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Identification of alloplasmic cytoplasmic male-sterile line of leaf mustard synthesized by intra-specific hybridization

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Abstract

The purpose of this study is to recognize the phenotypic behavior of cytoplasmic male-sterile (cms) line of leaf mustard synthesized by intra-specific hybridization and identify genetically transmitted molecular markers related to cytoplasm. Phenotypic variations of both vegetative and floral organs were observed in cms and its maintainer line. Pistil fertility was reduced correspondingly and recovered progressively with backcrossing. In BC_3, some flowers showed abnormality with pinnate, petaloidy, palm-like, carpelloidy or rudimentary silk-like stamens. The deformed floral phenotypes were also observed with closed or partially expanded corollas. By PCR-aided assay using specific primers from mitochondrial genes, *atpA* and one open reading frame (orf) were found to be distinctively present in the progenies of intra-specific F_1 hybrid, BC_2 and BC_3, which were proved to be maternally transmitted and genetically stable in various backcrossed progenies. The cms specific orf corresponds to a 663 bp-sized fragment with its own initiator (ATG) and terminator (TGA). Furthermore, it was deduced to encode for 220 amino acids, named *orf220* thereafter, and was shown to belong to the plant mitochondrial membrane YMF19-like protein family. Transcript of *orf* was exclusively present in cms and absent in its maintainer. Moreover, *atpA* gene was expressed in a larger transcript size in BC_3 progeny than in the maintainer.

Keywords: Leaf mustard; Cytoplasmic male-sterility; *orf220*; *atpA*; *atp6*; *atp9*; *cox* I

1. Introduction

Cytoplasmic male sterility (CMS), the maternally inherited trait of failure to produce viable pollens, is available in many higher plants and which is an ideal genetic system for utilization of hybrid vigor. CMS is frequently associated with chimeric open reading frame (orf) due to rearrangements of mitochondrial genes [1,2]. Besides, it is widely believed that the dysfunction of mitochondrial respiratory complex is responsible for cms, which is thought to be caused by interaction between orf-expressed product and respiratory complex [3].

Leaf mustard (*Brassica juncea* var. *multiceps* Tsen et Lee) is one traditional Chinese vegetable mainly for pickling. It is of great economic importance and its pickled products are favored by consumers in China and South-eastern Asian countries. However, mustard crop is a self-pollinated crop with up to 30% crossed pollination [4]. To date, all cultivars of leaf mustards in China are fixed ones and no F_1 hybrids have ever been released. Undoubtedly, cytoplasmic male sterility is an alternative way to produce hybrids of leaf mustard crop. In cms types, several cms cytoplasms, including '*nap*' [5], '*ogu*' [6], '*mur*' [7], '*oxy*' [8] and '*tour*' [9], have been identified in *Cruciferous* crops. However, the alloplasmic cytoplasms may dramatically influence flower morphological development in cms [10]. Recent research on alloplasmic cms tobacco carrying *N. repanda* cytoplasm showed a deficiency in energy metabolism during flower development [11,12]. We have previously

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bred a stable cms line of tuber mustard by distant hybridization and subsequent backcrossings[13].

In the present investigation, we were trying to recognize phenotypic behavior of cms line of leaf mustard synthesized by intra-specific hybridization using cms tuber mustard as donor. In addition, PCR-aided and RT-PCR assays were employed to identify molecular markers genetically related to cms and their differential expression at transcriptional levels.

2. Materials and methods

2.1. Introgression of cms

The donor of cms tuber mustard (*B. juncea* var. *tumida* Tsen et Lee) with an alloplasmic cytoplasm was developed by distant hybridization between *B. juncea* ($2n = 36$, AABB) and *Brassica rapa* ($2n = 20$, AA), followed by repeated backcrossings with fertile tuber mustard using as recurrent parents and as corresponding maintainers of cms. The cms hybrid crossed between *B. juncea* and *B. rapa* was of no economic value without swollen stem formation and poor-developed nectarines that caused severe limitation on bees visitation for cross-pollination. In the backcrossed progenies, plants with a relatively well-developed swollen stem, well-developed nectarines, and a higher degree of male-sterility were selected for successive backcrossings. In BC_7 of cms tuber mustard, it was successful to affect a stable male-sterile line of tuber mustard showing 100% in male-sterility regardless of temperature changes. Indeed, it was completely recovered for the trait of swollen stem formation, which was identical with its maintainer line [13]. For the aim of this investigation, the intra-specific hybridization and subsequent backcrossings were employed to synthesize cms leaf mustard (*B. juncea* var. *multiceps* Tsen et Lee). Plants of tuber mustard were taken as cms donor and fertile plants of leaf mustard were used as recurrent parents and resultant maintainers of cms as well.

2.2. Fertility assessment

Self-pollination of cms flowers by bagging was carried out to verify the disability of cms plants to produce and disperse functional pollens. Meanwhile, cms flowers were pollinated with pollens from fertile maintainers to test pistil fertility in the backcrossed progeny. Both male-sterility and pistil fertility were calculated in terms of fertile seeds per silique at maturity.

2.3. PCR-aided assay of mtDNA

Total DNAs were extracted by CTAB method [14] from leaves of cms tuber mustard and its maintainer, from F_1 hybrid obtained from hybridization between cms tuber mustard and leaf mustard and from the resultant BC_2 and BC_3 plants. Mitochondria-specific primers for *orf222*, *atpA*, *atp6*, *atp9* and *cox* I were designed and used to probe specific genes in cms leaf mustard (Table 1). Ten nanograms of DNAs were amplified in 25 μl of reaction buffer consisting of 10 μmol/l primers, 200 μmol/l each of dNTPs, 2.5 μl × PCR buffer, and 0.02 U Taq polymerase (Sangon Company, China). The PCR-running parameters were programmed for 2 min preheating at 94 °C, followed by 35 cycles each consisting 1 min at 94 °C for denaturation, 1 min at 50 °C for annealing and 2 min at 72 °C for extension, and finally ended by 1 cycle of 10 min at 72 °C for extension. The PCR products were separated on 1.0% agarose and visualized with ethidium bromide.

2.4. Transcription of specific genes by RT-PCR assays

Total RNA was prepared using Trizol reagents from young leaves and buds of BC_3 progeny and its maintainer. RNA samples were exhaustively treated with RNase-free DNasel, and afterwards 3 μg of total RNA was used for cDNA synthesis. Mitochondrial-specific primers for RT-PCR were used for transcriptional analysis. Besides, actin gene was assigned as a control for RT-PCR (Table 1). Additionally, a control without addition of reverse transcriptase was also carried out. PCR reactions were performed with 30 cycles for all genes tested. Meanwhile, the PCR-running parameters were identical to those for PCR-aided DNA analysis and visualization of amplified products as well.

2.5. Isolation of specific gene of cms

By probing with primers from mitochondria *orf222*, the cDNA fragment from young leaf of BC_3 leaf mustard was obtained and then directly ligated into the pBS-T vector, subsequently transformed into *E. coli* DH 5α, and cultured on agar LB medium containing IPTG and X-gal. The recombinant plasmid was sequenced by the Shanghai Biosia Company (China).

Table 1
Listing of mitochondria-specific primers used for PCR analysis

Gene	Primers
orf222	5′-ATGCCTCAACTGGATAAATT-3′ 5′-TCATCGAAATAGATCRAGKATYTCG-3′ (Y:C,T; R:G,A; K:G,T)
atpA	5′-GCTGCTTACAGGAGTTAGCC-3′ 5′-GTCCAATCGCTACATAGACA-3′
atp6	5′-ATGAATCAAATAGGGCTGGT-3′ 5′-TTAATGGAGATTTATAGCAT-3′
atp9	5′-ATGTTAGAAGGTGCAAAATC-3′ 5′-TCAGAATACGAATAAGATCA-3′
cox I	5′-ATGAAAAATCTGGTTCGATG-3′ 5′-TAACTTCACATAGCTTTTCG-3′
actin	5′-CGCCGAGCGGGAAATCGTC-3′ 5′-GGAAAGTGCTGAGGGATGC-3′