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Identification of cytoplasm types in rapeseed (*Brassica napus* L.) accessions by a multiplex PCR assay

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Abstract Cytoplasmic male sterility (CMS) has widely been used as an efficient pollination control system in rapeseed hybrid production. Identification of cytoplasm type of rapeseed accessions is becoming the most important basic work for hybrid-rapeseed breeding. In this study, we report a simple multiplex PCR method to distinguish the existing common cytoplasm resources, *Pol*, *Nap*, *Cam*, *Ogu* and *Ogu*-NWSUAF cytoplasm, in rapeseed. Cytoplasm type of 35 F_1 hybrids and 140 rapeseed open pollinated varieties or breeding lines in our rapeseed breeding programme were tested by this method. The results indicated that 10 of 35 F_1 hybrids are the *Nap*, and 25 the *Pol* cytoplasm type, which is consistent with the information provided by the breeders. Out of 140 accessions tested, 100 (71.4%), 21 (15%) and 19 (13.6%) accessions possess *Nap*, *Cam* and *Pol* cytoplasm, respectively. All 19 accessions with *Pol* cytoplasm are from China. Pedigree analysis indicated that these accessions with *Pol* cytoplasm were either restorers for *Pol* CMS, including Shaan 2C, Huiyehui, 220, etc. or derived from hybrids with *Pol* CMS as female parent. Our molecular results are consistent with those of the classical test-cross, suggesting the reliability of this method. The multiplex PCR assay method can be applied to CMS "three-line" breeding, selection and validation of hybrid rapeseed.

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Introduction

Cytoplasmic male sterility (CMS) is a widespread, maternally inherited trait that results in pollen abortion due to defects in mitochondrial function. Several CMS lines have been used in rapeseed F1 hybrid production, including alloplasmic type the Ogura cytoplasm of radish, i.e. *Ogu* CMS, endogenous cytoplasms such as *Nap, Pol,* and *Shaan 2A* cytoplasms found within species. A 19-kDa protein, ORF138, was correlated with the *Ogu* CMS (Bonhomme et al. 1992; Grelon et al. 1994). The expression of *orf224/apt6* locus in mitochondrial DNA (mtDNA) is highly correlated with the *Pol* CMS trait (Singh and Brown 1991; L'Homme and Brown 1993). The *Nap CMS* is associated with the expression of *orf222/nad5c/orf139* region in the mtDNA of male-sterile *Nap* (L'Homme et al. 1997). The discovery of *Pol* CMS line has greatly stimulated the rapeseed research and production. However, prevailing usage of the limited CMS resources could cause problems for substantial development of rapeseed production. Thus, the discovery of new cytoplasm resource has been the focus of rapeseed breeders. Development of efficient method to identify CMS types is most important task for rapeseed breeding.

The common classical method for identifying cytoplasm type in rapeseed is test-cross, which requires 3–4 years,

because rapeseed is biennial. Restriction fragment length polymorphism analysis of mtDNA is a fast method to distinguish cytoplasm types (Yang et al. 1998; Wei et al. 2005), but not suitable in high-throughput genotyping due to the requirement of large amounts of mtDNA, appropriate restriction endonucleases and suitable probes in southern hybridization. PCR-based markers developed from specific gene sequences are efficient and high-throughput tool for determining cytoplasm type. The sequences of CMS-associated genes in the *Pol* and *Nap* cytoplasm and the complete sequence of *Nap* mitochondrial genome are available in GenBank (Singh and Brown 1991; L'Homme and Brown 1993; L'Homme et al. 1997; Handa 2003), thus, it provide an opportunity to develop PCR markers based on these genes for rapidly identifying cytoplasm in rapeseed. Multiplex PCR enables simultaneous amplification of many target genes interested in one reaction by using multiple pairs of primers (Chamberlain et al. 1998). This technique has been used for identifying HMW-GS genes (Ahmad 2000; Ma et al. 2003) and waxy protein genes (Nakamura et al. 2002) in wheat, and distinguishing three cytoplasm types in onion (*Allium cepa* L.) (Kim et al. 2009). Multiplex PCR assay for identifying cytoplasmic types in rapeseed breeding programme has not been developed.

The objectives of this study were to (1) develop a multiplex PCR assay for identifying cytoplasm type in rapeseed, (2) evaluate the accuracy of the multiplex PCR assay by comparing the results of 35 F_1 hybrid cytoplasm types with the information provided by the breeders, (3) test the cytoplasm types of 140 open pollinated varieties or breeding lines by the multiplex PCR assay and (4) reveal the distribution of the cytoplasm types for efficiently utilization of these materials in rapeseed heterosis breeding programme.

Materials and methods

Plant materials

Two accessions of each cytoplasm type *Nap, Pol* and *Cam* were used as materials for DNA isolation and molecular marker development. One *Ogu* CMS accession and one improved *Ogu* CMS accession (*Ogu*-NWSUAF CMS) (Chang et al. 2010) were also included (Table 1). The seeds were germinated and cultured in trays in dark condition to get yellow seedlings for mtDNA and genomic DNA (gDNA) isolation.

Cytoplasm types of 35 F_1 hybrids in region test field of Shaanxi Province in 2007–2008 (Table S1), and 140 rapeseed accessions from different countries (93 open pollinated varieties and 47 breeding lines) (Table S2) were characterized using the method developed in this study. These rapeseed accessions were sown in the experimental field of Northwest A&F University, Yangling, Shaanxi, People's Republic of China in 2007–2008. Ten individual plants of three-leaf stage randomly chosen from each hybrid/accession were used for total gDNA isolation.

Table 1 Rapeseed accessions and their cytoplasm types used for molecular marker development in this study

Number	Nuclear origin	Cytoplasm type	Male fertility
1	8C	*Ogu*-NWSUAF	MS
2	Zhongshuang 2	*Ogu*	MS
3	Zhongshuang 2	*Pol*	MS
4	D89	*Pol*	MS
5	D89	*Nap*	MF
6	Westar	*Nap*	MF
7	Bronowski	*Cam*	MF
8	Zhongshuang 2	*Cam*	MF

MS male sterile, *MF* male fertile

mtDNA and total gDNA extraction

Highly purified mtDNA was extracted according to the method reported by Wang et al. (2008). Total gDNA was extracted following the protocol described by Edwards et al. (1991).

PCR primer selection

To identify each cytoplasm type of the *Nap, Pol, Cam, Ogu* and *Ogu*-NWSUAF, four pairs of primer specific to the mtDNA genes previously described by Wei et al. (2005) were used here (primer 1–4 in Table 2). To test if the mtDNA samples are mixed with gDNA, a nuclear male sterility gene *MS2Bnap*-specific primer in rapeseed (Hu et al. 2006) was also included (primer 5 in Table 2). All these primers were synthesized in Shanghai Sangon Biological Engineering Technology & Service Co. Ltd (http://www.sangon.com).

PCR amplification using single primer set

PCR amplification with single primer pair was performed using 50 ng of gDNA or mtDNA in a final volume of 20 µl, containing 0.5 µM of each primer, 150 µM of each dNTP and 0.25 units of *Taq* DNA polymerase (Tiangen Biotech. Co., Beijing, China). PCR was carried out with initial denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 2 min and a final extension at 72°C for 10 min. The PCR products were separated by flatbed electrophoresis using 1.5% agarose gels in 1× TAE buffer. The bands were stained with ethidium bromide and visualized under UV light.

Table 2 The sequences and information of the primers used in this study

Number		Sequence 5′–3′	Target gene	References
1	P11	GAAACGGGAAGTGACAAT	*Orf138*	
	P12	GCATTATTTTCTCGGTCCAT		
2	P21	AGCTGTCTGGAGGGAATC	*Orf222*	
	P22	GCGGTCTCACGCACTAATC		Wei et al. (2005)
3	P21	AGCTGTCTGGAGGGAATC	*Orf224*	
	P32	ACGACATCAAGGAGGAAC		
4	P41	ATGGGACTCACCCTTTACTTGA	*Orf139*	
	P42	CCGTTGCAGAAAAGAGATCC		
5	P51	CAGATTCAGCAGAGGAGATTGC	*MS2Bnap*	Hu et al. (2006)
	P52	GAAGCTCAGCTAAGTCCTCG		

Multiplex PCR

Multiplex PCR was developed for a simultaneous amplification of genes *Orf138, Orf224* and *Orf222* targeting the *Ogu, Pol* and *Nap* cytoplasm, respectively. The reaction components contained 50 ng of mtDNA/gDNA, 150 μM of each dNTP, 0.25 units of T*aq* DNA polymerase and 0.15 μM of each primer for gene *Orf138, Orf224* and *Orf222*, respectively, in total volume of 20 μl. The PCR amplification programme and electrophoresis was the same as that for the above-mentioned single primer set.

Results

The test of purification of mtDNA and gDNA isolated from rapeseed

To test if the mtDNA isolated from each of the eight accessions is mixed with its gDNA, PCR amplification of nuclear gene *MS2Bnap* was conducted with the primer 5 (Table 2), by using gDNA and mtDNA samples as templates, respectively. The patterns of PCR-products (Fig. 1a) showed that there were no PCR products for mtDNA as templates, indicating that the mtDNA isolated was free from gDNA.

Moreover, to check if the total gDNA samples are mixed with mtDNA, PCR amplification of mtDNA gene *orf139* was performed using the primer 4 (Table 2). The results showed that the target fragment of *orf139* was amplified in all the mtDNA as well as the gDNA samples (Fig. 1b), suggesting that the gDNA isolated contained mtDNA and could be used to amplify the gene specific to mtDNA.

PCR amplification using single primer set for detecting *orf224*, *orf222* and *orf138* in different cytoplasm types of rapeseed

To get molecular marker specific to each of cytoplasm type *Ogu, Nap, Pol* in rapeseed, PCR amplification was performed using mtDNA as templates with the single set primer 1, 2 and 3 (Table 2) targeting gene *orf138*, *orf222*, *orf224*, respectively. The patterns of PCR products were shown in Fig. 2a–c, respectively. A 465-bp band appeared in the accession with the *Ogu* cytoplasm or *Ogu*-NWSUAF cytoplasm (Fig. 2a). A 1,102-bp PCR product displayed in the two accessions with *Nap* cytoplasm and the accession with *Ogu*-NWSUAF cytoplasm (Fig. 2b), while a 747-bp fragment in the two accessions with *Pol* cytoplasm type (Fig. 2c). The 465-, 1,102- and 747-bp fragment was molecular marker specific to the *Ogu*, *Nap* and *Pol* cytoplasm types, respectively.

Development of molecular marker for different cytoplasm type in rapeseed by multiplex PCR

PCR amplification was performed using mtDNA as templates with the primer 1, 2 and 3 in each PCR reaction. The patterns of these multiplex PCR were shown in Fig. 3. It

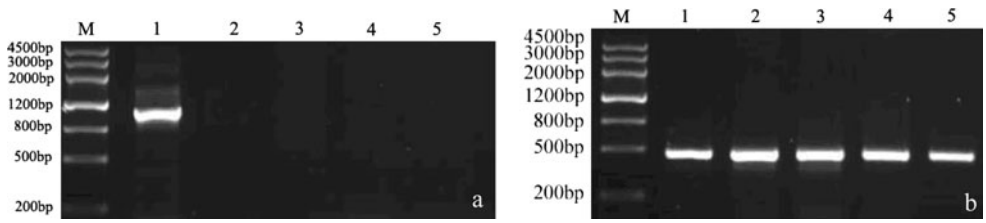

Fig. 1 The electrophoresis patterns of PCR products amplified with *MS2Bnap*-specific primer (**a**) and *orf 139*-specific primer (**b**). *M* DNA molecular weight standard. *Lane 1* gDNA as template, *lanes 2–5* mtDNA of *Nap, Pol, Cam* and *Ogu* cytoplasm type as template, respectively

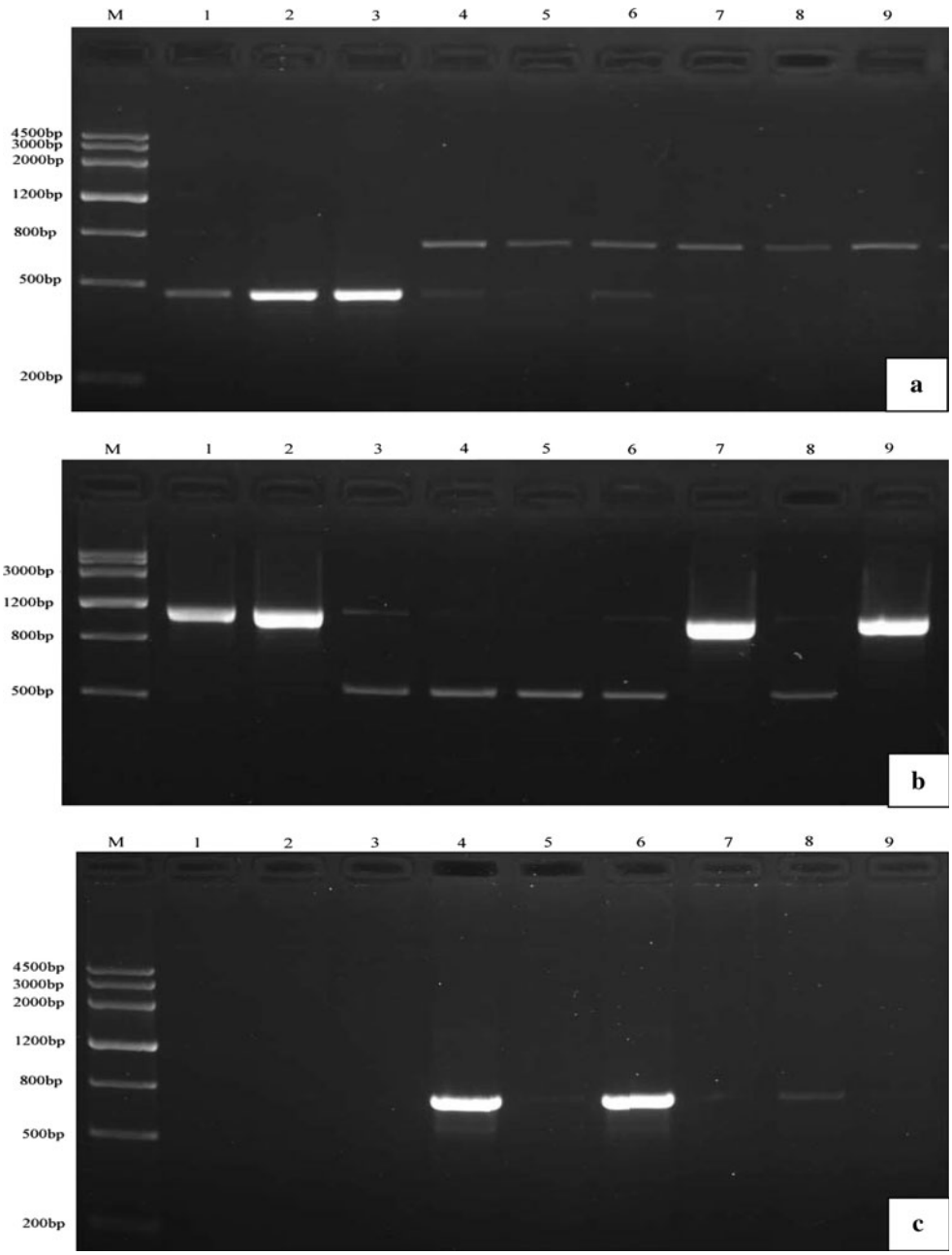

Fig. 2 The electrophoresis patterns of PCR products amplified with gene-specific primer pair 1 (**a**), primer pair 2 (**b**) and primer pair 3 (**c**), respectively. *M* DNA molecular weight standard. *Lanes 1 and 2 Ogu*-NWSUAF CMS cytoplasm, *lane 3 Ogu* CMS cytoplasm, *lanes 4 and 6 Pol* cytoplasm, *lanes 5 and 8 Cam* cytoplasm, *lanes 7 and 9 Nap* cytoplasm, respectively

appeared that the given cytoplasm type was associated with a specific combination of the respective PCR products. The combination of a 465- and 500-bp band was specific to the accession with *Ogu* cytoplasm, the combination of a 747- and 500-bp band specific to the two accessions with *Pol* cytoplasm, the combination of a 1,102- and 800-bp band specific to the two accessions with *Nap* cytoplasm, the combination of a 800- and a 500-bp band specific to the two accessions with *Cam* cytoplasm, and the combination of a 465- and 1,102-bp band specific to the two accessions with *Ogu*-NWSUAF cytoplasm. In order to check the repeatability and the sensitivity of this multiplex PCR method, five multiplex PCR reactions were separately conducted with different quantity of each of the eight DNA samples and finally same results were obtained, indicating that this method is highly specific, repeatable and sensitive. These results indicated that the cytoplasm type of the *Pol, Nap, Cam, Ogu* and *Ogu*-NWSUAF could be easily distinguished from each other by the specific pattern of multiplex PCR.

Further, in order to test if the purified mtDNA can be replaced by total gDNA as a template for amplifying the gene *orf224*, *orf222* and *orf138* in rapeseed, the total gDNA isolated from the eight accessions (Table 1) were used for

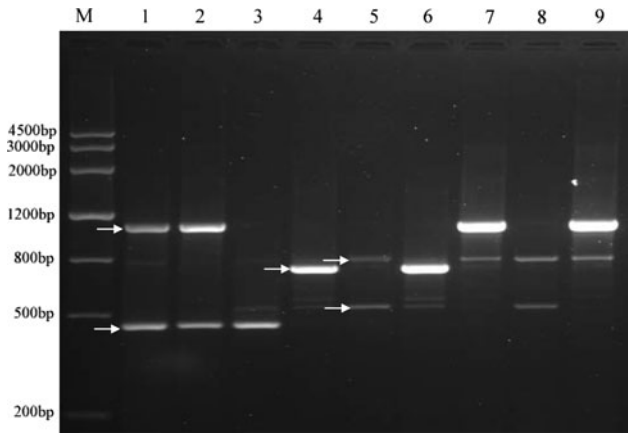

Fig. 3 The electrophoresis patterns of PCR products amplified with three pairs of primers for genes *orf138* (465 bp), *orf224* (747 bp), *orf222* (1,102 bp). *M* DNA molecular weight standard. *Lanes 1 and 2 Ogu*-NWSUAF CMS cytoplasm, *lane 3 Ogu* CMS cytoplasm, *lanes 4 and 6 Pol* cytoplasm, *lanes 5 and 8 Cam* cytoplasm, *lanes 7 and 9 Nap* cytoplasm, respectively

multiplex PCR and the PCR amplifications were repeated five times. The patterns of the PCR products were identical to those using mtDNA as templates (figure not shown here) suggesting that total gDNA instead of mtDNA can be used in the multiplex PCR.

Evaluation of 35 F_1 hybrids by the multiplex PCR

In order to verify the accuracy of the multiplex PCR assay developed above, 35 F_1 hybrids were evaluated. Ten of 35 hybrids were found to be the *Nap* cytoplasm type, while 25 to be the *Pol* cytoplasm type (Table S1). The results were consistent with the information provided by the breeders, suggesting that it was reliable to detect cytoplasm type in rapeseed by the multiplex PCR assay.

Identification of 140 open-pollinated varieties or lines by the multiplex PCR

Using the multiplex PCR developed here, 140 open pollinated varieties or breeding lines usually utilized in our rapeseed breeding programs were successfully identified. The cytoplasm types and their distribution of the 140 cultivars/lines were shown in Table S2 and summarized in Table 3. Among the 140 accessions tested, 100 accessions possess the *Nap* cytoplasm, 21 accessions *Cam* cytoplasm and 19 accessions *Pol* cytoplasm. All 19 accessions with the *Pol* cytoplasm came from China. Pedigree analysis indicated that all these accessions with the *Pol* cytoplasm were either restorers for the *Pol* CMS (Shaan 2A), including Shaan 2C, Huiyehui, 220, etc. or derived from hybrids with the *Pol* CMS (Shaan 2A) as female parent (Table 4).

Table 3 The distribution of cytoplasm types from 140 accessions investigated in this study

Resource	*Cam*	*Pol*	*Nap*	Total
Europe	2	0	27	29
America[a]	8	0	33	41
Asian (China)	11	19	40	70
Total	21	19	100	140

[a] Two accessions from Australia were included in this group

Discussion

Effectiveness of the multiplex PCR for cytoplasm identification in rapeseed

To date, the methods developed for identification of cytoplasm type in rapeseed include classical test-cross (Shiga et al. 1976, 1978), RFLP analysis of mtDNA (Yang et al. 1998) and PCR marker developed from gene sequence of mtDNA (Wei et al. 2005). Among these methods, test-cross is the most common but time-consuming, because winter rapeseed is biennial. While RFLP analysis of mtDNA is much faster than test-cross to distinguish the different cytoplasm types. However, because of the requirement of large amounts of purified mtDNA, appropriate restriction endonucleases and suitable probes in southern hybridization, RFLP analyses of mtDNA is still costly to perform as well as time-consuming. In contrast, the PCR marker allows a quick and efficient identification of the cytoplasm of individual plant interested. Several mitochondrial genes responsible for male sterility have been cloned in some plant species and most of them have been revealed as chimeric genes created through mtDNA rearrangement (Hanson and Bentolila 2004). The information of these chimeric genes has been used to design PCR primers to identify different cytoplasms in *B. napus* (Wei et al. 2005), onion (Kim et al. 2009; Engelke et al. 2003), radish (Kim et al. 2007; Lee et al. 2009) and other plant species (Engelke and Tatlioglu 2002; Cheng et al. 2009).

In the present study, a multiplex PCR assay was established, and the cytoplasm type of *Pol, Nap, Cam, Ogu* and *Ogu*-NWSUAF could be easily distinguished from each other in one PCR reaction by using three pair of primers. Our results also demonstrated that this novel PCR method is highly specificity, repeatability and sensitivity for identifying cytoplasm type in rapeseed, and that mtDNA can be replaced by total gDNA for multiplex PCR. The accuracy of the multiplex PCR assay was verified by the cytoplasm identification of 35 F_1 hybrids. Moreover, the identification results of 140 open-pollinated varieties or lines (Table S2) were consistent with those of the previous investigations by

Table 4 Pedigree of 19 rapeseed accessions with the *Pol* cytoplasm used in this study

Code[a]	Name	Pedigree
122	220	(7818*Marrno) F_2*Qva
123	227	Derived from 220
124	C103	Derived from hybrid with *Pol* CMS as female
125	C104	Derived from hybrid with *Pol* CMS as female
126	C128	Derived from hybrid with *Pol* CMS as female
127	C3	Derived from Shaan 2C
128	Ganza1-1offs	Derived from hybrid with *Pol* CMS as female
129	Huayehui	The restorer for *Pol* CMS
130	Huiyou 50S	7401*rapa
131	Huyouza1-1offs	Derived from hybrid with *Pol* CMS as female
132	L589	Derived from hybrid with *Pol* CMS as female
133	Pol A	*Pol* CMS(Shaan 2A)line
134	Qinyou 7-1Offs	Derived from hybrid with *Pol* CMS as female
135	Qinyou 8-1Offs	Derived from hybrid with *Pol* CMS as female
136	Qinza 2*Zhong 7	Derived from hybrid with *Pol* CMS as female
137	Shaan 2C	The restorer for *Pol* CMS/Shaan 2A
138	Shaan 2A	*Pol* CMS(Shaan 2A)line
139	Y6	Derived from hybrid with *Pol* CMS as female
140	Suyou 9	derived from hybrid with *Pol* CMS as female

[a] Same as Table S2

classical test-crossing method, exemplified by two accessions (huayehui and Shaan 2C) in Yang(1987), two accessions (Libra and Expander) in Hu et al.(1992) and three accessions (220, Libra, Zhongyou 821) in Wen et al.(2003). But one exception was the cytoplasm type of "Zhongshuang No. 4", which was identified as the *Nap* cytoplasm in the present investigation, but determined as sterile cytoplasm (S) by Wen et al. (2003). Pedigree information showed that Zhongshuang No. 4 was derived from the cross of Zhongyou 821 × Zhongshuang No. 2, thus, the cytoplasm type of Zhongshuang No. 4 and Zhongyou 821 should be the same, considering maternal inheritance of cytoplasm. Both accessions, Zhongshuang No. 4 and Zhongyou 821 were included in our experiment and Wen et al.'s (2003), and our molecular results showed that both accessions had the same cytoplasm type (*Nap*), but in the result of Wen et al. (2003), Zhongyou 821 was identified as fertile cytoplasm (N), Zhongshuang No. 4 as sterile cytoplasm (S). This may be caused by incorrect identity of Zhongshuang No. 4 used in Wen et al. (2003) experiment. All these results indicated that the multiplex PCR assay developed here is an accurate, rapid and efficient method for identifying the cytoplasm type in rapeseed.

This is the first effort to use three pairs of gene-specific primers to perform multiplex PCR for identifying cytoplasm types, *Nap, Pol, Cam, Ogu* and *Ogu*-NWSUAF in rapeseed. With more cytoplasm types being discovered and new CMS-associated gene being identified, multiplex PCR assay for identifying more targeting gene in mtDNA can be established.

Distribution of cytoplasm types in rapeseed

At present, the main cytoplasm types identified in rapeseed are *Pol* (or Shaan 2A), *Nap* and *Cam*. in China, 70% rapeseed seeds used in production are hybrids with CMS, mainly from the *Pol* system (or Shaan 2A) (Fu 2008). With variety "Isuzu" as the tester in test cross, Shiga et al. (1976), (1978) characterized the cytoplasm type of 162 rapeseed accessions from Japan and Europe, and found that among 129 accessions from Japan, 92 accessions (71.3%) possessed male sterile-inducing cytoplasm, 37 (29.7%) had fertile cytoplasm, while among 33 accessions from Europe, 17 accessions (51.5%) possessed male sterile-inducing cytoplasm, 16 (48.5%) had fertile cytoplasm. With variety "Bronowski" as the tester, which is maintainer for both *Pol* and *Nap* CMS, Rousselle and Renard (1978) found that, among 20 spring rapeseed accessions, 17 accessions (85%) had male sterile-inducing cytoplasm, only three accessions, including Bronowski, Brio and Ceska possessed fertile cytoplasm. Yang (1987) indicated that, among eight restorers for the *Pol* CMS tested, four accessions had male sterile-inducing cytoplasm, three had fertile cytoplasm and one undetermined. Using line "Xiangai B" as the tester, which is maintainer for both *Pol* and *Nap* CMS (Yang 1987), Hu et al. (1992) reported that, among 43 rapeseed varieties from 8 countries, 34 (79.1%)

possessed fertile cytoplasm, 9 (20.9%) male sterile-inducing cytoplasm. Wen et al. (2003) showed that, among 49 rapeseed varieties tested, 31 (63.3%) possessed fertile cytoplasm and 18 (36.7%) sterile cytoplasm.

RFLP assay of the cytoplasm type of 112 *Brassica* crops revealed that among 97 *B. napus* accessions tested, 45 accessions (48.9%) possessed *Nap* cytoplasm, 30 accessions (32.6%) *Cam* cytoplasm and 9 accessions (9.8%) *Pol* cytoplasm (Kemble 1987). Handa (2007) revealed that 55 of 69 *B. napus* accessions (79.7%) in Japan have the *Nap* cytoplasm (type I) and the remaining 14 accessions (20.3%) have the *Cam* cytoplasm (type II) by using four pairs of mitochondrial-specific primers. In the present investigation, among 140 accessions tested, 100 (71.4%) *Nap* cytoplasm, 21 (15%) *Cam* cytoplasm and 19 (13.6%) *Pol* cytoplasm accessions were detected. Generally, our results were in agreement with the previous results, that *Nap* cytoplasm was prevailing cytoplasm type in rapeseed (Kemble 1987; Handa 2007), and *Pol* cytoplasm only appeared in Chinese accessions (Kemble 1987). Considering *Pol* CMS was discovered in China (Fu et al. 1990), it is not surprising that all accessions with *Pol* cytoplasm came from China and existed in restorers for *Pol* CMS (Shaan 2A).

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