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RESEARCH PAPER

A male sterility-associated cytotoxic protein ORF288 in *Brassica juncea* causes aborted pollen development

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Abstract

Cytoplasmic male sterility (CMS) is a widespread phenomenon in higher plants, and several studies have established that this maternally inherited defect is often associated with a mitochondrial mutant. Approximately 10 chimeric genes have been identified as being associated with corresponding CMS systems in the family Brassicaceae, but there is little direct evidence that these genes cause male sterility. In this study, a novel chimeric gene (named *orf288*) was found to be located downstream of the *atp6* gene and co-transcribed with this gene in the *hau* CMS sterile line. Western blotting analysis showed that this predicted open reading frame (ORF) was translated in the mitochondria of male-sterile plants. Furthermore, the growth of *Escherichia coli* was significantly repressed in the presence of ORF288, which indicated that this protein is toxic to the *E. coli* host cells. To confirm further the function of *orf288* in male sterility, the gene was fused to a mitochondrial-targeting pre-sequence under the control of the *Arabidopsis APETALA3* promoter and introduced into *Arabidopsis thaliana*. Almost 80% of transgenic plants with *orf288* failed to develop anthers. It was also found that the independent expression of *orf288* caused male sterility in transgenic plants, even without the transit pre-sequence. Furthermore, transient expression of *orf288* and green fluorescent protein (GFP) as a fused protein in *A. thaliana* protoplasts showed that ORF288 was able to anchor to mitochondria even without the external mitochondrial-targeting peptide. These observations provide important evidence that *orf288* is responsible for the male sterility of *hau* CMS in *Brassica juncea*.

Key words: *Brassica juncea*, cytoplasmic male sterility, cytotoxic protein, mitochondrial-anchored protein, *orf288*, transgenic plants.

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited defect of higher plants in the production of functional pollen. Several studies have reported that chimeric open reading frames (ORFs) in the mitochondrial genome cause CMS in a variety of higher plant species (Dewey *et al.*, 1986; Chase, 2007). Several CMS-associated aberrant genes are located upstream or downstream of certain known mitochondrial genes and co-transcribed with them. The expression of these novel chimeric ORFs is usually inhibited at different levels by nuclear fertility restorer (*Rf*) genes (Sarria *et al.*, 1998; Wise *et al.*, 1999; Feng Liu *et al.*, 2001; Koizuka *et al.*, 2003; Kazama *et al.*, 2008; Yamamoto *et al.*, 2008). A survey of the literature shows that the expression of these CMS-associated ORFs in *Escherichia coli* can inhibit the growth of the host bacteria (Dewey *et al.*, 1988; Duroc *et al.*, 2005; Wang *et al.*, 2006). A series of experiments and observations have led to isolation and characterization of CMS-related genes in various higher plants.

urf13, an aberrant ORF in Texas (T)-cytoplasm maize, is the first identified CMS-related gene in a plant species

Abbreviations: *AP3*, *APETALA3*; BT, Boro II; CaMV, *Cauliflower mosaic virus*; CMS, cytoplasmic male sterility; *coxIV*, cytochrome oxidase subunit IV; CR, circularized RNA; GFP, green fluorescent protein; GUS, β-glucuronidase; IPTG, isopropyl-β-D-thiogalactopyranoside; OD, optical density; ORF, open reading frame; PET1, *Helianthus petiolaris* cytoplasm based male-sterile line in sunflower; *Rf*, fertility restorer; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcription-PCR; WA, wild abortive.

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(Dewey *et al.*, 1987, 1988; Wise *et al.*, 1987). Two nuclear genes, *Rf1* and *Rf2*, restore the male fertility of T-cytoplasm maize (Wise *et al.*, 1999; Feng Liu *et al.*, 2001). The CMS-BT (Boro II) is one of the most widely studied CMS/*Rf* systems in rice (*Oryza sativa* L.) (Akagi *et al.*, 2004; Wang *et al.*, 2006; Kazama *et al.*, 2008). It has been reported that *orf79*, which is a chimeric region located downstream of the *atp6* gene, is responsible for this type of CMS in rice (Wang *et al.*, 2006). In the BT-type CMS line, *orf79* co-transcribed with the *atp6* gene forms a 2.0 kb transcript and encodes a predicted transmembrane protein. In the presence of *Rf1a*, the 2.0 kb transcript is split into two transcripts of 1.5 kb and 0.5 kb by RNA processing (Wang *et al.*, 2006). A longer *orfB* transcript may be associated with the CMS-WA (wild abortive) system in rice (Das *et al.*, 2010). The transformation of *Arabidopsis* with mitochondrion-targeted *orf456* causes 45% of the first generation transformed (T_1) population to exhibit male sterility (Kim *et al.*, 2007). The expression of *orf129* with the assistance of a mitochondrial-targeting pre-sequence results in male sterility in transgenic tobacco plants (Yamamoto *et al.*, 2008). Male sterility in the *Helianthus petiolaris* (PET1)-CMS system of sunflowers is associated with a novel mitochondrial gene, *orfH522*, which results in sterile transformed tobacco (Nizampatnam *et al.*, 2009). These well-studied CMS genes have been investigated primarily on the basis of the differences in the DNA sequences of the mitochondrial genomes and in the expression pattern of mitochondrial genes among the CMS, maintainer, and restorer lines. In most cases, these early predictions substantiate further studies of these CMS-associated genes. Furthermore, transgenic plants with these chimeric ORFs failed to produce viable pollen, thereby providing additional important evidence that they are associated with male sterility.

Brassica is one of the most widely used genera for investigating the mechanisms of CMS. Approximately 10 types of CMS have been identified in *Brassica*, and most of them are due to rearrangements of the mitochondrial genome and expression of chimeric ORFs co-transcribed with functional genes in mitochondria (Landgren *et al.*, 1996; Ashutosh *et al.*, 2008). In the *polima* CMS system, *orf224*, which is co-transcribed with the *atp6* gene, forms dicistronic 2.2 kb and 1.9 kb transcripts (Singh and Brown, 1993). The CMS-associated *atp6*/*orf224* transcripts were shown to be dramatically reduced in the petals, stamens, and carpals, but not in the sepals of restored line flowers (Li *et al.*, 1998). The ogura CMS system has also been extensively studied owing to its high value in rapeseed breeding (Brown *et al.*, 2003; Gonzalez-Melendi *et al.*, 2008; Duroc *et al.*, 2009). An aberrant region of mitochondrial DNA in the ogura sterile line encodes a peptide of 138 amino acids the accumulation of which can be suppressed by the nuclear *Rfo* locus (Bellaoui *et al.*, 1999). *orf125* may be responsible for *Kosena* CMS, and the protein levels of the *Kosena* CMS-associated mitochondrial protein ORF125 were apparently reduced in plants in which male fertility was restored (Koizuka *et al.*, 2003). *orf222*, which shares 79% sequence similarity with the predicted *pol orf224* gene region, may be associated with *nap* CMS (L'Homme *et al.*, 1997). *orf263* co-transcribed with the *atp6* gene may be associated with *tournefortii* CMS and translated into a 32 kDa protein. This 32 kDa protein was only detected in alloplasmic lines (Landgren *et al.*, 1996). In the '*Tournefortii*–Stiewe' system, *orf193*, which is co-transcribed with *atp9*, probably impairs pollen development (Dieterich *et al.*, 2003). *orf108*, which is co-transcibed with the *atpA* gene, was found to be associated with *Moricandia arvensis* CMS, and the long *atpA* transcript is spliced within *orf108* in fertility-restored lines (Ashutosh *et al.*, 2008). A single nucleotide insertion mutation that results in the truncation of *atp6* may be associated with DCGMS CMS in radish (*Raphanus sativus* L.) (Lee *et al.*, 2009). However, these male sterility-associated ORFs are rarely transformed into fertile plants, which further confirm their functions.

Previously, *hau* was found to be a novel CMS system in *Brassica juncea*, and this system has been transferred to *Brassica napus*. Genetic, morphological, cytological, and molecular analyses showed that *hau* CMS is different from previously studied CMS systems in *Brassica* (Wan *et al.*, 2008). In the present work, a chimeric gene, *orf288*, has been found to be located downstream of the *atp6* gene and co-transcribed with it in the *hau* CMS line. The expression of the putative male sterility-associated gene *orf288* in *E. coli* and *Arabidopsis thaliana* indicated that *orf288* plays an important role in the male sterility of *hau* CMS in *B. juncea*.

Materials and methods

Plant materials

The *hau* CMS (00-6-102A) used in this study was originally discovered as a spontaneous male-sterile mutant in *B. juncea* in the experimental field of Huazhong Agricultural University in 1999 (Wan *et al.*, 2008). This type of CMS has been successfully transferred into *B. napus*. The sterile and maintainer lines of this CMS system in *B. napus* were also used to examine the expression differences of CMS-related genes. The maintainer line of *B. napus* was used to provide recurrent parents for 10 generations (BC_{10}) of crosses with *hau* CMS to establish this CMS system in *B. napus*.

DNA extraction and genome walking

Total genomic DNA samples were prepared from the fresh leaves of plants. To isolate the flanking sequence of the *atp6* gene and the whole sequence of *orf288*, genome walking was performed using the Genome-Walker Universal Kit (Clontech, USA) according to the manufacturer's protocol.

RNA isolation and RT-PCR

Total RNA was isolated from flower buds, roots, and fresh leaves using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed with DNase-treated total RNA samples using the RevertAid™ First-Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions for cDNA synthesis for reverse transcription-PCR (RT-PCR). Circularized RNA (CR)-RT-PCR (Kuhn and Binder, 2002) was performed to determine the 5' and 3' ends of *atp6* transcripts.

Northern blot analysis

The total RNA was fractionated on a 1.5% denaturing agarose gel containing 2% formaldehyde and transferred onto Hybond

N^+ membranes (Amersham, UK). Hybridizations were carried out at 68 °C overnight in hybridization solution (Toyobo). Filters were washed for 10 min in 2× SSC and 0.1% SDS at 37 °C. The membranes were exposed to a phosphor storage screen for 2–3 h, and the signals were scanned using the Typhoon FLA 9000 (Fujifilm, Japan).

Antibody preparation and western blotting

A peptide antigen corresponding to 17 residues of ORF288 was synthesized by a chemical synthesis method (Neweast, China) and used to immunize rabbits for antibody production. Crude mitochondrial proteins were extracted from the etiolated seedlings of male-sterile and the maintainer lines of *hau* CMS plants. The proteins were separated by 12% SDS–PAGE and transferred onto a membrane (PVDF type, Millipore). The membrane blots were incubated in blocking buffer (1% bovine serum albumin, 0.05% Tween-20, 20 mM TRIS-HCl, and 500 mM NaCl, pH 7.5) for 1.5 h and then incubated with primary antibody serum (1:500 dilution) for 3 h at room temperature. After three rinses (10 min each) with TBST, the blots were incubated in secondary antibody solution (affinity-purified phosphatase-labelled goat anti-rabbit IgG[HþL], 1:3000 dilution) for 1.5 h at room temperature and finally washed three times (10 min each) with TBST.

Expression of orf288 in E. coli

The whole fragment of *orf288* was amplified from the cDNA of flower buds using the primer pairs KpnI288STR and BamHI288ED; in Supplementary Table S1, available at *JXB* online, the *Kpn*I and *Bam*HI sites in the primer sequences are underlined. The corresponding enzymes were then used to clone this fragment into the bacterial expression vector PET32a (Novagen). The expression of ORF288 in *E. coli* BL21(DE3)plysS (Promega) was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical density (OD) of the samples reached ~0.6. Over the next 2.5 h, the OD of the samples was measured every 30 min at 600 nm using a UV-1601 spectrophotometer (Shimadzu, Japan)

Vector construction and expression of orf288 in A. thaliana

The *AP3* promoter fragment (base pairs –1 to –867 of the *AP3* gene) was amplified from *A. thaliana* using the oligonucleotide primer pairs AP3BR and AP3F; in Supplementary Table S1 at *JXB* online, the *Kpn*I and *Bam*HI sites in the primer sequences are underlined. The *Cauliflower mosaic virus* (CaMV) 35S promoter was amplified from *pCAMBIA2301* (CAMBIA) using the primers VNOF and VNOL. The pre-sequence of *coxIV* (mitochondrial transit peptide of the cytochrome oxidase subunit IV from yeast) (Köhler *et al.*, 1997) and the coding frame of *orf288* were also amplified using coxVIF, T-288L, T-288F, and 288L and fused by overlap extension PCR. In the absence of the mitochondrial-targeting peptide, an additional primer, VNO2, was used. The NOS sequence was amplified from *pCAMBIA2301* using the primer pairs NOSR and NOSF and was cloned to the 3' end of the expression fragment. All of the PCR products were inserted into the *pCAMBIA2300* vector (CAMBIA), and the *AP3* promoter fragment was also inserted into *pBI101* (Clontech) as a control. *Agrobacterium GV3101* and *A. thaliana* Columbia were used for the floral dipping, and the *Agrobacterium*-mediated transformation was performed according to the modified floral-dip method (Clough and Bent, 1998).

Transient expression and subcellular localization of ORF288

The full-length cDNA of *orf288* was PCR-amplified using primers HF and HL (Supplementary Table S1 at *JXB* online) and inserted into the pM999GFP (Supplementary Fig. S1) vector, that was provided by Dr Jian Xu (National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China), to generate an N-terminal green fluorescent protein (GFP) fusion product. This vector allows for the *in planta* expression of proteins under the control of the constitutive CaMV double 35S promoter. The mitochondria of protoplasts were marked using Mitotracker Red CMX-Ros staining solution (Molecular Probes, Invitrogen). The fusion construct was introduced into *Arabidopsis* protoplasts that were prepared from whole seedlings by polyethylene glycol (PEG)/calcium-mediated transformation (Yoo *et al.*, 2007). Fluorescence microscopy was performed using a confocal laser microscope.

Sequence analysis

Gene sequences were analysed using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and DNAStar software. Multiple alignments of DNA sequences were performed with ClustalX 2.0 (Larkin *et al.*, 2007) and GeneDoc (Nicholas and Nicholas, 1997), and the output was manually modified. The ORF288 transmembrane structure was predicted by the software TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM). The mitochondrial transit peptide was predicted by PredSL (http://hannibal.biol.uoa.gr /PredSL/) and SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

Results

A specific sequence located downstream of the atp6 gene in hau CMS cytoplasm

Previously, restriction fragment length polymorphisms (RFLPs) were detected for *atp1*, *atp6*, and *atp9* genes in the sterile and the maintainer lines (Wan *et al.*, 2008). Here, the flanking sequences of these genes in the male-sterile line were compared with those in the maintainer line. A chimeric fragment was found to be located downstream of the *atp6* gene (Fig. 1A). BLAST searches indicated that this fragment shares high similarity with *orf263* (Fig. 1B) in alloplasmic male-sterile *Brassica* lines (Landgren *et al.*, 1996). To identify the organization of this fragment, genome walking was carried out based on the previous results. The BLAST searches with a product of 629 nucleotides confirmed that the sequence similarity between *orf263* and the region downstream of *atp6* in the *hau* CMS mitotype was very high (99.5%). Four point mutations were detected: three were present in the coding region and one was located downstream of *orf263* (Supplementary Fig. S2 at *JXB* online). PCR amplification of *atp6–orf288* with the primers atp6288UP2 and atp6288DOWN2 (shown in Supplementary Table S1) also showed that the chimeric fragment was located downstream of the *atp6* gene. It was predicted that a deletion in these four point mutations would cause the length of the *orf263* product to increase to 288 amino acids. Therefore, this chimeric fragment was designated as *orf288*. Multiple alignments of *orf286*, *orf288*, and *orf263* showed that *orf286* (Fig. 1C) from the mitochondrial genome of *B. napus* (GenBank accession no. AP006444) shares 93% nucleotide sequence identity with *orf288* (Supplementary Fig. S2). *orf286* was identical to *orf288* at the sites of four single nucleotide differences between *orf288* and *orf263* (Supplementary Fig. S2). Sequence analysis showed that this *orf288* was a chimeric fragment located downstream of the *atp6* gene in the *hau*

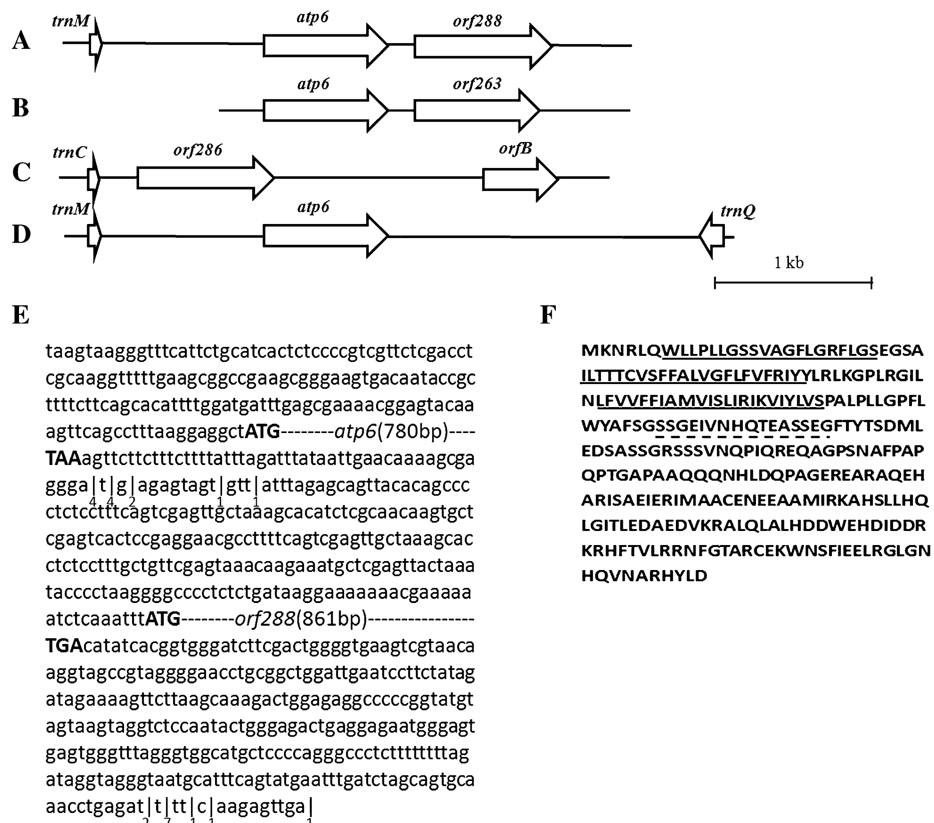

Fig. 1. (A–D) The organization of mitochondrial genome regions associated with the *orf288* gene for four different mitotypes. (A) The *trnM–atp6–orf288* region of the *hau* mitotype. (B) The *atp6–orf263* region of *tour* CMS: *orf263* is associated with this type of CMS (Landgren *et al.*, 1996). (C) The *trnC–orf286–orfB* region of the *nap* mitotype: *orf286* is an unidentified ORF in *nap* CMS (Handa, 2003). (D) The *trnM–atp6–trnQ* region of the *hau* CMS maintainer line. (E) Complete cDNA sequence of the *atp6/orf288* transcripts of the male-sterile line. The initiation and termination codon are highlighted in bold. The different 3' ends of transcripts are indicated by vertical bars, and the number under the bar indicates the number of the ends sequenced. **(**F) Amino acid sequence of ORF288: the transmembrane regions are underlined, and the dotted underlining indicates that the peptide was synthesized and used for antibody production. Arrows indicate the direction of transcription.

mitochondrial genome, but was not detected downstream of the *atp6* gene in the *hau* CMS maintainer line (Fig. 1D).

Two different transcripts of the atp6 *gene in the* hau *CMS sterile line*

Four mitochondrial gene-specific probes, *atpA*, *atp6*, *atp9*, and *orfB*, were used to analyse the expression differences of these gene transcripts between the male-sterile and the maintainer lines of *hau* CMS in *B. juncea*. Northern blotting results revealed no difference for the *orfB*, *atpA*, and *atp9* probes (Fig. 2A–C), but the *atp6* transcripts showed different banding patterns (Fig. 2D). There are two bands in the male-sterile line, and between them the shorter one was observed in the maintainer line (Fig. 2D). Therefore, the *atp6* gene and its flanking region were suggested together to be a strong candidate region of CMS-associated fragments in the *hau* CMS line. Because a chimeric fragment had been previously identified, northern blotting experiments with the *orf288* probe confirmed that the downstream region of *atp6* was expressed constitutively in the male-sterile line (Fig. 2E). The 2.3 kb transcript was also found in the *hau* CMS line of *B. napus* (Fig. 2F). The RT-PCR analysis also revealed that the *atp6* gene was co-transcribed with the *orf288* region in the *hau* CMS sterile line (Fig. 2G). To identify the RNA editing sites in *orf288*, the nucleotide sequences of the RT-PCR were compared with PCR products, and the alignment exhibited no evidence of RNA editing in the *orf288* coding region.

The 5' and 3' ends of the two different orf288*-related transcripts*

CR-RT-PCR was performed to identify the 5′ and 3′ ends of the *atp6* gene transcripts. The identified 5′ ends of the two *atp6* mRNAs were –165 nucleotides from the initiation codon (Fig. 1E). It was also observed that the mRNA ends of *atp6* could self-ligate without treating the RNA with tobacco alkaline phosphatase, which indicated that the 5' end of the *atp6* mRNA was generated by post-transcriptional processing. One *atp6* 3′ end was detected from 48 to 61 nucleotides downstream of the stop codon, and another was located from 276 to 289 nucleotides downstream of the *orf288* stop codon (Fig. 1E).

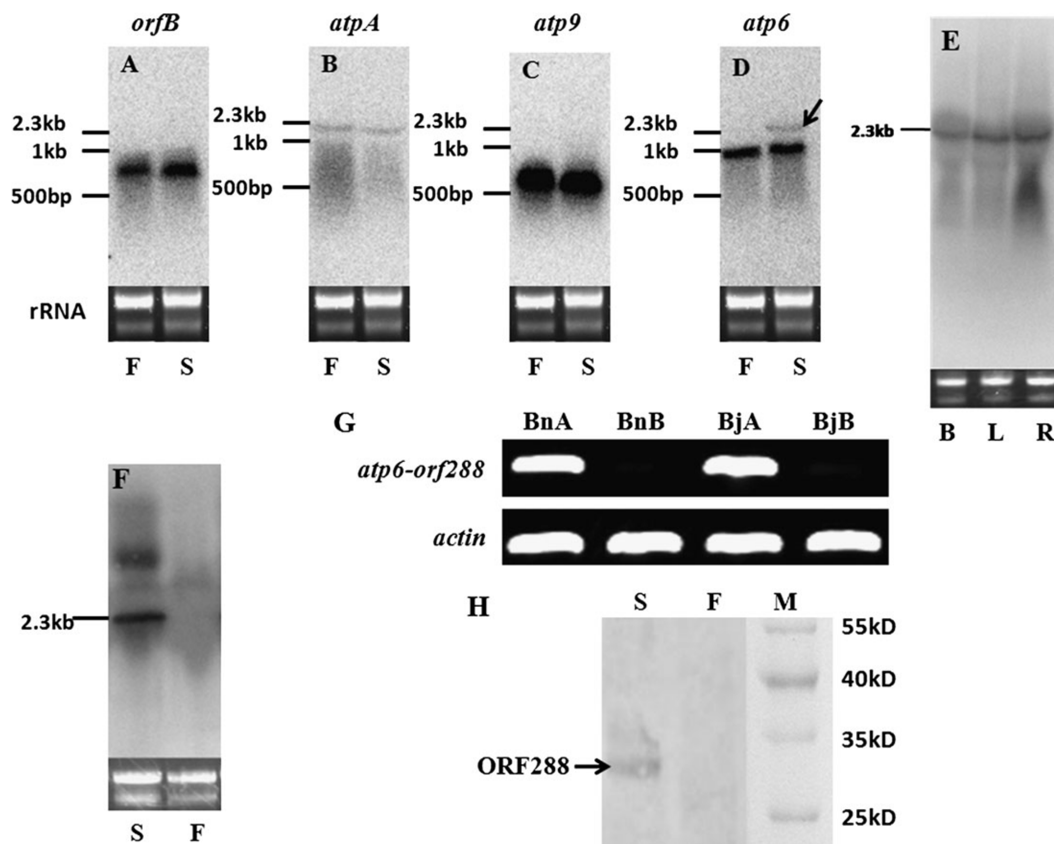

Fig. 2. (A–D) Northern blotting analysis of total RNA from buds of the male-sterile line (S) and the maintainer line (F) for four mitochondrial probes (*orfB*, *atpA*, *atp9*, and *atp6*). The *atp6* gene showed polymorphic band patterns of RNA transcripts (indicated by the black arrow). (E) Total RNA from the floral buds (B), leaf (L), and roots (R) of the *hau* CMS sterile line in *Brassica juncea* was blotted with the *orf288* probe. (F) The total RNA from the male-sterile line (S) and the maintainer line (F) of *hau* CMS in *Brassica napus* was detected using the *orf288* probe. (G) RT-PCR to demonstrate the expression of the *atp6–orf288* region in the *hau* mitotype, but not in its maintainer line. BnA, the male-sterile line in *Brassica napus*; BnB, the maintainer line in *Brassica napus*; BjA, the male-sterile line in *Brassica juncea*; BjB, the maintainer line in *Brassica juncea*. (H) Identification of ORF288 unique to the sterile line of *hau* CMS. Mitochondrial proteins were extracted from etiolated seedlings of male-sterile (S) and maintainer (F) lines and then were separated by 12% SDS–PAGE. The protein blots were then probed with an antibody to ORF288. The specific band (lane S) for ORF288 is indicated with an arrow. A pre-stained marker (M) was used for the detection of the transfer process and an evaluation of the molecular mass of the detected protein.

The chimeric gene orf288 *encodes a 32 kDa peptide in male-sterile mitochondria*

The northern blotting and CR-RT-PCR results showed that *orf288* was completely co-transcribed with the *atp6* gene in the male-sterile line. This gene was predicted to encode a 32 kDa protein with a triple transmembrane region (Fig. 1F). To confirm that this gene was actually translated into a protein, a peptide antigen corresponding to 17 residues (Fig. 1F) of ORF288 was synthesized and used to produce a polyclonal antibody. The mitochondrial proteins extracted from the etiolated seedling tissue of the fertile and sterile lines were detected using the above-mentioned antibody. A band of ~32 kDa was detected exclusively for the male-sterile sample (Fig. 2H).

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

To examine the function of *orf288*, its coding sequence was cloned into the expression region of a PET32a vector, and IPTG was used to induce its expression in *E. coli*. The growth of the host bacteria was repressed significantly with the expression of ORF288 (Fig. 3A). The growth of *E. coli* was normal when the tag peptide was highly expressed in the vector but was repressed when low levels of ORF288 fused with the tag peptide were expressed (Fig. 3B). This finding indicated that *orf288* encodes a peptide that is cytotoxic to host bacteria.

Expression of orf288 *in* Arabidopsis *significantly impairs the development of anthers*

To investigate the association of this chimeric gene with the male abortion of the *hau* CMS sterile line, four constructs were prepared and transformed into *A. thaliana* (Fig. 4A). The VNO construct contained a 35S promoter and *orf288*, which was fused to the mitochondrial transit peptide sequence of the nuclear *coxIV* gene of yeast for mitochondrial targeting. The fused ORF that was driven by the *AP3* promoter was specifically designed to investigate its effects on floral organs. To determine whether the chimeric gene

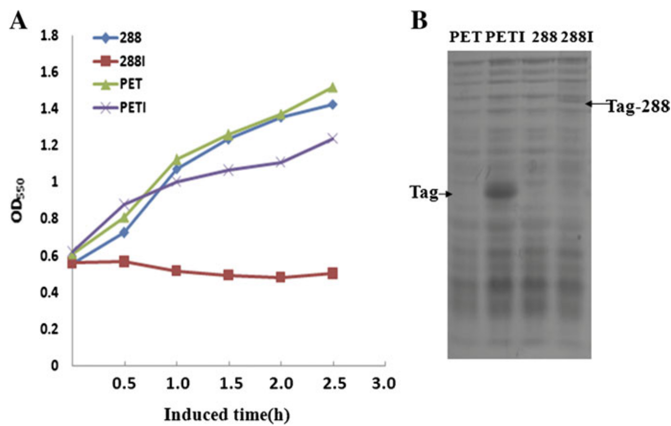

Fig. 3. (A) The effect of *orf288* expression on the growth of *E. coli* cells in liquid cultures with or without IPTG. IPTG was added when the cell growth reached OD_{550}=0.6. The expression vector PET32a was set as a control. 288, *orf288*-containing vector not induced by IPTG, 288I, induced with IPTG; PET, the control expression vector not induced by IPTG; PETI, the control expression vector induced by IPTG. (B) The expressed recombinant protein. Tag, the tag peptide in pet32a; Tag-288, the fusion peptide of tag and ORF288.

still functioned without a mitochondrial-targeting peptide, the VNII vector was constructed, in which *orf288* was driven by the *AP3* promoter but lacked the *coxIV* pre-sequence. A construct with the β-glucuronidase (GUS) gene driven by the *AP3* promoter was transformed into the plants as a control.

The statistical data showed that 80% of the T_1 plants with the VN3 construct developed abnormal flowers (Table 1; Fig. 4C–H). The pistil was surrounded by sepals but lacked stamens and petals (Fig. 4D, F, H; compare with the wild type, Fig. 4C, E, G). The stamens were instead represented only by one or two filaments with absent anthers for each flower (Fig. 4H). Surprisingly, ~80% of the transgenic plants with the VNII fragment showed phenotypes similar to those with the mitochondrial-targeting pre-sequence (Table 1). The transgenic plants that were transformed using the construct with the CaMV35S promoter were as fertile as the wild-type plants (Table 1). The control transformation with the AGUS construct did not change the male fertility of the transgenic plants. These transformation results indicated that the expression of *orf288* in *Arabidopsis* could lead to male sterility in the absence or presence of an external mitochondrial-targeting peptide, and an appropriate promoter is pivotal for this male sterility-associated gene to affect plant function significantly.

RT-PCR analysis was performed to examine the expression of the chimeric gene in the transgenic plants. Male-sterile T_1 plants were found to express the *orf288* gene strongly, but it was not expressed in fertile transgenic plants (Fig. 4B). To confirm that the male-sterile phenotype was transmitted to the second generation of transformed (T_2) plants, three male-sterile T_1 transformants were selected at random to assess the association of the *orf288* fragment with male sterility. These selected plants were cross-pollinated with wild-type pollen, and then the T_2 progeny were selected on kanamycin-containing medium and detected by the PCR amplification of *orf288* using specific primers. Each of the 158 T_2 plants with the transgenic fragments showed similar male-sterile phenotypes to their parents.

ORF288 is a mitochondrial-anchored protein

The above transformation experiments showed that the expression of *orf288* in *Arabidopsis* disrupted anther development even without a mitochondrial-targeting pre-sequence, and the morphologies of the transgenic plants were similar to those that possessed the external signal. This might suggest that *orf288* is functional in the cytoplasmic matrix or is targeted to the mitochondria without the assistance of an external transit peptide. PredSL (Petsalaki *et al.*, 2006) was used to predict the subcellular localization of ORF288, and the result showed that the N-terminal sequence could have its own signal function, because the mTP score was nearly 0.95126. The SignalP 3.0 results also revealed that the N-terminus of ORF288 may be a signal anchor peptide (signal anchor probability: 0.954) (Nielsen *et al.*, 1997; Nielsen and Krogh, 1998; Bendtsen *et al.*, 2004). This suggested that ORF288 is a mitochondrial-anchored protein that is originally expressed in the mitochondria of *hau* CMS cytoplasm and is capable of disrupting floral organ development.

To confirm that ORF288 is able to anchor to the mitochondria, the entire ORF was fused with GFP, and the fusion protein was expressed transiently in *Arabidopsis* protoplasts using the PEG transfection method (Fig. 5A–D). Mitotracker Red CMX-Ros dye was used to stain the mitochondria. The GFP fluorescence (Fig. 5A) and Mitotracker dye (Fig. 5B) images matched perfectly (Fig. 5C). These results demonstrated that without the external signal pre-sequence, ORF288 that is expressed in the cellular matrix additionally targets itself to the mitochondria.

Discussion

orf288*, a specific chimeric fragment located downstream of the* atp6 *gene*

The male-sterile *hau* CMS line was found to be a spontaneous male-sterile mutant in *B. juncea.* As a chimeric gene in the mitochondrial genome, the nucleotide sequence of *orf288* shared 99.5% similarity with the corresponding fragment of male-sterile *Brassica* lines containing *B. tournefortii* mitochondria (Landgren *et al.*, 1996). There were four point mutations: one deletion at base pair 764 and three nucleotide changes at base pairs 485, 785, and 802 in *orf288* (Supplementary Fig. S2 at *JXB* online). At these four positions, the nucleotides of *orf288* and *orf286* (located upstream of *orfB*) were identical. These observations might indicate that this region of *hau* CMS is evolutionarily closer to the homologous segment of the *nap* mitotype (Handa, 2003) than that of *B. tournefortii* CMS (Landgren *et al.*,

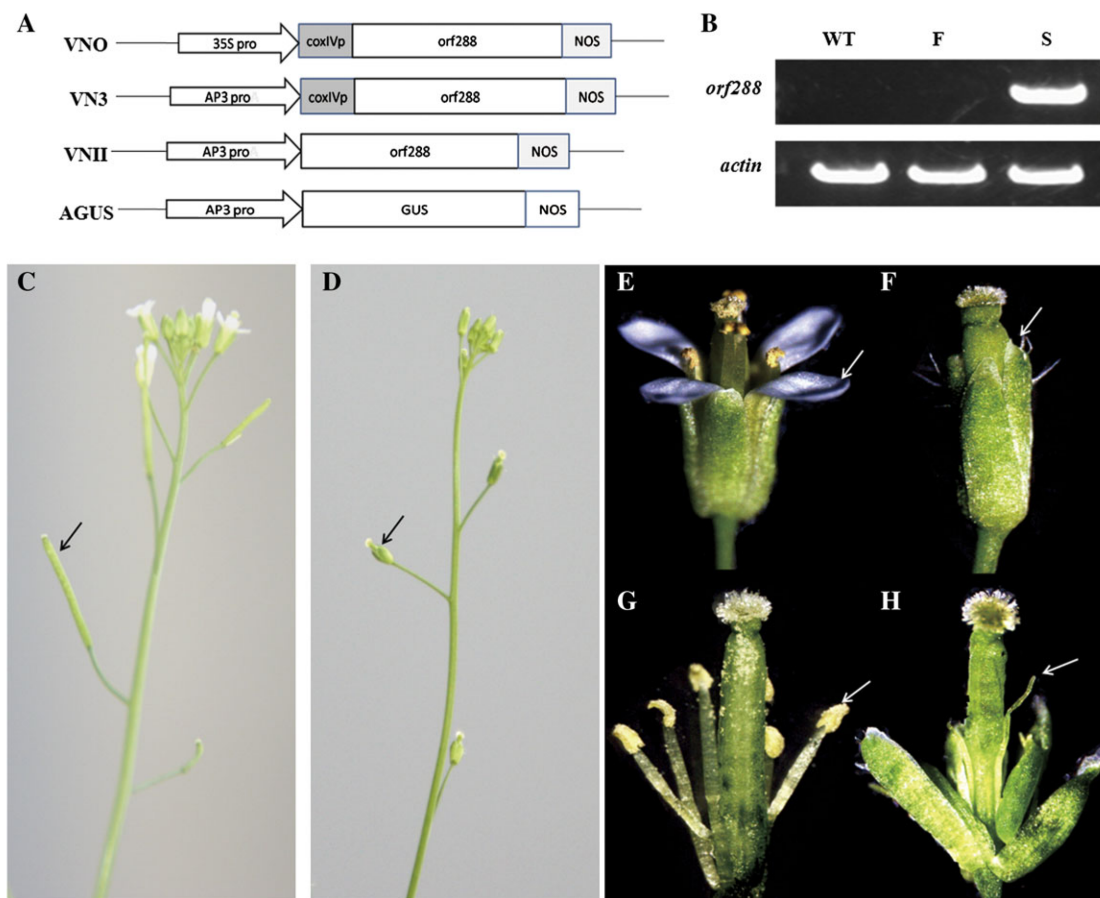

Fig. 4. (A) Schematic illustration of constructs used to transform *Arabidopsis*. The mitochondrial-targeting pre-sequence is the N-terminal 57 amino acids of the *coxIV* gene in yeast (grey boxes). ORFs are indicated by open boxes. Arrows indicate the CaMV35S or the promoter of the *Arabidopsis AP3* gene. The fragments were cloned to the expression sites of *pCAMBIA2300* (constructs: VN3, VNO, and VNII). and the *AP3* promoter fragment used was inserted into *pBI101* as a control. The direction of transcription is from left to right. (B) Expression analysis of *orf288* in transgenic plants with the VN3 construct. The transgenic plants showed male sterility (S) and full fertility (F). Wild buds (WT) were used as a control. (C–H) Phenotype analyses of male-sterile transformants with the VN3 construct. (C) Feature of a wild silique (black arrow); (D) feature of a male-sterile transgenic non-pollinated pistil (black arrow); (E) a wild flower with normal petals (white arrow); (F) a male-sterile transgenic flower without white petals; (G) wild anthers (white arrow); (H) no anther on top of a filament (white arrow).

Table 1. *Number of* A. thaliana *transgenic plants obtained with each construct*

Constructs	No. of PCR positive transgenic plants	No. of fertile plants	No. of sterile plants
VNII	20	4	16
VNO	22	22	0
VN3	15	3	12
AGUS	6	6	0

1996). *Tournefortii*–Stiewe CMS (*B. napus*) originated from a donor–recipient protoplast fusion of *B. tournefortii* and *B. napus* (Stiewe and Röbbelen, 1994). However, in this *B. tournefortii*-related male sterility system, the expression of the *atp6* gene is similar between the male-sterile line and its maintainer line (Dieterich *et al.*, 2003). *Brassica tour* CMS and *Tournefortii*–Stiewe CMS share a 1.58 kb *atp9* gene transcript (Dieterich *et al.*, 2003), which was not detected in the *hau* CMS mitotype and could contribute to the male sterility of the latter system. These observations indicated that the organization and expression of the CMS-associated genes were different in these three CMS systems.

The characterization of the transcriptional pattern of orf288

In most of the CMS systems that have been investigated, there is an association with mitochondrial genome rearrangement, and several CMS-associated genes are co-transcribed with functional mitochondrial genes (Dewey *et al.*, 1986; Hanson, 1991). Therefore, previous studies have routinely employed Southern and northern blotting analyses (Stahl *et al.*, 1994; Kim *et al.*, 2007). It was reported that three mitochondrial genes possessed RFLPs in the CMS and male-fertile lines (Wan *et al.*, 2008). In this study, northern blotting and RT-PCR analyses were used to detect transcriptional differences between fertile and sterile lines.

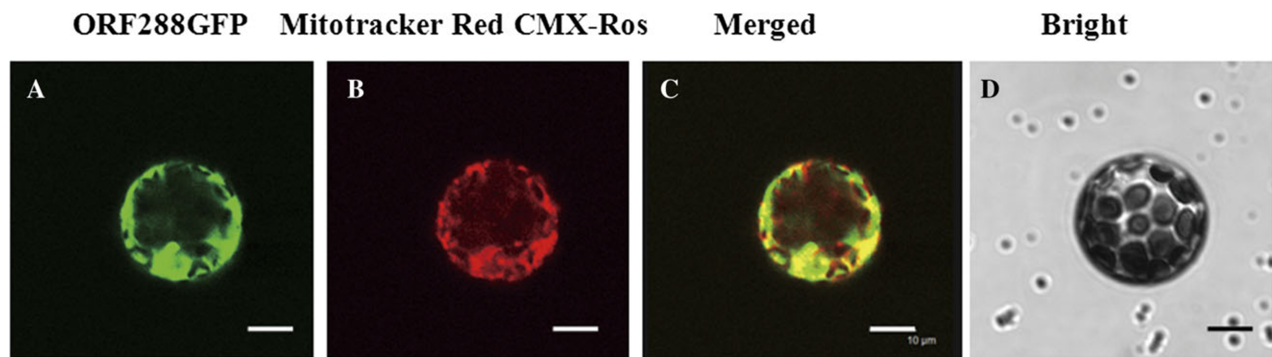

Fig. 5. Subcellular localization of ORF288. (A) The protoplast showed a green fluorescent signal at 488 nm; (B) the same protoplast showed a red fluorescent signal (stained by Mitotracker Red CMX-Ros) at 561 nm; (C) merged image of the green and red signals; (D) bright-field image. Data are representative of the transformed protoplasts. Green fluorescent signals were examined 16 h after transformation. Scale bars=10 μm.

A longer *atp6* transcript was found only in the male-sterile line, and this type of co-transcription was also found in several other CMS systems with known mitochondrial gene probes (Bonhomme *et al.*, 1992; Krishnasamy and Makaroff, 1993; Wang *et al.*, 2006).

Previous Southern blotting results indicated that the *atp6* gene is located at a single locus in the *hau* CMS mitochondrial genome (Wan *et al.*, 2008). Northern blotting with the *atp6* probe showed that two different transcripts were present in the sterile line. Furthermore, 12 independent CR-RT-PCR products of each of the above transcripts were sequenced, and two slightly scattering 3' ends were found (Fig. 1E). These observations suggested that the two *atp6* transcripts with different products originated from the same locus, and the insertion of the chimeric fragment *orf288* disrupted the transcriptional termination of the *atp6* gene in certain nuclear backgrounds.

ORF288 is a cytotoxic protein

BLASTP analysis showed that the N-terminus of ORF288 was identical to the NADH dehydrogenase subunit 5 and shared 68% identity with an uncharacterized ATP synthase C chain-like protein in *Arabidopsis*. The TMHMM result revealed that ORF288 is a multiple transmembrane protein with three transmembrane regions at its N-terminus. It was previously reported that ORF79 in Boro II cytoplasm and ORF129 in wild sugar beet are mitochondrial transmembrane proteins (Wang *et al.*, 2006; Yamamoto *et al.*, 2008), and the expression of several male-sterile genes is toxic or lethal to host bacterial cells (Dewey *et al.*, 1988; Duroc *et al.*, 2005; Wang *et al.*, 2006). The data showed that the expression of *orf288* in *E. coli* significantly repressed host cell growth (Fig. 3A). Therefore, it was suggested that ORF288 might also affect the development of floral organs by weakening mitochondria, as has been predicted by other researchers for several other CMS-associated genes (Wang *et al.*, 2006).

The multiple effects of ORF288 in transgenic plants

The *Arabidopsis APETALA3* gene is expressed specifically in stamens and petals during most floral development (Hill *et al.*, 1998), and its promoter has been successfully used to identify the CMS-associated gene *orf129* in sugar beet (Yamamoto *et al.*, 2008). The pre-sequence of *coxIV* (partial or full) in yeast is also efficient for mitochondrial localization (Köhler *et al.*, 1997; Kim *et al.*, 2007; Nizampatnam *et al.*, 2009). Therefore, in this study, the pre-sequence of *coxIV* was fused to the 5' end of *orf288* driven by the *AP3* gene promoter. For this promoter, ORF288 disrupted the development of the anther, and the male-sterile transformants with the VN3 and VNII constructs could not develop white petals. These phenotypes suggested that ORF288 not only strongly disturbs the differentiation of stamens but also affects the development of the petals in the transgenic plants. Previous experiments have shown that the anthers of the *hau* CMS line are transformed into thickened petal-like structures that lack anthers and filaments. The stamen primordia deviated from normal polarization and formed petal primordia, which developed into petal-like structures where the stamen would have been located (Wan *et al.*, 2008). Although the flower morphologies of the male-sterile transgenic plants were different from those of the male-sterile *hau* CMS line, both reached their male abortion stages earlier than those of other previously reported CMS types in *Brassica*. The male-sterile phenotype was also found to be co-segregated with a transgenic fragment in the T_2 progeny of male-sterile plants that were pollinated by wild pollen.

It was initially hypothesized that the transgenic plants with *orf288* driven by CaMV35S would be male sterile, but the result showed that the male fertility was not affected. In fact, several other researchers also found the same phenomenon, when they used CaMV35S to drive their CMS-associated genes (Chaumont *et al.*, 1995; Duroc *et al.*, 2006; Nizampatnam *et al.*, 2009). According to present knowledge, it was suggested that this might be due to the weak promotion of the CaMV35S promoter in anther/floral tissues and the male-sterile genes were not expressed in sufficient amounts in the appropriate anther/floral cells. In addition, several CMS-associated genes were also detected in vegetative tissues (Dewey *et al.*, 1987; Bellaoui *et al.*, 1999), but the growth of most vegetative tissues is not being

notably affected. This may be due to the vulnerability of anther/floral tissues or specific interaction of CMS-associated genes with some factors in floral organs.

The subcellular localization of ORF288 expressed in the cytoplasmic matrix

For the first time, a CMS-associated gene expressed in the cellular matrix of transgenic plants was found to be targeted to the mitochondria without the assistance of an external transit peptide. In chili pepper and wild beet, similar transgenic experiments with the CMS-associated gene did not result in male-sterile plants when a targeting pre-sequence was not used to assist in the mitochondrial targeting process (Kim *et al.*, 2007; Yamamoto *et al.*, 2008). However, the expression of the common bean CMS-associated gene (*orf239*) caused male sterility in transgenic tobacco plants even when ORF239 was not targeted to the mitochondria (He *et al.*, 1996). Subsequently, it was found that ORF239 accumulated within the callose layer of the pre-meiotic pollen mother cell wall and the primary cell wall (Abad *et al.*, 1995; He *et al.*, 1996; Sarria *et al.*, 1998). In this study, the male-sterile transgenic plants obtained with the VNII construct showed similar phenotypes to those with the mitochondrial-targeting peptide. Therefore, considering the targeting function and subcellular localization of these CMS-associated genes, the function of *orf288* in male sterility in the *hau* CMS system may be distinct from that of *orf239* in the common bean or *orf129* in wild beets.

Restoring genes are imperative for the production of hybrid seeds in breeding and are useful for the characterization of cytoplasmic sterile genes. *orf288* has been detected in the mitochondrial genome of a wild *B. tournefortii* plant that is male fertile. Therefore, it was predicted that this plant may possess *hau* CMS-restoring genes. This work provides convincing evidence that *orf288* disrupts stamen development in transgenic plants, but the mechanism that results in this phenotype currently is not clear. Therefore, the nuclear gene expression profiles of flowers of the *hau* CMS line are now being compared with those of the maintainer line using microarray analysis to investigate further the mechanism of male sterility in *hau* CMS.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Map of the pM999GFP vector.

Figure S2. Multiple alignment of DNA sequences of *orf288*, *orf286*, and *orf263*

Table S1. Primers used in the study.

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