

SHORT COMMUNICATION

Sequence Analysis of the mtDNA Region Correlated with *Shaan 2A* Cytoplasmic Male Sterility in Rapeseed (*Brassica napus* L.)

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Abstract: Until now in Europe has not been cultivated any hybrid cultivar of oilseed rape based on the *Shaan 2A* cytoplasmic male sterility (CMS), a widely used CMS system in China. The aim of Czech breeders now is to produce new, improved cultivars of rapeseed based on this CMS system. Sterile *Shaan 2A* CMS line (S; *rf/rf*), its corresponding maintainers (N; *rf/rf*) and fertility restorers (S; *Rf/Rf*) were analyzed on molecular level for the presence of functional CMS cytoplasm. Two new primer pairs covering CMS-associated gene (so called *orf224-1*) present in *Shaan 2A* CMS line were developed and selection capability of the developed primers was successfully evaluated. These primers can be used for early selection of plants with functional *Shaan 2A* CMS system in breeding programmes.

Keywords: cytoplasmic male sterility (CMS); marker-assisted selection (MAS); *orf224-1*; rapeseed (*Brassica napus* L.); *Rf*-restorer gene; *Shaan 2A* CMS line

Rapeseed is one of the most important oil crops grown in the world. Its yield and many quality parameters were improved, particularly through hybrid breeding. Hybrid seed production of rapeseed is mostly based on cytoplasmic (CMS) or genic (GMS) male sterility and rarely on self-incompatibility (SI) (KUČERA *et al.* 2010). CMS is a widespread maternally transmitted trait in higher plants that results in the failure of pollen formation, while nuclear genes called fertility restorers (*Rf*) have the ability to suppress the male-sterile phenotype. CMS sterile line results from the expression of novel and often chimeric open reading frames (ORFs) in the mitochondrial genome (HANSON & BENTOLILA

2004; PELLETIER & BUDAR 2007) and represents a condition in which plants fail to produce functional pollen (CHASE 2007). Several CMS systems as *Ogu-INRA*, *MSL* and *Pol* are already used in hybrid breeding of *Brassica napus* and commercial cultivars based on *Ogu-INRA*, *MSL* and *Safecross MS* are registered and cultivated in the Czech Republic. In China, 70% rapeseed cultivars are based on CMS, mainly the *Pol* or *Shaan 2A* systems that both arose spontaneously within the species (ZHAO *et al.* 2010). The *orf224-1* gene has been isolated as a *Shaan 2A* CMS-associated gene (WANG *et al.* 2002). Comparison of the CMS associated genes in *Pol* (*orf224*) and *Shaan 2A* (*orf224-1*) showed

99.9% and 99.6% homologies of sequences in nucleotide and amino acid respectively and the only difference in nucleotide sequence between them is an A in *Shaan 2A* instead of a G found in the *Pol* at position +398 (WANG *et al.* 2002). The *orf224-1* gene encoded 224 amino acids and is located at the upstream of the *atp6* gene. Available *orf224-1* gene sequence with its flanking regions was used to design new original and specific primers. These primers were subsequently tested on the *Shaan 2A* CMS based Czech breeding materials.

Shaan 2A CMS lines (CMS1, CMS2 and CMS3), (S; *rf/rf*), their corresponding maintainers (mCMS1, mCMS2 and mCMS3), (N; *rf/rf*) and fertility restorers (*Rf1*, *Rf2* and *Rf3*), (S; *Rf/Rf*) were analysed for CMS performance. Young leaves from individual plants were collected for genomic DNA (gDNA) extraction. Genomic DNA was isolated according to CTAB procedure described by DOYLE and DOYLE (1987). Total gDNA has been used for PCR because it has already been confirmed that gDNA is mixed with mtDNA and PCR amplification of mtDNA *orf* genes is possible (ZHAO *et al.* 2010). The concentration and quality of DNA was determined with a BioMate 5 spectrophotometer.

Primer design: two new primer pairs based on known mtDNA sequences from *B. napus* with *Shaan 2A* type of CMS were designed using Primer 3 Software (ROZEN & SKALETSKY 2000). PCR amplification was performed using primers S2A178F and S2A178R amplifying region of *orf224-1* gene. Another couple of primers S2A1.3F and S2A1.3R amplifying part of the intron between *orf224-1* gene and region for tRNAm^{et} were used (for primers see Table 1). PCR was performed with 10 ng of genomic DNA as template, mixed with 1.25 µl (10µM) of each primer, 12.5 µl PPP MM (TopBio) and distilled water to make a final volume of 25 µl. PCR reaction involved pre-denaturation for 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 60/57°C (for primer T_m see Table 1) and 1.5 min at 72°C,

finally, 10 min at 72°C. PCR products were subjected to electrophoresis on 1.5% agarose gel in 1 × TBE buffer, and detected by staining with ethidium bromide. The DNA bands of interest were excised from the gel and purified with QIAquick Gel Extraction kit (Qiagen) and the DNA was sequenced using 3730XL DNA sequencer.

CAPS analysis was performed at 37°C for 2 h using *MseI* restriction enzyme with the following restriction digestion conditions: 10 µl PCR products, 1.5 µl of 10 × buffer and 1 unit of restriction enzyme in a final volume of 15 µl. The products were resolved on 2.5% agarose gel in 1 × TBE buffer. The bands were stained with ethidium bromide and visualised under UV light.

The *orf224-1* co-segregating marker S2A178F/R, although it provided a genetic anchor to the *orf224-1* genomic region, did not allow this region to be genetically defined by PCR technique. PCR products of approximately 530 bp were detected in all samples, but sequencing analyses showed differences between sequences obtained from plants with functional mitochondrial *orf224-1* gene and plants with fertile cytoplasm (maintainers of sterility; N; *rf/rf*) (Figure 1). Unlike in study WANG *et al.* (2002) we found in plants carrying the functional mitochondrial *orf224-1* gene another allele differing in nucleotide in position +398 (G). Sequence alignment showed 82% identities between sequences from plants with functional mitochondrial *orf224-1* gene and plants with fertile cytoplasm and in variable region many cleavage sites for different enzymes were detected and in this study use of *MseI* was verified. Plants carrying a functional *orf224-1* gene did not have a cleavage site for this restriction enzyme, whereas its dysfunctional ortholog was cleaved into two fragments of 217 and 316 bp (Figure 2).

PCR primers S2A1.3F and S2A1.3R amplified non-coding region of mtDNA located at the upstream of the *orf224* (–1319 to –32bp; nucleotides are numbered from start codon ATG). Analysis with

Table 1. Characteristic of the primers used in this study

Primer	T _m (°C)	Nucleotide sequence	GenBank accession No. of original gene sequence
S2A178F	60	5'-TGGAGTACTTGGGATCAGCA-3'	EU254234.1
S2A178R	60	5'-ATCTCGCAGACGATCAAGGT-3'	EU254234.1
S2A1.3F	57	5'-AGTGGACCGGGTAGTGCTTA-3'	EF600034.1
S2A1.3R	57	5'-CCTCCAGACAGCTTCACTCC-3'	EF600034.1

T_m – primer melting temperature

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CMS      TGGAGTACTTGGGATCAGCAGAATTCTAAAACCTATGGAACCAACTGCTTTCACACCGGGG 60
Rf       TGGAGTACTTGGGATCAGCAGAATTCTAAAACCTATGGAACCAACTGCTTTCACACCGGGG 60
mCMS    TGGAGTACTTGGGATCAGCAGAATTCTAAAACCTACGGAACCAACTGCTTTCACACTGGGG 60
*****

CMS      GAAGACCCTCCTGAGCAAGGGAAGGCTTGG---AAAAAATCGTAGTTCAGATTCAAGTCG 117
Rf       GAAGACCCTCCTGAGCAAGGGAAGGCTTGG---AAAAAATCGTAGTTCAGATTCAAGTCG 117
mCMS    TAAGACCATCCAGAGCAAGCTAAAGCTTGGTGGAAAAGATCGTA-----CAAGTAA 111
*****

CMS      GTTCGAGGTATCAGCGTTGGCCGCCCATATTTTATCATTTCGTTGGTCCCAAAATTGGG 177
Rf       GTTCGAGGTATCAGCGTTGGCCGCCCATATTTTATCATTTCGTTGGTCCCAAAATTGGG 177
mCMS    GTTCGGGGTCTTAGCGTTGCCACGCGCTATTTCTCATGTTGTTGGTCCCAAAATGCG 171
*****

CMS      ACCAGTTTTCTACATTATATATAAATTT--TTTTGTTTGTGGGGTTGAAATGGGGGGTA 235
Rf       ACCAGTTTTCTACATTATATATAAATTT--TTTTGTTTGTGGGGTTGAAATGGGGGGTA 235
mCMS    GCTAGCTATATATCTAATATATATGTTTGAATTTTATTT-TTGGGATT-AAATGGGGGTTG 229
*****

CMS      TTAGGAAATGAAATTTGTCAATTCGGCGTCGGACCAGATGGCGTCGCGCCCCCAGCGCTG 295
Rf       TTAGGAAATGAAATTTGTCAATTCGGCGTCGGACCAGATGGCGTCGCGCCCCCAGCGCTG 295
mCMS    CTAGGAAATGAGATATTTAGTTTCGGCGTCGGACCAGATGGCGTCGCGCCCCCAGCTCTA 289
*****

CMS      GATCTCAACGAGCGCCCCGCTCTGCATCTTTTGTACGCGGATGTTGAGAGTCCGACTCT 355
Rf       GATCTCAACGAGCGCCCCGCTCTGCATCTTTTGTACGCGGATGTTGAGAGTCCGACTCT 355
mCMS    GATCTCAACGAGCGCCCCGCACTGCATCTTTTGTACGCGGATGTTGAGAGTCCGACTCT 349
*****

CMS      CAACAAGCGCGAAATAATGACATGTACGCGCATCTTAGGCGCGTACAGGAGATCACCCAA 415
Rf       CAACAAGCGCGAAATAATGACATGTACGCGCATCTTAGGCGCGTACAGGAGATCACCCAA 415
mCMS    CAACAAGCGCGGAATGCTGATATGCTAGCGCATATTAGCCGAGTGCAAGAGATAACCCGT 409
*****

CMS      AACTAGAGGGTGTAGCGGATATCGTGGCGCGTCAAGCCCTCCTGGATATAATGAAATGG 475
Rf       AACTAGAGGGTGTAGCGGATATCGTGGCGCGTCAAGCCCTCCTGGATATAATGAAATGG 475
mCMS    GACCTAGAGGGTGTAGCATGATATCGCGCGCGTCAAGCCCTCCTGATATCATGAAGTGG 469
*****

CMS      GAGGTCAGAA----GCCTTCAGGAGCACTTTCGGATCTTTCGGCACCTTGATCGTCTGCG 531
Rf       GAGGTCAGAA----GCCTTCAGGAGCACTTTCGGATCTTTCGGCACCTTGATCGTCTGCG 531
mCMS    GAGGTCAGGATGCTGCTTGGATCACCACCTTCCGGGTCTTTCGGTACCTTGATCGTCTGCG 529
*****

CMS      AGAT 535
Rf       AGAT 535
mCMS    AGAT 533
*****

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Figure 1. Nucleotide sequences of functional *orf224-1* gene in CMS and *Rf* line and its dysfunctional ortholog present in maintainers of sterility (mCMS); cleavage site for restriction enzyme *MseI* is underlined

designed primers S2A1.3F/R detected all accessions with *Shaan 2A* CMS cytoplasm by presence of approximately 1300 bp bands (Figure 3).

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 & BENTOLILA 2004). The information

of chimeric *Shaan 2A* mitochondrial gene has been used to design PCR primers to identify plants with sterile cytoplasm (S). Surprisingly specific primers S2A178F/R amplifying part of the region of *orf224-1* detected the presence of similar sequences also in maintainers of sterility. Anyway the difference between the sequences was large enough to be separated by PCR-RFLP technique, because

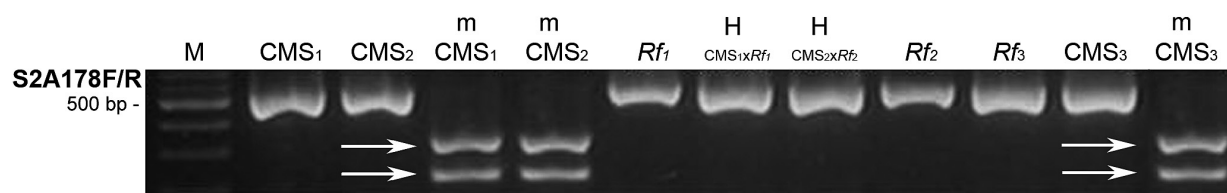


Figure 2. Specific CAPS polymorphism was detected between accessions of *Shaan 2A* CMS (CMS, *Rf*, mCMS and F₁ hybrids – H) from internal region amplified with S2A178F/R primers by treating *Mse*I endonuclease; the arrows indicate polymorphic amplicons in maintainers of sterility (mCMS); M 100 bp gene ladder

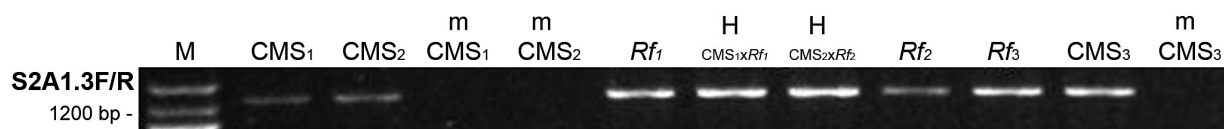


Figure 3. Screening of all accessions of *Shaan 2A* CMS (CMS, *Rf*, maintainers of sterility (mCMS) and F₁ hybrids (H)) with PCR marker S2A1.3; M 100 bp gene ladder

many different regions for restriction enzymes were detected. From amino acid sequence it is likely, that sequence obtained with these primers from maintainers of sterility can be transcribed also into putative protein with unknown function. In contrast primers S2A1.3F/R designed in non-coding region of mtDNA did not amplify any PCR product from maintainers of sterility. This may be due to differences in sequence in the primer annealing site or absence of this sequence. Two new primer pairs for *Shaan 2A* CMS were developed and validated in this study. These primers can be used to distinguish plants with functional *Shaan 2A* CMS cytoplasm in breeding programmes.

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References

- CHASE C.D. (2007): Cytoplasmic male sterility: A window to the world of plant mitochondrial-nuclear interactions. *Trends in Genetics*, **23**: 81–90.
- DOYLE J.J., DOYLE J.L. (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, **19**: 11–15.
- HANSON M.R., BENTOLILA S. (2004): Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *Plant Cell*, **16**(S): 154–169.
- KUČERA V., VYVADILOVÁ M., KLÍMA M. (2010): Utilization of cytoplasmic male sterility *Shaan 2A* in a hybrid breeding of winter oilseed rape. *Agricultura Tropica et Subtropica*, **43**: 182–185.
- PELLETIER G., BUDAR F. (2007): The molecular biology of cytoplasmically inherited male sterility and prospects for its engineering. *Current Opinion in Biotechnology*, **18**: 121–125.
- ROZEN S., SKALETSKY H.J. (2000): Primer3 on the WWW for general users and for biologist programmers. In: KRAWETZ S., MISENER S. (eds): *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, 365–386.
- WANG Y., MA S., WANG M., ZHENG X., GU M., HU S. (2002): Sequence analysis of the gene correlated with cytoplasmic male sterility (CMS) in rapeseed (*Brassica napus*) Polima and *Shaan 2A*. *Chinese Science Bulletin*, **47**: 124–128.
- ZHAO H.X., LI Z.J., HU S.W., SUN G.L., CHANG J.J., ZHANG Z.H. (2010): Identification of cytoplasm types in rapeseed (*Brassica napus* L.) accessions by a multiplex PCR assay. *Theoretical and Applied Genetics*, **121**: 623–650.

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