RESEARCH PAPER

Expression of the nuclear gene TaFAd is under mitochondrial retrograde regulation in anthers of male sterile wheat plants with timopheevii cytoplasm

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Abstract
Alterations of mitochondrial-encoded subunits of the FoF1-ATP synthase are frequently associated with cytoplasmic male sterility (CMS) in plants; however, little is known about the relationship of the nuclear encoded subunits of this enzyme with CMS. In the present study, the full cDNA of the gene TaFAd that encodes the putative FAd subunit of the FoF1-ATP synthase was isolated from the wheat (Triticum aestivum) fertility restorer ‘2114’ for timopheevii cytoplasm-based CMS. The deduced 238 amino acid polypeptide is highly similar to its counterparts in dicots and other monocots but has low homology to its mammalian equivalents. TaFAd is a single copy gene in wheat and maps to the short arm of the group 6 chromosomes. Transient expression of the TaFAd–GFP fusion in onion epidermal cells demonstrated TaFAd’s mitochondrial location. TaFAd was expressed abundantly in stem, leaf, anther, and ovary tissues of 2114. Nevertheless, its expression was repressed in anthers of CMS plants with timopheevii cytoplasm. Genic male sterility did not affect its expression in anthers. The expression of the nuclear gene encoding the 20 kDa subunit of Fo was down-regulated in a manner similar to TaFAd in the T-CMS anthers while that of genes encoding the 6 kDa subunit of Fo and the γ subunit of F1 was unaffected. These observations implied that TaFAd is under mitochondrial retrograde regulation in the anthers of CMS plants with timopheevii cytoplasm.

Key words: CMS, FAd subunit, FoF1-ATP synthase, retrograde regulation, wheat.

Introduction
Cytoplasmic male sterility (CMS), a maternally inherited trait characterized by the inability to produce functional pollen but maintain female fertility, has been observed in >150 plant species (Kaul, 1988), for instance the wild abortion (WA)-type male sterility in rice (Oryza sativa), the T-type male sterility in maize (Zea mays), and the polima-type male sterility in oilseed rape (Brassica napus). A few CMS systems, such as T-CMS (Wilson and Ross, 1962), V-CMS, and K-CMS (Mukai and Tsunewaki, 1979; Lucken, 1987), have been reported in common wheat (Triticum aestivum), most of which were generated from interspecific hybridizations. The T-CMS was first obtained by replacing the cytoplasm of common wheat cultivar ‘Bison’ with T. timopheevii cytoplasm through backcross (Wilson and Ross, 1962), and was most widely used in the studies of wheat heterosis utilization. Incompatibility of the cytoplasm and nucleus in an alloplasmic line was viewed as the cause of sterility (Zubko, 2004); however, the exact mechanisms of CMS are still unclear. Introducing fertility-restoring genes into the alloplasmic lines restores the male fertility of the CMS lines.

Mitochondrial DNA (mtDNA) polymorphism of CMS lines is often associated with CMS (Hanson and Bentolila, 2004). The most frequently observed mtDNA alterations...
occur in regions encoding subunits of the F$_{0}$F$_{1}$-ATP synthase (EC 3.6.1.34) or/and cytochrome c oxidase (EC 1.9.3.1). Qiu et al. (2001) reported restriction fragment length polymorphism of atp6, nad3, and cox1 genes between the wheat T-type CMS line ‘75-3369A’ and its maintainer. Atp6 is a component of the F$_{0}$F$_{1}$-ATP synthase, a key enzyme for ATP synthesis by using the proton gradient generated from the respiratory chain (Lehninger et al., 1993). This enzyme comprises three sections: F$_{0}$, F$_{1}$, and F$_{A}$ (Nagley, 1988; Lehninger et al., 1993; Walker and Dickson, 2006), where F$_{0}$ is the membrane-spanning section and F$_{1}$ is hydrophilic. F$_{A}$ has not been well defined; it may be the physical link between F$_{1}$ and F$_{0}$ (Walker and Dickson, 2006). GmfF$_{A}d$ from soybean is the only plant F$_{A}$ subunit gene cloned so far (Smith et al., 1994). Beside nuclear genes such as GmfF$_{A}d$, mitochondrial genes encode some of the subunits in this enzyme complex, for instance, ATP6, ATP8, ATP9, and ATPA. Expression of an unedited wheat mitochondrial atp9 gene in tobacco nucleus resulted in male sterility (Hernould et al., 1993).

The nuclear–cytoplasmic interactions in CMS have been investigated at the expression level. Some nuclear genes in the alloplasmic wheat CMS lines with Aegilops crassa cytoplasm showed altered expression patterns (Murai et al., 2002). In anthers of the alloplasmic CMS B. napus carrying rearranged Arabidopsis mtDNA, dozens of nuclear genes are down-regulated (Carlsson et al., 2007). Mitochondrial regulation of nuclear genes was termed ‘mitochondrial retrograde regulation’ (MRR) in animals and yeast (Liao and Butow, 1993). Expression of the fertility-restoring genes in CMS lines cleaves or degrades the mitochondrial abnormal transcripts (Wang et al., 2006) or the resulting products (Bellaoui et al., 1999), which in turn could alleviate or eliminate the undesirable MRR in CMS lines. The only exception to date is the maize Rf2 gene, which restores the fertility of maize T-CMS plants by complementing the deficiency of aldehyde dehydrogenase (Cui et al., 1996).

In previous studies, an expressed sequence tag (EST) Y546 homologous to the F$_{A}d$ subunit of wheat F$_{0}$F$_{1}$-ATP synthase was cloned, which shows differential expression between anthers from euplasmic wheat lines and anthers from plants carrying the timopheevii cytoplasm and the fertility-restoring gene Rp6 (Ma et al., 1991). In the present study, the full cDNA of this gene was isolated and evidence was provided that F$_{A}d$ of wheat (TaF$_{A}d$) could be under MRR in CMS anthers.

**Materials and methods**

**Plant materials**

The plant materials used in this study included the restorer line 2114 carrying Rp6, the F$_{2}$ progenies derived from the cross of 2114 as the female parent with the wheat variety ‘Sumai No. 3’ that does not have fertility-restoring genes. Sumai No. 3, ‘Yangmai No. 5’ (Y5), and the generic male sterile (GMS) line GMS-Y5, which differs from Y5 by possessing the dominant generic male sterility gene Ms$_{2}$. Wheat cultivar ‘Chinese Spring’ (C.S.), C.S. nulli-tetrasomic lines, and ditelosomic lines were used in chromosome localization analysis. All these materials are hexaploid common wheat and have three subgenomes, each of which is derived from a different diploid progenitor.

The plants were grown in a field in Nanjing, China in the normal growing season. Leaves, stems, spikelets, anthers, and ovaries at the meiosis stage were collected for RNA analysis. To check male fertility, the spikes were bagged to self before flowering and the seed-set in the two basal florets per spikelet was counted. Those plants without seed-set were classified as sterile.

**DNA and RNA isolation**

Genomic DNA was extracted according to the procedures described by Ma and Sorrells (1995). RNA was extracted using the Trizol reagent (Invitrogen, USA) following the manufacturer’s protocol and quantified with the spectrometer (Ultraspexc 2100 pro; Amersham Pharmacia, UK).

**Full cDNA isolation and chromosome mapping**

The 540 bp Y546 sequence was queried against the wheat dbEST (580 000 ESTs, the 154th release of GenBank, 2006). Using the criteria of >95% identity and E$^{-100}$, over 400 homologous EST hits were retrieved and used in contig assembly with the parameter settings of >40 bp overlap and >95% identity after removing possible vector sequence contamination. Based on the contig, primers (F, 5′-CGGAGGAGAAGAAGGAGCCGAGAGGC-3′; R, 5′-ATACCATCCTGGTGCTATACGACA-3′) for the full cDNA amplification were designed.

The first-strand cDNA was synthesized with 3 µg total RNA using the M-MLV reverse transcriptase (Promega, USA) according to the manufacturer’s instructions. The PCR was performed in a 25 µl mixture using ~5 ng first-strand cDNA as the template, 5 pmol of each of the primers, 5 nmol of each of the dNTPs, 37.3 nmol MgCl$_{2}$, 0.5 U rTaq DNA polymerase (Takara, Japan), and 1× PCR buffer supplied together with the enzyme. The thermal cycle profile is 94°C for 3 min, 36 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min, and a final extension of 5 min at 72°C.

The target band excised from the gel was purified and ligated into the pUC19-based TA cloning vector pX-T. The ligation mixtures were then used in transformation of the competent JM109 cells. The positive clone was sequenced in Shanghai Invitrogen on an Applied Biosystems 3730 DNA analyser.

**ORF identification, protein translation, molecular mass calculation, and amphipathic helix prediction** were conducted with the software Macvector 9.0 (Accelrys, Oxford, USA). Prediction of the signal peptide was carried out with the CBS prediction server (http://genome.cbs.dtu.dk/services/SignalP).

The primer pair 5′-CACCCGAGATGACCTATTGAAAGG-3′ and 5′-CTCCTCCCTCTTATGACCCCTC-3′ were designed based on the isolated full cDNA sequence and used in amplifying nulli-tetrasomic line DNA. The PCR product was digested with AlulI in the standard procedure and separated with 8% PAGE gel (arc:bi:s=19:1) to reveal polymorphism.

**Southern hybridization**

Southern hybridization was conducted following the procedure of Ma and Sorrells (1995). After hybridization, the filters were washed once with 2× SSC, 0.1% SDS (v/v), and once with 1× SSC, 0.1% SDS, each for 15 min, and then were exposed to X-ray films for 7 d.
Vector construction, transient expression, and microscopy

The full coding DNA sequence was amplified using pfu DNA polymerase (Dingguo Biotech, China) with the primer pair 5’-TATAATCTGAGACGAGAAAGGAAAGCGAGAAGA-3’ and 5’-CATGCCAGCTCAAGGAATGTGCTTCGTCAATGGCCAA-3’ that contains an XbaI restriction site (underlined) at the 5’-ends. After digestion with XbaI, the products were inserted into the XbaI site of a pUC19-based expression vector with the CaMV35S-EGFP-NOS cassette to generate a CaMV35S promoter-driven GFP-fusion construct. The ligation products were transformed into JM109 competent cells.

Using the PDS-1000 particle delivery system (Bio-Rad, Hercules, CA, USA), the sequence-confirmed fusion construct was introduced through 1.1 µm tungsten particles into the epidermal cells of onion bulbs on agar plates for transient expression in the procedure recommended by the manufacturer. After 24 h culture, the bombarded epidermal cells were treated with 50 nM of the mitochondrion-specific dye MitoTracker Red CM-H2XRos (Molecular Probes, Leiden, The Netherlands) for 20 min and then rinsed three times with PBS buffer (137 mM NaCl, 1.4 mM KH2PO4, 4.3 mM Na2HPO4, 2.7 mM KCl, pH 7.4). Fluorescent signals in the transformed cells were viewed using a confocal scanning microscope system (TCS NT; Leica, Germany) with 488 nm exciting wavelength of GFP and 578 nm of MitoTracker Red. The cellular structure was visualized under bright-field optics.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was carried out in 12.5 µl reactions with ~2.5 ng of each of the template cDNA, 2.5 pmol of each primer, 2.5 nmol of each dNTP, 18.6 nmol MgCl2, 0.2 U rDNA polymerase (Takara, Japan), and 1× PCR buffer supplied together with the enzyme. The PCR cycles were 94°C for 3 min, 22 cycles of 94°C for 20 s, 58°C for 30 s, 72°C for 30 s, and a 5 min 72°C extension. The primers (5’-TGAAGGGCACTCTTITTTT-GAGGTTG-3’ and 5’-TTCTCAGTGCTGTAAGGGATTC-3’) for the wheat FAd gene were designed based on the 3’-end cDNA sequence. Primers for the 20 kDa subunit, 6 kDa subunit, and γ subunit genes of ATP synthase, i.e. 5’-TCCCAAGGAACCA-AAGC-3’ and 5’-CTGCTGCTGCTGCTAATGGC-3’, 5’-CGCA-CCAGGAGTATCACC-3’ and 5’-GGATCTAACGATCC-3’ were designed based on EST sequences retrieved from wheat dbESTs with the criteria of >85% positive identity in a 33 amino acid (aa) overlap. RT-PCR of the housekeeping β-tubulin gene with the primer pair 5’-CTGTTGTAGTGTTGTGCGCAAG-3’ and 5’-ACCTCCCTCATAGCTTCCTGCAGG-3’ was used as the control. The PCR products were resolved in 1.5% agarose gels and viewed under UV light after staining with ethidium bromide.

Polyclonal antibody preparation and western blot

The coding region for the N-terminal aa 25 to aa 238 of the TaFAd protein was amplified using pfu with the primer pair 5’-TATACCATGGGTCCATACTCGCTGCTC-3’ and 5’-TGTG-GTCGGCCTAAAGGAAATGTGCTTCATGGCCAA-3’ with a NcoI or a Sall restriction site (underlined), respectively. After digestion with both enzymes, the PCR products were inserted into the pET-32a expression vector through the NcoI and Sall restriction site (Merck, USA). The His-TaFAd fusion protein was expressed in Escherichia coli strain ‘ROS’ after induction with 1 mM IPTG (Merck). The ~50 kDa target band excised from the gel containing ~200 µg antigen was crushed and mixed with 0.7 ml Freund’s complete adjuvant (Dingguo Biotech, China). The mixture with 200 µg antigen was injected into a rabbit, followed by injection of the same amount of antigen every 3 weeks. Four weeks after the final injection, antiserum was collected and antibody specificity against TaFAd was checked by western blot.

Tissues were completely homogenized in liquid nitrogen and sonicated for 2 min after adding the extraction buffer (8 M urea, 4% CHAPS, 65 mM DTT, and 150 µM PMSF) to ensure rupturing the mitochondria completely. Eighty micrometers of proteins were separated with 12% SDS-PAGE and were then transferred onto the PVDF transfer membrane using a semi-dry electroblotting apparatus (Transblot SD, Bio-Rad, USA). Western hybridization with the anther proteins was conducted twice. The first one used antibody purified by mixing 1 ml antiserum with an equal volume of cell lysis products from the bacteria carrying only the pET-32a vector and centrifuging at 100 000 g for 20 min at 4 °C. The membrane was pre-hybridized for 1 h in 10 ml TBST buffer (100 mM NaCl, 25 mM TRIS, 0.1% Tween) with 1% BSA, then incubated for 2 h in 10 ml TBST hybridization buffer added with the antibody in a dilution factor of 500. After washing with the TBST buffer three times, the membrane was then incubated with the peroxidase-labelled goat anti-rabbit IgG (Promega, USA) with a dilution factor of 4000 for 2 h. Hybridization signals were visualized using the Super Signal ECL system according to the manufacturer’s instructions (Pierce, USA).

For the second western hybridization, 700 µl antiserum was purified by mixing with 1750 µl total bacterial lysates, and the pre-hybridization was conducted for 6 h in 10 ml TBST buffer with 3% BSA. The hybridization with the antibody was carried out at 4 °C. The incubation time with anti-rabbit IgG was 3 h.

The TaFAd sequence described in this article has been submitted to the GenBank data library under the accession number AY614716.

Results

Cloning and characterization of TaFAd

More than 400 ESTs homologous to Y546 with over 95% homology in the 154th release of GenBank were identified. This result indicates that this expression is in high abundance. A contig with a predicted molecular mass of 27 kDa and a theoretical pI of 8.42. It is similar to the putative rice (BAD07747) and Arabidopsis FAd (AAM64665) proteins and has a 59 aa C-terminal extension compared with the reported soybean GmFAd (X79058) (Fig. 1). However, a soybean EST (CSS457581) was identified that encodes this missing C-terminus through mining the NCBI soybean dbESTs, which is consistent with the fact that X79058 does not have a stop codon (Smith et al., 1994). Y546 was hereafter renamed as TaFAd. The similarity of the FAd proteins from the above-mentioned four plants is over 80%. They have only ~20% similarity to their bovine equivalent.

Southern hybridization using TaFAd cDNA as the probe detected no more than three bands when the DNA was digested with the restriction endonucleases EcoRI and...
EcoRV (Fig. 2), suggesting that TaFAd is a single copy gene as the hexaploid wheat has three subgenomes.

Mitochondrial localization of TaFAd
TaFAd has the characteristics of soluble mitochondrial proteins; overall hydrophilicity, amphiphilic helix structures, a high portion of positively charged amino acids, and an N-terminal hydrophobic domain. It contains a conserved N-terminal mitochondrial signal peptide (RLVSRS) from aa 6 to aa 11. This was proved by the co-localization of TaFAd–GFP fusion with the mitochondrion-specific MitoTracker (Fig. 3).

Chromosomal mapping of TaFAd
Amplification of DNA from C.S. nulli-tetrasomic lines using the TaFAd-specific primers yielded a monomorphic band. However, after AluI digestion of these PCR products, a missing band appeared in nulli-tetrasomic lines lacking chromosome 6A and 6B and ditelosomic lines lacking the short arms of these two chromosomes (Fig. 4). Thus, it was concluded that TaFAd genes are on the short arms of the group 6 chromosomes. The rice FAd homologue was on chromosome 2, which is syntenic to group 6 wheat chromosomes.

Differential expression of TaFAd in anthers of CMS lines and GMS lines
The TaFAd gene is expressed abundantly in anthers at the meiosis stage of the restorer line 2114 (Fig. 5A). This was also true in the fertile F2 plants; however, in anthers of the sterile F2 plants, the expression of TaFAd was hardly detected and this deficiency of expression occurred pre-meiosis (Fig. 5C). In the initial western blot with antibody raised against TaFAd, two weak bands ~25 kDa in size, likely to be the mature TaFAd and its cytoplasmic precursor, were visualized in extracts of the fertile anthers, but not in extracts from sterile anthers (Fig. 5D, a). To verify this result, western blot using antibody purified with stronger stringency was conducted. It resulted in a much stronger intensity of the two ~25 kDa bands (Fig. 5D, b) and disappearance of a few non-specific bands detected in the first hybridization (data not shown). Thus, consistent with the RT-PCR results, the TaFAd protein was undetectable in sterile anthers.

To examine if TaFAd is expressed in genic male sterile lines, RT-PCR was performed with anthers of Y5 and GMS-Y5. It was found that TaFAd was expressed equally...
in anthers from both lines (Fig. 5B). Thus, the repression of TaFAd’s expression is not a universal feature of all kinds of male sterile lines.

Similar to TaFAd, the gene encoding the 20 kDa subunit of the Fo section of FoF1-ATP synthase was also down-regulated in the CMS anthers. But the genes encoding the 6 kDa subunit of Fo and the c subunit of F1 were not affected by T-CMS (Fig. 6).

Discussion

FoF1-ATP synthase is a mitochondrial protein complex critical for cell life since it generates most of the cellular energy. Not all of its component subunits are encoded by mitochondrial genes. The nuclear TaFAd gene characterized in the present study encodes the d subunit of the FA segment of this complex. The primary structure of the FAd subunits is highly conserved among higher plants, implying that it has conservative functions. In Arabidopsis, rice, and wheat, FAd is a single copy gene, but there may be two FAd copies in soybean (Smith et al., 1994). ATP synthase is rich in cells (Lehninger et al., 1993). Consistent with this, the TaFAd gene is expressed actively in various tissues. Mining EST databases of rice and Arabidopsis indicated that the FAd gene was expressed ubiquitously and abundantly in these two plants.

Microsporogenesis is an energy-demanding process, which is prone to disruption by deficiency of cellular energy metabolism. In CMS plants, the Fo,F1-ATP synthase is specifically reduced in both quantity and enzyme activity (Bergman et al., 2000; Zhang et al., 2007). Zhang et al. (2007) recently reported that the dysfunction of the Fo,F1-ATP synthase in HL-CMS rice is caused by alteration of the ATP6 subunit. It was found that the FAd gene in the T-CMS wheat was strongly repressed in immature anthers. However, the 6 kDa and c subunit nuclear genes of ATP synthase were stably expressed in the same tissues. These results suggested that the anther degeneration could not be the cause of the expression repression of the FAd gene. Another piece of evidence for this is that the FAd gene expression in GMS-Y5 was also abundant in anthers and not different from Yangmai No. 5. The differential expression pattern between the T-CMS lines and the GMS line implied that the anther-specific repression of TaFAd in T-CMS anthers occurred before meiosis (Fig. 5C). The assembly and stoichiometry of ATP synthase in sterile anthers is very likely to be disrupted since the TaFAd subunit was not detectable (Fig. 5D) and expression of the 20 kDa Fo subunit was reduced (Fig. 6). Considering the fact of abnormal mitochondrial ORFs in T-CMS cells (Qiu et al., 2001), it is proposed that the incompatibility of alloplasmic genes with the nuclear genes results in the impairment of ATP synthase assembly and activity in anthers in ways that include alternation of the expression of its component subunit genes, and eventually leads to male sterility. However, it is still not known how this impairment is caused by the cyto-nuclear incompatibility. The cytotoxic effect of the chimeric mitochondrial peptide (Wang et al., 2006), the accumulation of undesirable metabolites (Cui et al., 1996), and the premature programmed cell death (Balk and Leaver, 2001) have all been related to CMS. It is necessary to clarify the functional pathways of CMS to understand its mechanisms and make better use of it in crop improvement.

MRR in CMS anthers have been reported a few times (Murai et al., 2002; Carlsson et al., 2007). However, how the mitochondria transduce the relevant signal to the nucleus and affect the viability of flowers/pollen is still not understood. Kuzmin et al. (2004) showed that the retrograde signals to the nucleus originate from a reduced
mitochondrial trans-membrane potential. MRR may first alter the expression of nuclear transcription factors, which in turn regulate the transcription of other genes (Linke and Börner, 2005). A promoter region necessary for MRR has been identified in the AtAOX1a gene of Arabidopsis (Dojcinovic et al., 2005). Genes involved in floral/microspore formation, programmed cell death, energy metabolism, signal transduction, and protein transport have been suggested as potential targets for MRR (Murai et al., 2002; Carlsson et al., 2007; Fujii et al., 2007). Even though the TaFAd gene might be the MRR target in the T-CMS line, it is not yet known whether it is one of the primary targets of MRR or a downstream component of the signalling cascade.

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References


