseed CMS is caused by a kind of mutual action of karyoplasms and influenced by environment, its mechanism is still at the stage of exploring. *Pol* CMS is associated with expression of the novel open reading frame (ORF), or$F_2$24, situated upstream of and co-transcribed with a normal mitochondrial gene, atp6 (Singh and Brown, 1993; Singh and Brown,1991) while *nap* CMS is associated with a different but related ORF, or$F_2$22, that is co-transcribed with nad5c, the central exon of a trans-spliced gene, and a short ORF of unknown function (L'Homme et al. 1993; L'Homme et al.1997). Anand CMS was breeded in *B. juncea* by Rawat and Anand in

India (Rawat and Anand 1979). This sterile cytoplasm was regarded to be originated from *Brassica Tourefortii*, which was produced by far hybridization between *B.juncea* and *B. Tourefortii*, and was a kind of alloplasmic male sterility. a chimeric orf$_2$63 was in the vicinity of the atp6 gene, which may be responsible for the expression of the *tour* CMS phenotype.(Landgren et al. 1996;Stiewe G et al.1994).Tournefortii-Stiewe CMS is associated with a special gene arrangement around a novel atp9 gene.Rearrangements upstream for one of these genes have generated a chimeric 193-codon ORF, which is designated as orf$_1$93. (J.-H. Dieterich et al .2003).Orf$_1$38 is ogura CMS-associated region by comparing the CMS cybrid with its fertile revertant(Bonhomme S et al 1991; Bonhomme S 1992).

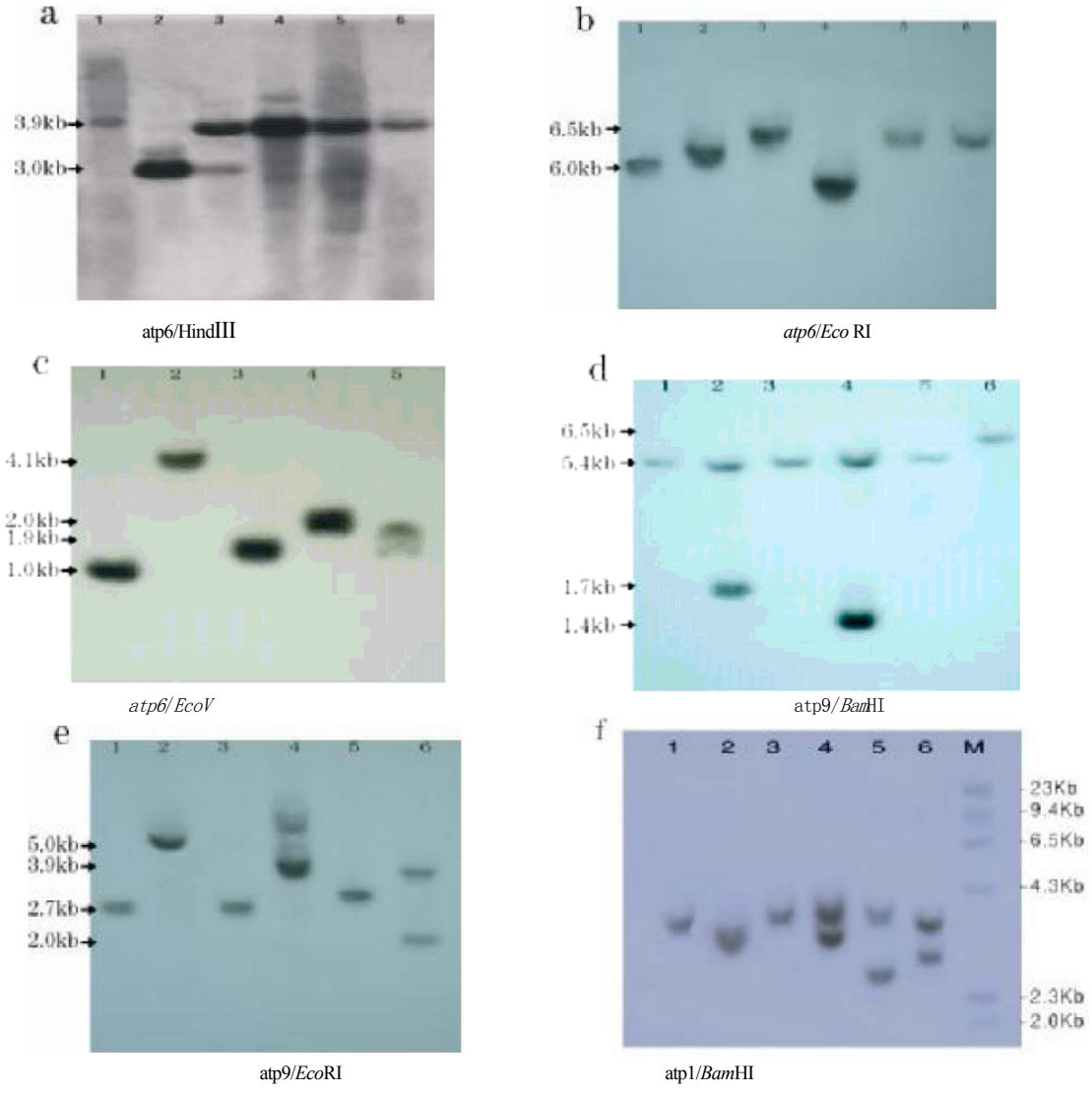


Fig. 3 Southern analysis betwe en five CMS and one maintainer lines. Total DNAs were digested with EcoRⅤ,EcoRI, HindⅢ, BamHI,blotted onto a nylon membrane and hybridized with a gene-specific atp9, atp1 and atp6 mitochondrial probe.**a** atp6/ HindⅢ,**b** atp6/EcoRI,**c** atp6/EcoRⅤ,**d** atp9/ BamHI,**e** atp9/ Eco RI,**f** atp1/BamHI.**a-f** Lanes: 1, pol CMS; 2, 6-102A ; 3, 6-102;4, tour CMS; 5,nap CMS;6,ogu CMS ;M, size markers.

Concerning to this novel sterile type *hau* CMS, we need to isolate the related gene area of *hau* CMS for more detailed study of its controlling mechanism. We also need to find the restoring line, isolate and clone rapeseed *Rf* gene(s)., It will be very interesting to study the expression of *Rf* gene and the interaction between *Rf* gene and sterility associated mt DNA location. For the long run, we will aim at discovering the mechanism of CMS fertility, maintainance and restoration.

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