A Novel *orf288* Co-transcripted with *Atp6* is Associated with *hau* Cytoplasmic Male Sterility in *Brassica juncea*

Jing Bing; Fu Ting-dong; Yi Bin; Wen Jing;
Tu Jin-xing; Ma Chao-zhi; Shen Jin-xiong(✉)
(National Key Laboratory of Crop Genetic Improvement/National Engineering Research Center of Rapeseed, Huazhong Agricultural University, Wuhan 430070, P. R. China)

Abstract
Genome walking and sequence analysis showed that a chimeric fragment was located downstream of *atp6* gene. Northern blotting and RT-PCR results gave convincely evidences that this fragment was co-transcripted with *atp6* gene, and it could be associated with male sterility in *hau* CMS, a novel CMS in *B. juncea* reported before. The fragment encoded an open reading frame (*orf*) with 288 amino acids. To elucidate the function of this putative *orf*, the fragment was cloned to the bacteria expression vector and transferred into *Arabidopsis*. The result showed that the protein ORF288 was toxic to *E.coli* cells, and the transgenic plants with *orf288* also failed to produce functional pollen (data not showed here). These observations indicated that this novel *orf288* gene in *hau* CMS cytoplasm might be one of the CMS-associated genes in *Brassica* family

Key Words: *hau* CMS, northern blotting, *atp6*, *orf288*

Introduction

Cytoplasmic male sterility (CMS) is the maternally inherited defect of higher plants to produce functional pollen. In recent years, several studies have reported that some open reading frames (*orfs*) in mitochondrion (mt) possibly caused CMS in various higher plant species. These *orfs* are located downstream or upstream of certain known mt genes and co-transcripted with these natural genes, and usually inhibited by nuclear restorer genes at different levels.

Previously, we reported that *hau* was a novel CMS in *B. juncea* and it had been successfully transferred to *B. napus* (Wan, Jing et al. 2008). Genetic, morphological, cytological and molecular analysis showed that *hau* CMS is different from previously studied CMSs in *Brassicas*. In this study, we investigated the differences of organization and expression of several mithochondrial genes between male sterile and maintainer lines, and the expression of *orf288* in *E.coli*.

Materials and Methods

Plant materials

Both sterile and maintainer lines of *hau* CMS in *B. juncea* and in *B. napus* were used in this study (Wan, Jing et al. 2008).

DNA extraction and Genome walking

Total genomic DNA samples were prepared from the fresh leaves. The Universal Genome-walker Kit (Clontech) was used to isolate the flanking sequence of *atp6* gene and the whole sequence *orf288*.

RNA isolation and RT-PCR

Total RNA was isolated from flower buds using Trizol (Invitrogen). Reverse transcription was performed with DNase-treated total RNA samples by using RevertAid^{tm} First Strand cDNA Synthesis Kit (Fermentas).

Northern blot analysis

Northern blot analysis was followed the protocols from Amersham, UK, Toyobo and Fujifilm Japan.

Expression of orf288 in E.coli

The whole fragment of *orf288* was amplified from cDNA of flower buds using the primer pairs KpnI288STR and BamHI288EDL, and then cloned into the bacterial expression vector pet32a. The expression of *orf288* in *E.coli* was induced by adding 0.5mM IPTG. The OD value of the samples was measured each 0.5hr using UV1601.

Results

A specific sequence located down-stream of atp6 gene in hau CMS cytoplasm

Compared the flanking sequences of gene *atp1*, *atp6* and *atp9* between male sterile line and maintainer line of *hau* CMS, a chimeric fragment, contained 876 nucleotides, was identified downstream of *atp6* gene. Blast results indicated that the fragment shared high similarity (99.6%) with *orf263* in *tour* CMS (Landgren et al. 1996). There were three point mutations in the coding region of *orf263*. With a deletion in these mutations, we predicted that the open reading frame will lengthen to 288 amino acids. We designated this chimeric fragment *orf288*. The nucleotide sequence identity of *atp6* gene and its 101bp after coding region was completely similar between male fertile line and *hau* mitotype. Multiple alignments of *orf286 (Handa 2003), orf288,* and *orf263* showed that *orf286* shared 93% nucleotide sequence identity to that of *orf288*. Fig. 1 illustrates that *orf286* located downstream of *orfB* gene, as a putative *orf,* the localization was different from *orf263* and *orf288*, but considering the three point mutations, the *orf288* was closer to *orf286* than *orf263* evolutionarily.

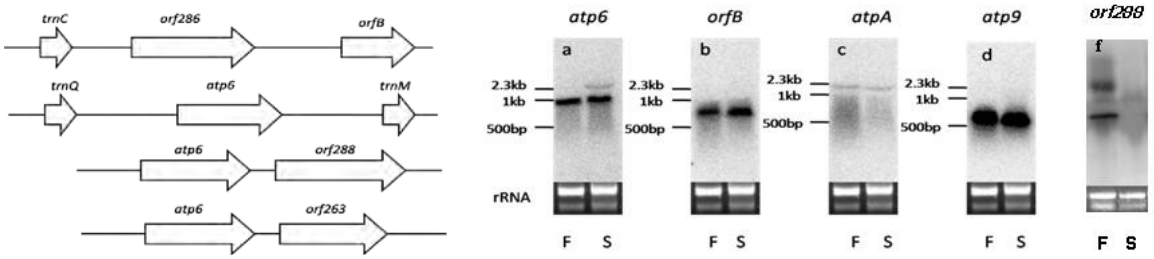


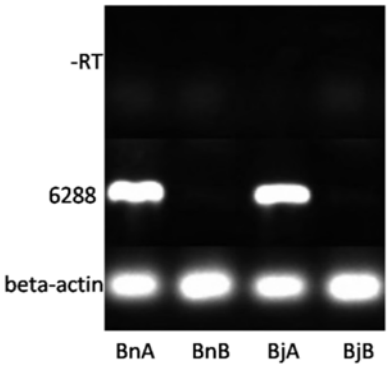
Fig. 1 (the left) The organization of mitochondrial genome regions associated with *orf288* gene in four different mitotypes

a: *trnC-orf288-orfB* region of *nap* mitotype; b: *trn-atp6-trnM* region of *hau* CMS maintainer line; c: *atp6-orf288* region of *hau* mitotype; d: *atp6-orf263* region of *B. tournefortii.*

Fig. 2 (the right) Northern blot analysis of RNA from buds of male sterile line(S) and maintainer line(F) with four mitochondrial probes (*atp6, orfB, atpA, atp9*). The *atp6* gene showed polymorphic band patterns of RNA transcripts.

Two different transcripts of atp6 gene in hau CMS sterile line

Four of mitochondrial gene specific probes, *atpA, atp6, atp9, orfB* were used to analyze expression difference of these gene transcripts between male sterile and maintainer lines of *hau* CMS in *B. juncea*. Our data revealed no difference with the *atpA, atp9, and orfB* probes (Fig. 2b, c, d), but the *atp6* transcripts showed different pattern of bands (Fig. 2a). As showed in Fig. 2a that there were two bands in male sterile line and the shorter one appeared in maintainer line as well. We therefore focused on the transcription pattern of *atp6* gene and its flanking region. Comparing the DNA sequences of *atp6* gene 5` region between CMS and male fertile lines, and no difference was found. As a chimeric fragment was identified before, northern blot experiment with *orf288* probe confirmed that the downstream region of *atp6* expressed in male sterile line specifically (Fig. 2f). The RT-PCR analyses also revealed that *atp6* gene co-transcripted with *orf288* region specifically in *hau* CMS line (Fig. 3). It was depicted that a total of 427 C to U conversions were identified in ORFs of mitochondrion (Handa 2003). To identify the RNA editing sites in the *orf288*, the nucleotide sequence of RT-PCR and PCR products were compared and the alignment showed there was no RNA editing events in the *orf288* coding region (data not show).



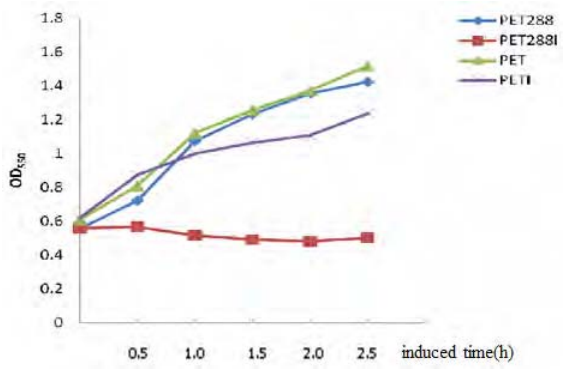


Fig. 3 (the left) RT-PCR to demonstrate expression patterns of *atp6-orf288* region in *hau* mitotype.

BnA: male sterile line in *B. napus*; BnB: maintainer line in *B. napus*; BjA: male sterile line in *B. juncea*; BjB: maintainer line in *B. juncea*.

Fig. 4 (the right) Effect of *orf288* expression on the growth of *E. coli* cells in liquid cultures with or without isopropylthio-b-D-galactoside (IPTG). The expression vector pet32a was as a control.

PET288: without induced by IPTG; PET288I: induced with IPTG; PET: the control expression vector not induced by IPTG; PETI: the control expression vector induced by IPTG.

The expression of orf288 significantly represses the growth of E.coli

Previously, we had confirmed that the putative *orf* was transcript specifically in male sterile line of *hau* CMS cytoplasm. To examine the function of *orf288*, the coding sequence of the fragment was cloned to the expression region of pet32a vector, and IPTG was used to induce expression of this *orf* in *E.coli*. It was observed that the growth of host bacteria was repressed significantly with the expression of *ORF288*. The growth of *E.coli* was normal with high level expression of tag peptide in the expression vector. It was indicated that *orf288* encodes a toxic peptide for host bacteria.

Discussion

The male sterile line of *hau* CMS was found as a spontaneous male sterile mutant in *B. juncea.* As a chimeric putative gene on its mitochondrial genome, the nucleotide sequences of *orf288* region showed high similarity to the corresponding fragment in *B. napus* (*tour*). There were only three point differences with one deletion at position 764, and two nucleotide changes at 485 and 785 of *orf288* respectively. But, at the three positions, the nucleotide of *orf288* and *orf286* located downstream of *orfB* was identical. This might mean that this region of *hau* CMS was evolutionarily closer to *the* CMS *Tournefortii*-Stiewe originated from a donor-recipient protoplast fusion of *B. tournefortii* and *B. napus* described by Stiewe and Röbbelen (1994). But, in this *B. tournefortti*-related male sterile system, the expression of *atp6* gene showed no difference between male sterile line and its maintainer line (Dieterich, Braun et al. 2003). *Brassica* tour CMS and CMS 'Tournefortii-Stiewe' shared a 1.58kb transcript of *atp9* gene, which was not observed in *hau* CMS mitotype, might contribute to male sterility of the latter system.

A number of studies on various CMS systems have demanstrated that CMS is associated with mitochondrial genome rearrangement, and a number of CMS-associated genes are co-transcripted with functional genes in mitochondrion. Therefore, southern and northern blotting analyses were mostly used in researches before. Previously, we had reported there were two of mitochondrial genes showed by RFLPs in the CMS and male fertile lines. In this study, northern blotting and RT-PCR analysis were used to detect transcriptional differences between male sterile and maintainer lines, and then multiple bands of *atp6* genes were found only in male sterile line. This phenomenon was also found in several CMS lines (Bonhomme, Budar et al. 1992; Wang, Zou et al. 2006; Kim, Kang et al. 2007). With these results above, We inferred *orf288* might be an important candidate gene related with *hau* CMS.

It is well known that mitochondria have been evolved from an α-proteobacterial endosymbiont into vital eukaryotic organelles. The products of several CMS-associated genes were transmenbrane proteins, and when expressed in *E.coli* were toxic to the host bacterial cells. In this study, we predicated that the chimeric gene *orf288* encoded a transmenbrane protein that was cytotoxic to *E. coli.* It was previously reported that the expression of CMS-associated genes *orf138* from radish (Duroc, Gaillard et al. 2005) and *orf79* from rice (Wang et al, 2006) were lethal to *E. coli.* We would focus on *orf288* since this putative chimeric gene shared these similarities with other CMS-associated genes.

Reference

Bonhomme, S., F. Budar, et al. (1992). Sequence and transcript analysis of the Nco2.5 Ogura-specific fragment correlated with cytoplasmic male sterility in *Brassica* cybrids. Mol Gen Genet **235**(2-3): 340-348.

Dieterich, J. H., H. P. Braun, et al. (2003). Alloplasmic male sterility in *Brassica napus* (CMS '*Tournefortii*-Stiewe') is associated with a special gene arrangement around a novel atp9 gene. Mol Genet Genomics **269**(6): 723-731.

Duroc, Y., C. Gaillard, et al. (2005). Biochemical and functional characterization of ORF138, a mitochondrial protein responsible for Ogura cytoplasmic male sterility in *Brassiceae*. Biochimie **87**(12): 1089-1100.

Handa, H. (2003). The complete nucleotide sequence and RNA editing content of the mitochondrial genome of rapeseed (*Brassica napus* L.): comparative analysis of the mitochondrial genomes of rapeseed and *Arabidopsis thaliana*. Nucleic Acids Research **31**(20): 5907-5916.

Kim, D. H., J. G. Kang, et al. (2007). Isolation and characterization of the cytoplasmic male sterility-associated orf456 gene of chili pepper (*Capsicum annuum* L.). Plant Mol Biol **63**(4): 519-532.

Landgren, M., M. Zetterstrand, et al. (1996). Alloplasmic male-sterile *Brassica* lines containing *B. tournefortii* mitochondria express an ORF 3' of the atp6 gene and a 32 kDa protein off. Plant Mol Biol **32**(5): 879-890.

Stiewe, G., G. Röbbelen (1994). Establishing cytoplasmic male sterility in *Brassica napus* by mitochondrial recombination with *B. tournefortii*. Plant Breeding **113**(4): 294-304.

Wan, Z., B. Jing, et al. (2008). Genetic characterization of a new cytoplasmic male sterility system (*hau*) in *Brassica juncea* and its transfer to *B. napus*. Theor Appl Genet **116**(3): 355-362.

Wang, Z., Y. Zou, et al. (2006). Cytoplasmic male sterility of rice with boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. Plant Cell **18**(3): 676-687.