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Isolation and *in silico* functional analysis of *MtATP6*, a 6-kDa subunit of mitochondrial F_1F_0-ATP synthase, in response to abiotic stress

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ABSTRACT. Mitochondrial F_1F_0-ATP synthase is a key enzymatic complex of energy metabolism that provides ATP for the cell. Subunits of this enzyme over-express under stress conditions. Little is known about the structure and regulatory mechanism of the F_0 portion of this enzyme. We isolated the full-length coding sequence of the *RMtATP6* gene from rice and wheat, and partial sequences from *Aegilops crassa* and *Triticum monococcum* (Poaceae). We found that the sequence of rice *RMtATP6* is 1965 bp long and contains two exons and one intron in 3'-UTR. Then, we analyzed the 2000-bp upstream region of the initiation codon ATG of the *RMtATP6* and *AtMtATP6*, as promoter. The *RMtATP6* coding sequence was found to be much conserved in the different plant species, possibly because of its key role under stress conditions. Promoter analysis demonstrated that *RMtATP6* and *AtMtATP6* include *cis*-acting elements such as ABRE, MYC/MYB, GT element in the

upstream region, which respond to abscisic acid stress hormone and might show vital its roles in biotic and abiotic tolerance as an early-stress responsive gene. A mitochondrial signal peptide of 30 amino acids in length and an N-terminal cleavage site between amino acids 20 and 21 were discovered in *RMtATP6*. In addition, we found a transmembrane domain with an alpha helix structure that possibly passed through the mitochondrial inner membrane and established the 6-kDa subunit in the F_0 portion of the enzyme complex. Apparently, under stress conditions, with increasing ATP consumption by the cell, the 6-kDa subunit accumulates; by switching on F_1F_0-ATP synthase it provides additional energy needed for cell homeostasis.

Key words: MtATP6; F_1F_0-ATP synthase; Wheat; Abiotic stresses; *In silico* functional analysis

INTRODUCTION

Higher plants are usually sensitive to environmental stresses, such as high salinity, draught, high temperature, intense light, and chilling (Suda et al., 2009). Drought and salinity are the major abiotic stresses that severely threaten the food supply on Earth (Nevo and Chen, 2010). To survive under stress conditions, plants respond and adapt to these stresses at the molecular and cellular levels and also at the biochemical and physiological levels (Shinozaki and Yamaguchi-Shinozaki, 2000).

The mitochondrion is referred to as the "power house" of the cell because it is responsible for the synthesis of the universal energy "currency" of the cell, i.e., adenosine triphosphate (ATP) (Manatt and Chandra, 2011). The inner membrane of the mitochondrion includes the protein complexes of the electron transport chain. Oxidation/reduction reactions together with components of the electron transport chain produce a proton gradient that is used by F_1F_0-ATP synthase to phosphorylate adenosine diphosphate (ADP), thereby producing ATP (Figure 1) (Manatt and Chandra, 2011).

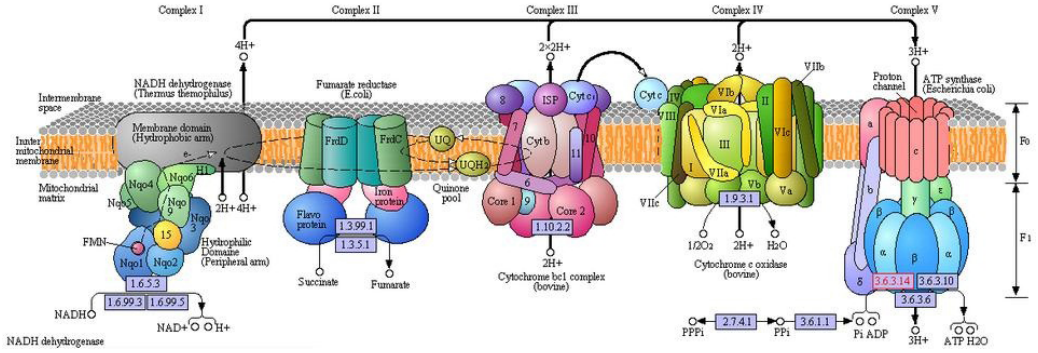

Figure 1. Electron transfer chain in the inner-membrane of mitochondrion. The electron transfer chain contains five complexes designated as complex I, II, III, IV, and V (F_1F_0-ATP synthase). The electrochemical H^+ gradient provided by these membrane-bound complexes serve as energy source for ATP synthesis from ADP and inorganic phosphate by an F_1F_0-ATP synthase. Available at [http://www.genome.jp/kegg/].

F_1F_0-ATP synthase seems to play a fundamental role in the synthesis of ATP in all living organisms (Agarwal, 2011). This enzyme is present in the plasma membrane of bacteria, the thylakoid membrane of chloroplast, and the inner membrane of mitochondria. Two functionally distinct parts have been identified in the F_1F_0-ATP synthase molecule: the F_1 portion, which performs ATP synthesis and hydrolysis reactions, and the F_0 portion, which mediates the proton transport (Soontharapirakkul et al., 2011). Prokaryotes and eukaryotes have similar F_1 structures, which consist of 5 subunits designated α, β, γ, δ, and ε (von Ballmoos et al., 2010; Manatt and Chandra, 2011). However, F_0 structures are rather different in these 2 types of organisms (Spinazzola and Zeviani, 2007).

The subunits F_1 and F_0 are controlled by 2 different genetic systems in plant cells, i.e., the nuclear and the mitochondrial genomes. Plant mitochondrial genome mostly encodes the hydrophobic subunits (Meyer et al., 2008), but the F_1 subunits are encoded only by the nucleus (Arnold et al., 1998). In contrast, a 6-kDa subunit of hydrophobic F_0 in rice (*RMtATP6*, GenBank accession No. AB055076; LOC. Os03g0606200) has been characterized; it was encoded by the nucleus, and its protein is transferred to the mitochondrial inner membrane (Zhang and Liu, 2003; Zhang et al., 2006). This subunit was identified by analysis of the rice mitochondrial proteome (Heazlewood et al., 2003). Further, another gene orthologous to *RMtATP6*, *AtMtATP6*, was identified in *Arabidopsis thaliana* (2 paralogues, including GenBank accession No. AK117680; LOC. At3g46430 and GenBank accession No. NM_148152; LOC. AT5G59613) (Meyer et al., 2008). In another research, a full-length mitochondrial F_1F_0-ATP synthase 6-kDa subunit gene (*JcMtATP6*, GenBank accession No. FJ619050) derived from *Jatropha curcas*, was identified. This gene was obtained from yeast functional genetic screen tolerant to abiotic stress and it could confer abiotic stress tolerance (Eswaran et al., 2010).

Further, Hamilton et al. (2001) reported that the exposure of *Triticum aestivum* to aluminum increased the mitochondrial F_1F_0-ATP synthase activity only in an aluminum-tolerant wheat variety. They recommended that the upregulation of ATP synthase activity was an adaptive response in aluminum tolerance of wheat. Correspondingly, in wild-type yeast, NaCl stress causes increased activity of the mitochondrial F_1F_0-ATP synthase activity and increased expression of the F_1F_0-ATP synthase α-subunit (Hamilton et al., 2001).

Accordingly, *RMtATP6* (Zhang et al., 2006) and *AtMtATP6* (Zhang et al., 2008) were overexpressed under various stresses. They suggested that increased expression of the MtATP6 must play a role in maintaining or intensifying the activity of the F_1F_0-ATP synthase under stress conditions (Zhang et al., 2008). Consistent with this suggestion, overexpression of the 6-kDa subunit gene (*RMtATP6* or *AtMtATP6*) in transgenic yeast, *Arabidopsis*, and tobacco increased the resistance to salts, drought, and oxidative and cold stresses (Zhang et al., 2006, 2008).

Of course, in a new study, we analyzed the expression of the MtATP6 under NaCl stress in wheat and wild wheat relatives: *Aegilops crassa* and *Triticum boeticum* (Moghadam AA, Ebrahimie E, Taghavi SM, Niazi A, et al., unpublished results). Results showed that this gene is overexpressed in the first few hours of salt stress and is involved in early responses to salt stress. Therefore, we concluded that the MtATP6 is probably involved in the regulation of early responses to salt stress.

In this research, we isolated the MtATP6 not only in wheat and rice but also in wild wheat relatives (NCBI accession Nos. HM173320 for *RMtATP6*; GQ503255 for *WMtATP6*; GU183146 for *AcMtATP6*, and GU183145, for *TmMtATP6* in rice, wheat, *A. crassa*, and *Triti-*

cum monococcum subsp *aegilopoides* or *T. boeticum*, respectively; Moghadam et al., 2009). We chose wild wheat relatives (Triticeae) because they represent a useful source for investigating genetic variation in abiotic stress tolerance (Farooq, 2009; Ramezani et al., 2012), and they are well adapted to dry environments (Nevo and Chen, 2010). Accordingly, *A. crassa* and *T. monococcum* of the Triticeae tribe are very interesting since gene transfer from this stress-adapted species to wheat is rather easy (Valkoun et al., 2004).

Thus far, however, little is known about the role of F_1F_0-ATP synthase in different environmental stresses, and the mechanism underlying its regulatory activity (Kinosita et al., 1998). The molecular mechanism of this enzyme is one of the most basic processes in biology and should therefore be properly understood (Agarwal, 2011). We hypothesized that the 6-kDa subunit, encoded by the nucleus, plays a role in the regulation of the function of mitochondrial F_1F_0-ATP synthase under environmental stresses. Most of the organelle gene-regulatory processes are under the control of the nucleus genome (Woodson and Chory, 2008).

On the basis of our hypothesis, the following 3 purposes were defined for this study: 1) to isolate the MtATP6 from rice, wheat, and the wild wheat relatives *A. crassa* and *T. boeticum*, as adapted genetic resources to abiotic stresses; 2) to characterize and analyze *in silico* a putative stress-inducible promoter for the MtATP6 in rice (*RMtATP6*) and *Arabidopsis* (*AtMtATP6*), which would enable the identification of specific *cis*-acting elements and thereby enable the prediction of the metabolic pathway that the 6-kDa subunit is involved in, and 3) to study *in silico* the cell localization and protein structure of the 6-kDa subunit in rice. Structure and localization analysis of the 6-kDa subunit will enable the elucidation of its location and, possibly, cellular activity.

MATERIAL AND METHODS

Plant materials

Cultivars of Iranian rice (*Oryza sativa indica* 'Neda'), Iranian wheat (*T. aestivum* 'Alamut'), and wheat wild relatives - *A. crassa*, and *T. boeticum* (*T. monococcum* subsp *aegilopoides*) - were used. Seeds were cultured in pots and grown under 16/8 photoperiod at 25°C in greenhouse prior to DNA and RNA extraction (3-4 leaves/stage).

DNA and RNA extraction

DNA and genomic DNA of abovementioned genotypes were extracted from seedlings with 3-4 leaves. Genomic DNA was extracted by the modified CTAB method (Knapp and Chandlee, 1996). Total RNA was extracted using the RNXTM (-Plus) reagent kit (Cinnagen, Tehran, Iran), according to manufacturer instructions. The quality of the extracted RNA and genomic DNA was verified by agarose gel electrophoresis. Then, the quantity and concentration of RNA and DNA were measured using a nanodrop set (Thermo Fisher Scientific, USA). Before use, DNA and RNA samples were stored at -80°C. The integrity and quantity of RNA were checked by visual observation of 28S and 18S rRNA bands on 1.2% agarose gel.

cDNA synthesis

cDNA from all treated samples was synthesized using the first-strand cDNA synthesis

kit (Fermentas, Germany) according to manufacturer instructions. DNA-free total RNA (1 µg) was reverse transcribed by using the oligo-dT primers (Fermentas). The cDNA samples were stored at -20°C before use.

Primer design

Primer for the MtATP6 was designed using the Vector NTI Suite 9 software. On the basis of the only available sequence in GenBank for Poaceae rice (Zhang et al., 2006; *RMtATP6*, GenBank accession No. AB055076) primers were designed as MtATP6-F1/MtATP6-R1 covering 5'-UTR up to 3'-UTR of rice *RMtATP6* mRNA, in order to amplify the full-length cDNA and the full-length genomic of *RMtATP6* in the rice cultivar Neda. Since the amplified genomic fragment was quite large (2379 bp), 2 new sets of primers, namely, MtATP6-F2/MtATP6-R2 and MtATP6-F3/MtATP6-R3, were designed to amplify this sequence as smaller products (Table 1).

A pair of specific primers was designed as MtATP6-F4/MtATP6-R4 on the basis of the *RMtATP6* coding DNA sequence (CDS) in rice in order to amplify and isolate MtATP6 CDS and its genomic sequence from wheat. Moreover, primer pairs of MtATP6-F5/MtATP6-R5 were designed according to the conserved CDS of rice *RMtATP6* to amplify and consequently isolate the partial CDS of orthologous *MtATP6* in *A. crassa* and *T. boeticum* (Table 1).

Table 1. Primers designed in this research for *MtATP6* using the Vector NTI Suite 9 software based on *AtMtATP6* (GenBank accession No. AK117680), *RMtATP6* (AB055076).

Primer name	Anneling temperature (°C)	Amplified fragment length (bp)	Forward and reverse sequences (5'→3')
MtATP6-F1	60	2379	ATCCGCTTCTTCCCCCTTCG
MtATP6-R1			ATACAACTGGAGCGGTTGTCCAC
MtATP6-F2	60	1652	GGTTCTTTCCGCTGTCCTG
MtATP6-R2			GCTTGATTTGATCATTGCAGTGC
MtATP6-F3	60	1077	TTGCTCCGTGATCAAGATAGCC
MtATP6-R3			GAGATAATACTCCCAGCCCCAG
MtATP6-F4	59	353	AGGAGGAGATCGAGGATGTT
MtATP6-R4			ATGCCCAGCTTAAAGCAGG
MtATP6-F5	62	145	CCGTGGCCGGTGTTCTTCC
MtATP6-R5			TGTGCGCCTGGACGAACTTG

PCR and cloning

For the PCR, 1 µL synthesized cDNA or 100 ng/µL genomic DNA was used as the template. The PCR was performed in a total volume of 20 µL containing the following: 1 µL of each primer to the final concentration of 10 µM, 2 µL 10X PCR buffer, 0.7 µL 50 mM $MgCl_2$, 0.5 µL 10 mM dNTPs, and 1 U Taq DNA polymerase (5 U/µL) (Cinnagen). The PCR program was carried out under the following conditions: the template was denatured at 94°C for 5 min, followed by 32 cycles of amplification, including denaturation at 94°C for 1 min, annealing (at proper temperature, as mentioned in Table 1) for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

After PCR using various primer pairs, amplified fragments were sub-cloned into vector pTZ57R/T (Fermentas), using the *Escherichia coli* strain DH5α as the host bacterium, as per manufacturer instructions. Plausible successful recombinant plasmids were extracted us-

ing a plasmid DNA extraction kit (Fermentas). DNA sequencing was carried out using M13 forward/reverse primers, with ABI 3730xl DNA sequencer of Macrogen Inc. (Korea). All obtained sequences in this study were submitted to the NCBI database (Moghadam et al., 2009). Sequence alignment and homology searches were performed using the BLAST-N search algorithm in GenBank at the NCBI database.

In silico functional analysis of *RMtATP6* and *AtMtATP6*

Promoter identification

To identify the promoter region of the MtATP6 in rice *RMtATP6* and *Arabidopsis AtMtATP6* (AK117680) and its paralogue *AtMtATP6* (NM_148152), deposited at the NCBI CDS of *MtATP6* were searched against the genomic data of rice and *Arabidopsis* by using the phytozome database (http://www.phytozome.net/). After identifying the gene on the chromosome using the BLAST-N algorithm, the region 2000 bp upstream of the transcriptional start point (ATG) of rice (*RMtATP6*) and *Arabidopsis* (*AtMtATP6* and its homologue *AtMtATP6*) 6-kDa subunit was considered the promoter.

Promoter motif analysis

The upstream region of the 6-kDa subunit encoding a genomic fragment was analyzed using the PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/) database. For this purpose, upstream region sequences of *RMtATP6* and 2 *AtMtATP6* paralogues were applied to predict their key *cis*-acting elements and the precise location of these elements.

The PLANTCARE promoter database focuses on *cis*-regulatory elements rather than the core promoter structure, aiming to reveal the regulatory mechanism that underlies the expression profiles (Lescot et al., 2002).

Protein analysis

The 6-kDa subunit has been identified as an F_0-proton subunit channel of mitochondrial F_1F_0-ATP synthase; however, little information is available on its structure (Agarwal, 2011). The analysis of protein properties would help understand the biochemical and physiological role of this subunit. Computational biology can extract a large number of protein properties for an amino acid sequence (Ebrahimi et al., 2011; Tahmasebi et al., 2012). Various properties of the 6-kDa subunit, such as subcellular localization, signal peptide prediction, topology prediction, secondary structure, domains/patterns/motifs prediction, and 3-D structure prediction, were analyzed using the protein databases.

The protein sequence (accession ID: BAB21526) was obtained by the conceptual translation of the *RMtATP6* open reading frame of the *O. s. japonica* genome (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). ProtParam was employed in order to perform primary sequence analysis (http://expasy.org/cgi-bin/protparam). The ProtParam computes properties, such as molecular weight, theoretical pI, instability index, and grand average of hydropathicity (GRAVY).

Moreover, similarity was assessed using different programs in NCBI, such as BLAST-P and PSI-BLAST (http://BLAST.ncbi.nlm.nih.gov/BLAST.cgi). Multiple sequence alignment was performed using Vector NTI Suit 9.

Subcellular localization

The subcellular localization and functional classification of the 6-kDa subunit were predicted using Utoronto (http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi), iPSORT, PRED-CLASS, Predotar (http://www.inra.fr/predotar/), and TargetP (Emanuelsson et al., 2007). iPSORT predicts the subcellular localization. PRED-CLASS uses cascading neural networks to classify proteins into various classes. Predotar is a prediction service for identifying putative *N*-terminal-targeting sequences. The TargetP algorithm identifies mitochondrial targeting pre-sequence of putative *N*-terminal-targeting sequences.

Signal peptide prediction

We used diverse pattern-searching applications to foretell the significant signal peptides in the 6-kDa subunit. Signal peptide prediction was achieved using PrediSi, Phobius, TargetP (http://www.cbs.dtu.dk/services/TargetP), and iPSORT.

PrediSi predicts signal peptide sequences and their cleavage sites on the basis of a position-weight matrix. Phobius is a combined transmembrane protein topology and signal peptide predictor that uses a well-trained hidden Markov model.

Topology prediction

The topology of the 6-kDa subunit was predicted using TSEG, TMpred, TMHMM, PHDhtm, HMMTOP, SPLIT, DAS, and TopPred II.

TSEG uses a discriminator function to predict the transmembrane segments. TMpred algorithm is based on the statistical analysis of transmembrane proteins and their helical membrane-spanning domains. TMHMM predicts transmembrane regions based on the hidden Markov model. PHDhtm uses a neural network to predict the locations of the transmembrane helices. HMMTOP 2.0 prediction is based on the hypothesis that the differences in the amino acid distribution in different structural parts define the localization of the transmembrane regions. SPLIT 4.0 predicts the location of transmembrane helices by performing an automatic selection of optimal amino acid attributes and corresponding preference functions. DAS uses dense alignment surface method to predict transmembrane regions. TopPred II predicts the topology of a protein on the basis of its hydrophobicity profile and positive-inside rule.

Secondary structure

The SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) consensus secondary structure server was employed to run the protein sequence against several different secondary structure prediction tools and generates a consensus secondary structure out of them.

Moreover, SOPMA and SMART were used to identify patterns and the presence of any domains in the 6-kDa subunit. In domain-search databases of ScanProsite and SMART, the presence of the domains has been screened by the multiple sequence alignments used to make the corresponding domain profile (Geer et al., 2002).

Three-dimensional structure prediction

Initial attempts to predict the tertiary structure of the 6-kDa subunit were made using different approaches like homology modeling. PDBsum (http://www.ebi.ac.uk/pdbsum/) provides a glance overview of every macromolecular structure deposited in the Protein Data Bank (PDB) and provides schematic diagrams of the molecules in each structure and of the interactions between them (Laskowski et al., 2005).

Protein-protein interaction network

Because of a well-defined protein-protein interaction network in *Arabidopsis*, the 6-kDa subunit (AtMtATP6, GenBank accession No. BAC42332) was employed. STRING 8.3 (http://string-db.org) was used to predict all the proteins that interact with the 6-kDa subunit. The interactions include direct (physical), and indirect (functional) associations.

RESULTS AND DISCUSSION

Isolation of full-length cDNA of *RMtATP6* from rice

In order to isolate *RMtATP6* from rice, PCR was performed using the primer pair MtATP6-F1/MtATP6-R1. After amplification, a 414-bp long fragment from cDNA and a 2379-bp long fragment from genomic DNA were identified. The difference may well indicate the presence of a large intron in the *RMtATP6* genomic sequence (Figure 2). Genomic and cDNA bands were subsequently cloned and sequenced.

Further, the 2379-bp band was sequenced in 2 stages. In the first stage, 800 bp was sequenced from both the fragment ends. Next, the entire 2379-bp fragment was sequenced using 2 other primer pairs, namely, MtATP6-F2/MtATP6-R2 and MtATP6-F3/MtATP6-R3, i.e., primer pairs 2 and 3, respectively (Table 1). Then, in the second stage, genomic DNA was used as the PCR template and amplified by the primer pairs 2 and 3. After sequencing, a 1652-bp band was formed by amplifying primer 2, and a 1077-bp band, from primer 3. Consistent with the results of Southern blotting in the study by Liu and Zhang (2004), sequencing and subsequent BLAST-N search showed that the 414-bp cDNA and 2379-bp fragment were most similar to the rice *RMtATP6* on the chromosome 3 locus of Os03g0606200. Finally, they were submitted with the GenBank accession Nos. GQ429012 and HM173320, respectively, in the NCBI database. Thus, we concluded that the *RMtATP6* gene is harbored by the nucleus and that its protein product is transferred inside the mitochondrial inner membrane and placed in the F_0 portion of the F_1F_0-ATP synthase enzyme (Zhang et al., 2006).

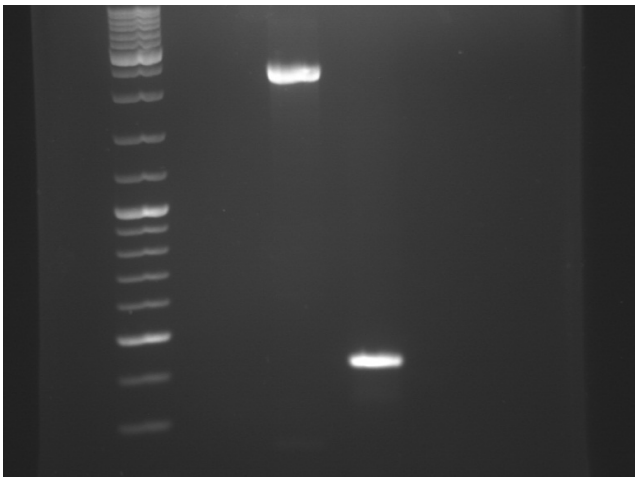

Figure 2. Ethidium bromide gel electrophoresis for *RMtATP6* amplified by MtATP6-F1/MtATP6-R1 primer pairs with annealing temperature of 60°C. From left to right: Ruler™ DNA Ladder Mix (Fermentas, Germany), PCR negative control (H_2O), genomic band 2379 bp, and cDNA band 414 bp, respectively.

Intron-exon boundary identification of rice gene *RMtATP6*

After isolation of full-length genomic *RMtATP6*, we compared the cDNA with the genomic DNA of the rice *RMtATP6* and identified the exon and the intron sequences (Figure 3). For the first time, our results showed that full-length genomic *RMtATP6* is 2379 bp and that it has 2 exons and 1 intron. The first exon is placed at 1-301, and the second exon is placed at 2267-2379 on the genomic sequence. In addition to this, a long intron is placed at 302-2266 within the 3'-UTR and had a length of 1965 bp. Analysis of the boundary points of intron-exon showed a splice donor site with the consensus GT at 302 after the first exon and the splice acceptor site with the consensus AG at 2266 before the second exon. These results demonstrate that the single intron is of the splicing GT/AG type; hence, it is the most frequent type of intron in the genome (Matlin et al., 2005).

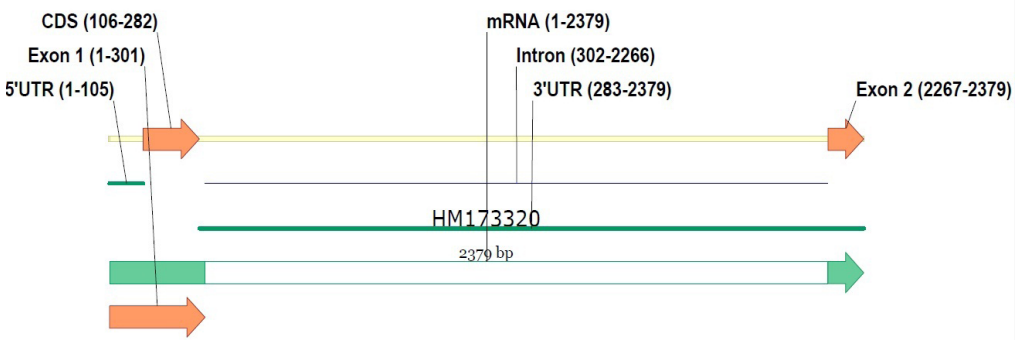

Figure 3. Intron-exon boundary identification of *RMtATP6* isolated from Iranian rice (*Oryza sativa indica* 'Neda') (*RMtATP6*, GenBank accession No. HM173320). As is shown, this gene has two small exons and a large intron inside of 3'-UTR region. Numbers in parentheses characterize the length of the region. Arrows and lines identify the gene regions.

Nucleotide comparison of *RMtATP6* and 2 *AtMtATP6* paralogues

We compared the length and sequence of introns, exons, and UTRs between rice *RMtATP6* and 2 *Arabidopsis AtMtATP6* paralogues. Genomic sequence analysis of rice *RMtATP6* showed that it contains 2 exons with lengths 301 and 113 bp and one intron of 1965 bp in length. However, *AtMtATP6* (AK117680) gene had 2 introns with lengths 162 and 481 bp and 3 exons with lengths 214, 43, and 337 bp.

Further, *AtMtATP6* (NM_148152) paralogue had 2 exons with lengths 47 and 121 bp and 1 intron with 183 bp in length. Although *AtMtATP6* and *RMtATP6* had different gene structures and were not from plants of the same family, the results showed that the CDS was intensively conserved between them (Figure 4). These results demonstrated that the 6-kDa subunit was conserved in all the plants and possibly plays a key role in the regulation of energy metabolism in plants under stresses.

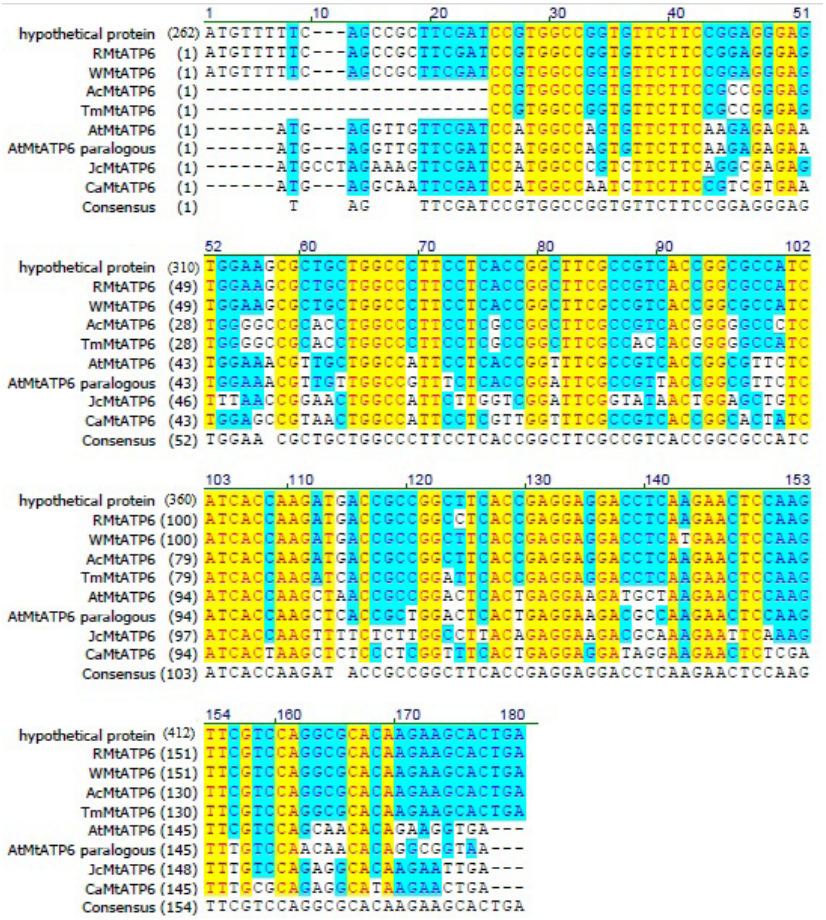

Figure 4. A multiple alignment of isolated *MtATP6* CDS sequence of Poaceae and comparison with another orthologous *MtATP6* that was performed by the vector NTI suit 9 software. The *MtATP6* CDS sequences showed highly conservative nucleotides, suggesting a key role for this protein in various plants.

Isolation of full-length CDS of the wheat 6-kDa subunit gene (*WMtATP6*)

In order to isolate the *WMtATP6* CDS, PCR was performed using the MtATP6-F4/RMtATP6-R4 primers. A specific band with 353 bp in length was amplified by using the genomic DNA of wheat. The sequenced fragments had most similarity with the *RMtATP6* gene of rice. Finally, the wheat amplification fragment was submitted to the NCBI database with the accession No. GQ503255. One CDS at 85-261 with a length of 177 bp was detected. These results corresponded with those reported by Zhang et al. (2006). Alignment results of rice and wheat CDSs (Figure 4) showed that the CDS region of *RMtATP6* is conserved to a great extent in wheat and rice.

Isolation of partial CDS of the 6-kDa subunit gene in *A. crassa* (*AcMtATP6*) and *T. monococcum* subsp *aegilopoides* (*TmMtATP6*)

Using the MtATP6-F6/MtATP6-R6 primer, we isolated a part of the CDS of *AcMtATP6* and *TmMtATP6* in *A. crassa* and *T. monococcum* subsp *aegilopoides* or *T. boeticum*, respectively. Primers were employed to amplify a 156-bp fragment by using genomic DNA and cDNA. These 2 fragments were submitted to the NCBI database with accession Nos. GU183146 (*AcMtATP6*) and GU183145 (*TmMtATP6*). In addition, these fragments had heist similarity with *RMtATP6* in GenBank analysis, indicating that the MtATP6 was largely conserved among different plants. Alignment of the 6-kDa subunit CDS sequences in the Poaceae family showed that it was highly conserved between the members of this family (Figure 4). Therefore, we concluded that the MtATP6 probably has a structure similar to that of this family. Further, *RMtATP6* CDS and *AtMtATP6* (Figure 4) showed a high percentage of similarity (77%). From these findings, we inferred that the 6-kDa subunit of the F_0 portion plays a key role in the energy metabolism of plants.

Promoter analysis

Identification of the 6-kDa subunit promoters in rice and Arabidopsis

Promoter analysis revealed 2 putative basic *cis* elements, namely, TATA and CAAT boxes, located upstream of the initiation ATG codon in *RMtATP6*, *AtMtATP6*, and *AtMtATP6* paralogues. A putative "TATA" box motif, "TATAAA", was found 389 bp upstream of the initiation codon ATG of the *RMtATP6* open reading frame. Further, a "TATA" box motif was found 195 bp (with TATATATA sequence) and 537 bp (with TATATA sequence) of the initiation codon ATG of *AtMtATP6* (AK117680) and its paralogue *AtMtATP6* (NM_148152), respectively. The *RMtATP6* promoter motifs along with 2 TATA and CAAT boxes are shown in Figure 5.

Several hormone- and environment-related cis-elements were predicted on RMtATP6 and AtMtATP6 promoter sequences

Using the PLANTCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), we analyzed the region 2000 bp upstream of ATG of *RMtATP6* (AB055076),

AtMtATP6 (AK117680), and its paralogue *AtMtATP6* (NM_148152) considering it as the promoter and predicted the key *cis*-acting elements and their location (Table 2). Thus, 3 promoters were shown to harbor multiple stress-related *cis*-acting elements (Table 2). As shown in Figure 5, a number of regulatory motifs that are potentially related to environmental signals were found in the *RMtATP6* promoter upstream of the 2 putative basic *cis* elements TATA (-389) and CAAT (-510) boxes.

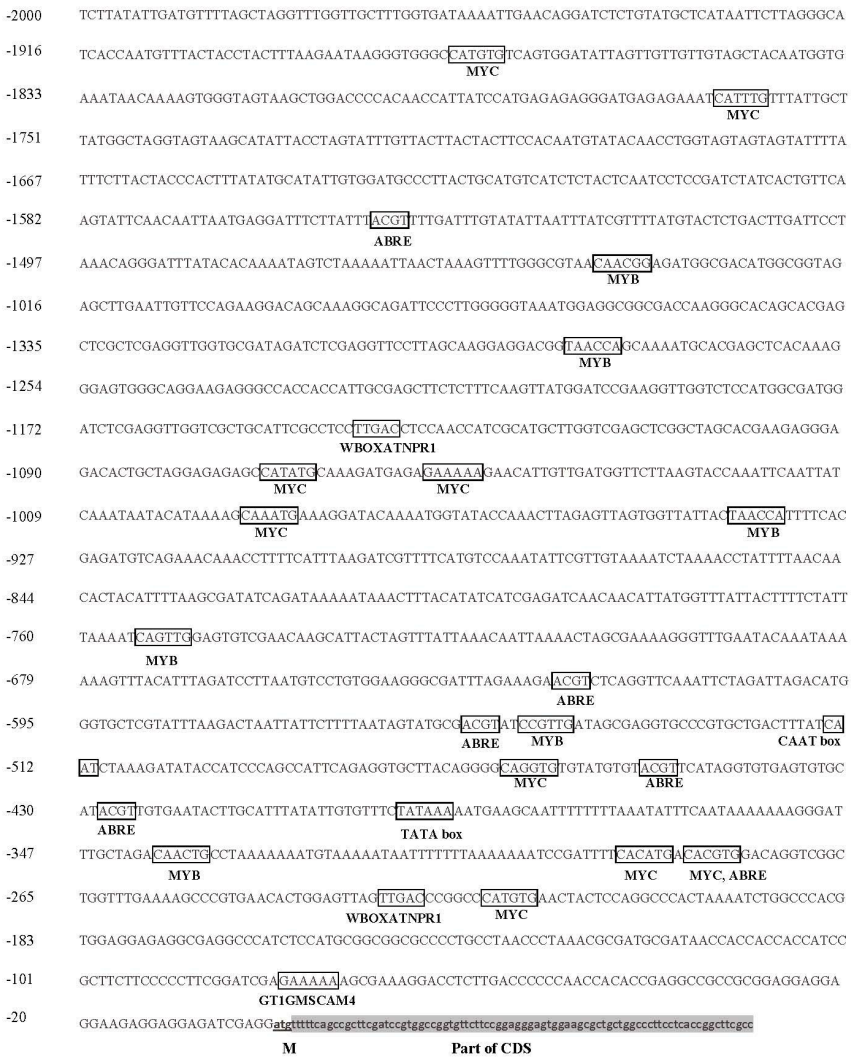

Figure 5. Sequence of the putative promoter region of the *RMtATP6* (HM173320) in rice. Gray sequence: part coding region of *RMtATP6*. The M (methionine) in the translation initiation codon (ATG) was designated as +1. The putative CAAT and TATA boxes are shaded and underlined. The ABRE-, MYB- and MYC-responsive elements that are potentially involved in stress responsiveness are shown shaded and boxed. Also salicylic acid (WBOXATNPR1) and pathogenesis and salt-related (GT1GMSCAM4)-responsive elements are boxed. The *cis*-acting elements are annotated beneath the figure.

Table 2. Prediction of specific and general *cis*-acting elements of the *RMtATP6*, *AtMtATP6*, and paralogue *AtMtATP6* promoters with the use of the PLANTCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/).

Element	Element core sequence	Element No.			Function
		RMtATP6	*AtMtATP6*	Paralogue *AtMtATP6*	
ABRE	ACGT	6	4	4	Response to ABA
IBOXCORE	GATAA	3	2	1	Response to light
GATABOX	GATA	9	10	7	Response to light
MYB	CNGTTR/WAACCA/YAACKG	6	4	7	Response to drought and ABA
MYC	CANNTG/CATGTG/CACATG	8	5	9	Response to ABA and cold
NTBBF1ARROB	ACTTTA	4	0	0	Response to auxin
GT1GMSCAM4	GAAAAA	2	5	3	Pathogenesis and salt-related
CURECORECR	GTAC	3	4	3	Copper and oxygen
WBOXHVISO1	TGAC	6	1	20	Response to sugar
WBOXATNPR1	TTGAC	3	0	3	Response to SA
TAAAGSTKST1	TAAAG	2	0	2	Regulate guard cell-specific gene
WBOXNTERF3	TGACY	2	0	9	Response to wound
ABRERATCAL	MACGYGB	3	0	0	Response to calcium ion
ARE	TGGTTT	2	0	2	Response to anaerobic induction
CAAT-box	CAAT	10	6	12	Enhancer regions
TATA-box	TATA	9	10	13	Element around -30 of transcription start

In the *RMtATP6* promoter, we found that the ABRE elements were involved in the response to abscisic acid (ABA); MYB-binding sites, in drought inducibility, and ABA signals and MYC-binding sites, in response to drought, ABA, and cold; NTBBF1ARROB elements, in response to auxin and light, and GT1GMSCAM4 motif, in response to pathogenesis and salt. The existence of the putative elements ABRE, MYB, MYC, and GT1GMSCAM4 suggested that the promoter region of *RMtATP6* might respond to several types of stresses, especially abiotic stresses, via a complex mechanism and might show vital its roles in biotic and abiotic tolerance especially early hours after stress.

Interestingly, these results confirm that the overexpression of *AtMtATP6* and *RMtATP6* in transgenic yeast and *Arabidopsis* plants (Zhang et al., 2006, 2008) or *JcMtATP6* in the salt-hypersensitive (shs-2) mutant of yeast (Eswaran et al., 2010) increases the resistance to salt, drought, oxidative, and cold stresses. Put together, these results suggest that the induction of F_1F_0-ATP synthase probably plays a role in stress tolerance (Soontharapirakkul et al., 2011). We postulated that this complex maintains the ATP levels of cells by electron-transfer chain in the stress conditions (Soontharapirakkul et al., 2011).

As previously mentioned, little is known about the expression response of F_1F_0-ATP synthase subunit genes against environmental stresses and the underlying regulatory mechanism. It appears that investigation of the upstream regulatory portion of the MtATP6 would contribute to the understanding of the molecular mechanisms involved in the regulation of energy metabolism under stress conditions.

Promoter analysis of these genes will enable the elucidation of the underlying molecular mechanisms involved in stress conditions. Studies showed that genes responsive to stresses contain regulatory *cis*-acting elements of ABRE and MYBRS/MYCRS (Figure 6) (Mahajan and Tuteja, 2005). Promoter analysis of *RMtATP6*, *AtMtATP6*, and paralogue *AtMtATP6* showed that they have ABRE and MYC/MYB elements that induced by ABA and environmental stresses (Mahajan and Tuteja, 2005), similar to *Arabidopsis NHX1* promoter (Shi and Zhu, 2002) and promoters of late-embryogenesis-abundant and *ROS* (Chinnusamy et al., 2005).

ABA is known to be an important phytohormone that plays a critical role in response to various stress signals. The application of ABA to plant mimics the effect of a stress condition. Since many kinds of abiotic stresses ultimately result in the desiccation of the cell and osmotic imbalance, there is an overlap in the expression pattern of stress-related genes after exposure to cold, drought, high salt levels, or ABA. This suggests that various stress signals and ABA share common elements in their signaling pathways and that these common elements cross talk with each other, to maintain cellular homeostasis (Shinozaki and Yamaguchi-Shinozaki, 2000).

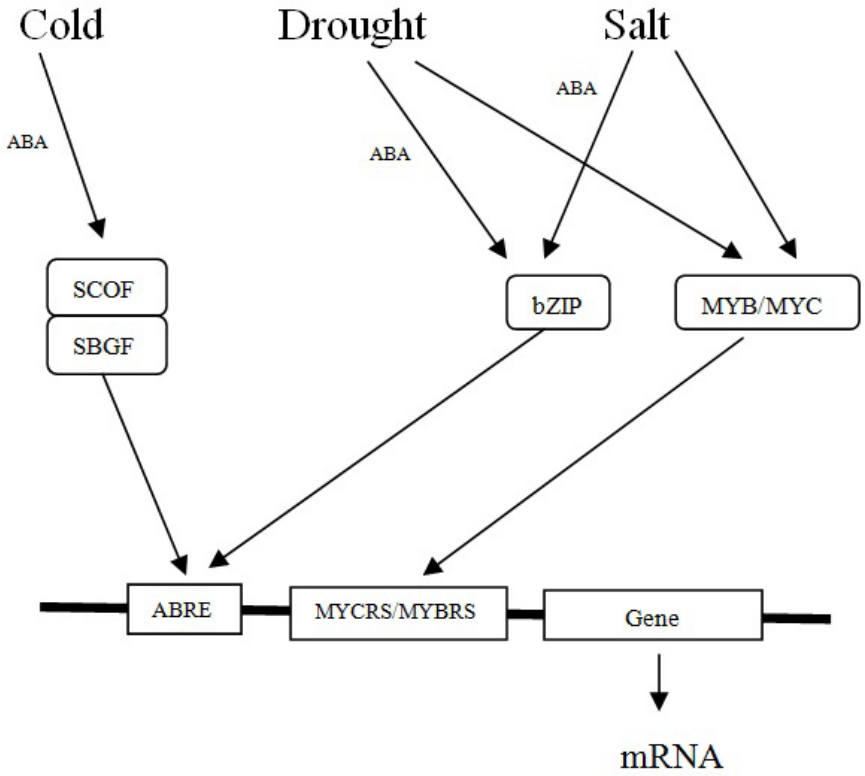

Figure 6. Different responsive transcription factors to stresses that apply their influence on stress genes via abscisic acid (ABA)-dependent and ABA-independent pathways. Osmotic stress signaling created by salinity and drought stress seems to be mediated by transcription factors such as ABRE, MYC and MYB and transcription activators, which interacts with ABRE or MYCRE/MYBRE elements in the promoter of stress genes (Mahajan and Tuteja, 2005).

The main function of ABA seems to be the regulation of water balance and osmotic stress tolerance in plants. ABA-induced gene expression often relies on the presence of the *cis*-acting element ABRE (Shinozaki and Yamaguchi-Shinozaki, 2000). The promoters of stress responsive genes have typical *cis*-regulatory elements, such as DRE/CRT, ABRE, and MYCRS/MYBRS, and are regulated by various upstream transcriptional factors. Since cold, salinity, and drought stress ultimately impair the osmotic equilibrium of the cell, these transcription factors as well as the major stress genes probably cross talk with each other for their maximal response and help in reinstating the normal physiology of the plant.

In salt and osmotic stress conditions, plants maintain high K^+ concentration and low Na^+ concentration within the cytosol, and both ionic and osmotic homeostasis must be restored, which is an energy-consuming process (Zhu, 2002). Because ATP energy is very essential for processes such as Na^+ efflux by Na^+-ATP synthase, Na^+-K^+/H^+ transport across the plasma membrane, and control of K^+ uptake and Na^+ flow by vacuolar V-ATP synthase (Srivastava et al., 2009; Soontharapirakkul et al., 2011), ATP is the current rate of energy in the cell and this ATP is provided chiefly by mitochondrial F_1F_0-ATP synthase (Srivastava et al., 2009; Soontharapirakkul et al., 2011).

Studies have shown that under ionic stress, both F_1F_0-ATP synthase and V-ATP synthase subunits are simultaneously overexpressed (Hamilton et al., 2001). While the functions of these enzymatic complexes are related with each other (Dietz et al., 2001), the function of V-ATP synthase under ionic stress depends on ATP production by F_1F_0-ATP synthase to maintain cell homeostasis (Dietz et al., 2001; Hamilton et al., 2001). The 6-kDa subunit possibly regulates the function of F_1F_0-ATP synthase under conditions of stress. The 6-kDa subunit could induce the expression of other subunits, thereby increasing the amount of F_1F_0-ATP synthase complex in the mitochondrial membrane (Dietz et al., 2001).

The F_1F_0-ATP synthase complex plays a central role in the ATP production, and the 6-kDa subunit of the F_0 portion (Zhang et al., 2006, 2008) may represent a functional subunit. On the basis of our conclusions, overexpression of *RMtATP6*, *AtMtATP6*, and *JcMtATP6* in transgenic cells will increase the F_1F_0-ATP synthase complex activity for producing more ATP under osmotic and salt stress, and therefore, high levels of ATP in the cell will maintain the K^+/Na^+ ratio and osmotic balance (Zhang et al., 2006, 2008; Eswaran et al., 2010; Soontharapirakkul et al., 2011). This clearly explains why stress-tolerant plants have higher yield than sensitive plants under salt and osmotic stresses (Kong et al., 2001). It appears that the 6-kDa subunit is involved not only in maintaining the function of F_1F_0-ATP synthase but also confers tolerance to environmental stresses. Taken together, it can be concluded that the 6-kDa subunit possibly acts as a regulatory factor and has a key role in the regulation of F_1F_0-ATP synthase under stress conditions.

Protein analysis

Primary sequence analysis

The conceptually translated the 6-kDa subunit is made of 58 amino acids with a predicted molecular weight of 6.9371 kDa and an isoelectric point of 10.01. Although the GRAVY index value (-0.297) shows that it is probably a soluble protein, previous experiments have shown that this protein is a hydrophilic F_0 subunit; however, its role is not clear at present (Zhang et al., 2006, 2008). All these results demonstrate that the 6-kDa subunit is a hydrophobic protein and may be located in the F_0 portion of F_1F_0-ATP synthase. This inference is consistent with experimental results described previously (Liu and Zhang, 2004).

Homology and similarity

The 6-kDa protein is highly conserved among the following plant species: rice (*RMtATP6*; GenBank accession No. BAB21526), *Arabidopsis* (AtMtATP6: BAC42332),

A. crassa (AcMtATP6: ACZ64509), *T. monococcum* (TmMtATP6: ACZ64508), wheat (WMtATP6: ACV90429), potato (PMtATP6: P80497), *J. curcas* (JcMtATP6: ACV50432), and *Capsicum annuum* (CaMtATP6: ACX53672) (Figure 7A).

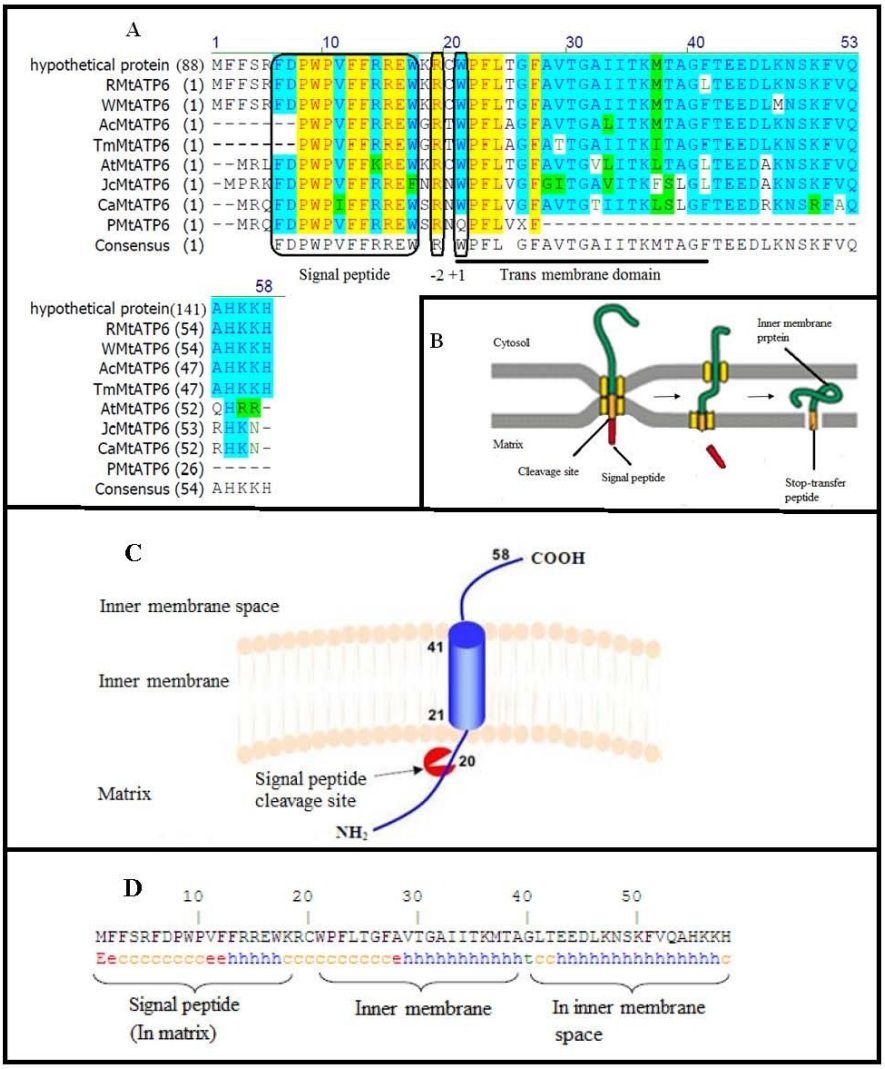

Figure 7. A. Multiple alignment of amino acid sequences of the 6-kDa subunit of F_0 part by vector NTI suit 9 was performed on the different plants listed above. The 6-kDa subunit amino acid sequence alignment showed highly conservative residues, suggested a key role for this protein in various plants. Signal peptide and trans-membrane domain were shown. The amino acid W boxed at position 21 is as protected removal site +1 and the conserved amino acid R boxed at position 19 is a putative consensus amino acid at position -2 of removal site. **B.** Protein transfer by stop-transfer mechanism to the mitochondrial inner-membrane. **C.** Predicted model for the 6-kDa subunit showing structural and probably functional features in the mitochondrial inner-membrane. **D.** Consensus secondary structure predictions for the 6-kDa subunit. One matrix segment, an inner-membrane part, and an inner-membrane space part are predicted.

As shown in Figure 7A, interestingly, highly conservative residues that potentially relate to the fundamental functions of the 6-kDa subunit were found in the different plant species. Another intriguing result was that when BLAST-P was performed by the 6-kDa subunit amino acid sequence, we found another hypothetical protein (145 amino acids) in rice (hypothetical protein, AAR87233), which was longer than the 6-kDa subunit (58 amino acids). The 6-kDa subunit amino acid sequence had 100% similarity with the *N*-terminal of the hypothetical protein (from amino acid 88-145). This finding seems to suggest that the 6-kDa subunit may be the result of the splicing of this hypothetical protein (Figure 7A).

Localization predictions

All the tools used to predict the cellular location of the 6-kDa subunit protein indicated that it is a putative mitochondrial transmembrane protein (Table 3). This prompted us to examine the sequence for the presence of the signal peptide and potential cleavage sites in the 6-kDa subunit sequence. Seven signal peptide prediction tools indicated the presence of a potential signal peptide in the 6-kDa subunit (Table 3; Figure 7A).

Table 3. Signal peptide, localization and signal peptide length prediction for the rice RMtATP6 (GenBank accession No. BAB21526) protein by several protein analysis databases.

Database	Signal peptide	Cell location	Signal peptide length (aa)	Cleavage position
Phobius	Present	No prediction	20	20\21
Predisi	Present	No prediction	32	32\33
SignalP	Present	No prediction	32	32\33
iPSORT	Present	Mitochondrion	30	No prediction
Inter pro scan	Present	No prediction	32	No prediction
Terminator3	Present	No prediction	20	20/21
TargetP	Present	Mitochondrion	30	20/21

Zhang et al. (2006) demonstrated that the 6-kDa subunit was associated with the entry of green fluorescent protein into the mitochondrion and that it was placed in the F_0 hydrophobic portion. If the final position of the protein is the mitochondrion, chloroplast, or secretary trade, it must contain a special signal peptide in the *N*-terminal end to recognize by translocase machine (Schatz and Dobberstein, 1996; Tahmasebi et al., 2012). Consistent with these reports, the majority of the programs predicted a mitochondrial signal peptide of length around 30 amino acids in the *N*-terminal end of the 6-kDa protein (Table 3).

Further, studies have also proven that mitochondrial signal peptides have a greater concentration of positive amino acids, such as Arg, Ala, and Ser, than negative amino acids, such as Asp and Glu, and are almost 20-80 amino acids in length (Gavel et al., 1988; Chacinska and Rehling, 2004). Similarly, the signal peptide of the 6-kDa subunit with length of 30 amino acids contains a greater extent of positive amino acids, such as Arg, and hydrophobic amino acids, such as Phe and Val. The existence of these amino acids explains the amphipathic structure of the protein (Figure 7A).

The majority of the programs also predicted an *N*-terminal cleavage site between the amino acids 20 and 21 (Table 3). Most of the protein initially enters the matrix and after the removal of the signal peptide, it is conducted to the inner membrane (Chacinska and Rehling, 2004).

Therefore, the existence of 2 signal peptides is predictable. However, one weak con-

sensus sequence was detected, by the presence of an Arg residue at -2 or -3 of removal site, which corresponds to the removal site of the mitochondrial signal peptidase (Gavel et al., 1988). Interestingly, we detected an Arg at the amino acid position 19, before the transmembrane and after the signal peptide (Figure 7A). This putative Arg is at -2 near the removal site (Gavel et al., 1988).

Thus, from our results, the signal peptide with a length of 30 amino acids was detected by databases and includes 2 signal peptides of lengths 20 and 10 amino acids. Corresponding to the stop-transfer mechanism, the first signal peptide was removed after the transfer of the 6-kDa subunit to matrix and the second hydrophobic signal peptide is a non-removal signal peptide that conducts the 6-kDa subunit within mitochondrial F_1F_0-ATP synthase (F_0 part) (Table 4; Figure 7B). We present a predicted model for the 6-kDa subunit showing its structural and functional features (Figure 7C).

Table 4. Subcellular location for the rice RMtATP6 (GenBank accession No. BAB21526) protein by using several protein analysis databases.

Database	Mitochondrion	Chloroplast	Endoplasmic reticulum
Predotar	Yes	No	No
TargetP	0.892	0.022	0.202
iPSORT	Yes	No	No
Utoronto	Yes	No	No

Topology predictions

The 6-kDa subunit amino acid sequence (*RMtATP6*) was analyzed using topology-prediction databases. The majority of the results showed a transmembrane around the amino acids 20-40 (Table 5). This transmembrane can pass throughout the inner mitochondrial membrane with the F_0 portion. Even though most programs fail to recognize the signal peptide, a consensus topology emerged (Table 3). The location of the *N*-terminal was predicted to be the mitochondrial matrix, while that of the *C*-terminal was predicted as the mitochondrial inner membrane space (Table 5; Figure 7C).

Table 5. Topology predictions for the rice RMtATP6 (GenBank accession No. BAB21526) protein by using several protein analysis databases.

Database	N-terminal	Trans-membrane position
TMHMM	IN	20-42
Inter pro scan	-	21-41
PHDhtm	-	25-37
HMMTOP	OUT	23-42
SMARTT		20-42
TSEG	-	20-41
DAS	-	23-37
TMpred	IN	20-39
TopPred	OUT	20-40
SMART	-	20-42
Phobius	-	21-41

2-D structure prediction and analysis

We searched for the presence of domains, patterns, and motifs in the 6-kDa subunit to gain insight into its function and structure. The SMART results showed the presence of the structural domain that we earlier identified using topology-prediction programs and signal peptide prediction programs. Further, the secondary structure predicted using the SOPMA program showed that the 6-kDa subunit includes 53.45% alpha helix and 36.21% random-coil structure (Figure 7D).

3-D structure prediction and analysis

Homology-based prediction of the tertiary structure of the 6-kDa subunit failed because of the lack of homologous structures. We used the PDBsum server to model the tertiary structure of the 6-kDa subunit. Visualization and analysis of the predicted structure showed that it is correlated with the bacteriophage T_4 ATP synthase (the ATP synthase that powers DNA packaging into bacteriophage T_4 procapsids) (Table 6). This enzyme includes 2 T_4 gp17 ATP synthase and terminase-6 domains. The 6-kDa protein transmembrane domain had 30% similarity with the amino acids 94-115 of the T_4 gp17 ATP synthase domain *N*-terminal. The amino-terminal domain of gp17 has an ATP-binding site with ATP synthase activity, whereas the carboxy-terminal domain has nuclease activity (Sun et al., 2007). The 6-kDa subunit transmembrane domain is probably an ATP-binding site that regulates ATP production levels.

In addition, the T_4 gp17 ATP synthase domain had been identified in the α and β subunits (PDB accession No. 1H8H) of bovine heart mitochondria F_1-ATP synthase (Abrahams et al., 1994) and PcrA helicase from *Bacillus stearothermophilus* (Subramanya et al., 1996). On the basis of these results, the plant mitochondrial F_0 6-kDa subunit can be considered as having originated from bacteria or bacteriophages (according to the endosymbiotic hypothesis). It is also possible that several 6-kDa subunits participate in the activities of the F_0 portion of the enzyme F_1F_0-ATP synthase, but the role of the 6-kDa subunit still remains unclear.

Table 6. Homology 3-D structure prediction and analysis of rice *RMtATP6* (GenBank accession No. BAB21526) by PDBsum server (http://www.ebi.ac.uk/pdbsum/).

PDB code	Model	Length	%-Tage	A.A. Overlap	Z-score	Ligand	Protein name
2o0h	(A) X-ray 1.88Å	357	30%	40	114.5	ATP	T_4 gp17 ATP synthase domain mutant complexed with ATP
2o0j	(A) X-ray 1.80Å	360	30%	40	114.4	ADP	T_4 gp17 ATP synthase domain mutant complexed with ADP

Protein-protein interaction network analysis

Using the STRING server, we searched for proteins interacting with the *Arabidopsis* 6-kDa subunit (AtMtATP6: BAC42332). The search revealed that this protein interacts with the following mitochondrial proteins: uncharacterized protein (locus name, At4g32470.2), ubiquinol-cytochrome-c reductase-like protein (putative uncharacterized protein At3g52730), cytochrome c_1 (AT5G40810.1), putative ubiquinol-cytochrome C-reductase complex ubiquinone-binding protein (QP-C) (At3g10860), ubiquinol-cytochrome C reductase complex ubiquinone-binding protein (At5g05370), ubiquinol-cytochrome C reductase-like protein

(At5g13430), probable mitochondrial-processing peptidase subunit alpha-1, cleaved presequences (transit peptidase, At1g51980), ubiquinol-cytochrome c_1 reductase cytochrome c_1-like protein (At3g27240), putative uncharacterized protein (At2g40765), ubiquinol cytochrome-c reductase (At2g40760), and annotation not available (ATMG00220.1). Further, interaction with the mitochondrial transit peptidase confirms that the *Arabidopsis* 6-kDa subunit has at least 1 removal signal peptide.

The 6-kDa subunit possibly correlates the F_0 function with the F_1 function in the ATP production level regulatory. On the basis of these predictions, we put forward a model for the 6-kDa subunit (Figure 7C). This model also prompted our hypothesis that the 6-kDa subunit might function as a novel regulatory subunit in the mitochondrial F_0 part. This model will be used to design and execute experiments to confirm the functions and topology of the 6-kDa subunit and further our understanding of its role in the tolerance of plants to stresses.

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