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RESEARCH

How the Nucleus and Mitochondria Communicate in Energy Production During Stress: Nuclear *MtATP6*, an Early-Stress Responsive Gene, Regulates the Mitochondrial F_1F_0-ATP Synthase Complex

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Abstract A small number of stress-responsive genes, such as those of the mitochondrial F_1F_0-ATP synthase complex, are encoded by both the nucleus and mitochondria. The regulatory mechanism of these joint products is mysterious. The expression of 6-kDa subunit (MtATP6), a relatively uncharacterized nucleus-encoded subunit of F_0 part, was measured during salinity stress in salt-tolerant and salt-sensitive cultivated wheat genotypes, as well as in the wild wheat genotypes, *Triticum* and *Aegilops* using qRT-PCR. The *MtATP6* expression was suddenly induced 3 h after NaCl treatment in all genotypes, indicating an early inducible stress-responsive behavior. Promoter analysis showed that the *MtATP6* promoter includes *cis*-acting elements such as ABRE, MYC, MYB, GTLs, and W-boxes, suggesting a role for this gene in abscisic acid-mediated signaling, energy metabolism, and stress response. It seems that 6-kDa subunit, as an early response gene and nuclear regulatory factor, translocates to mitochondria and completes the F_1F_0-ATP synthase complex to enhance ATP production and maintain ion homeostasis under stress conditions. These communications between nucleus and mitochondria are required for inducing mitochondrial responses to stress pathways. Dual targeting of 6-kDa subunit may comprise as a mean of inter-organelle communication and save energy for the cell. Interestingly, *MtATP6* showed higher and longer expression in the salt-tolerant wheat and the wild genotypes compared to the salt-sensitive genotype. Apparently, salt-sensitive genotypes have lower ATP production efficiency and weaker energy

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management than wild genotypes; a stress tolerance mechanism that has not been transferred to cultivated genotypes.

Keywords *MtATP6* · Salt stress · Organelle communication · Energy homeostasis · Wheat · qRT-PCR

Abbreviations

MtATP6	Mitochondrial F_1F_0-ATP synthase 6-kDa subunit
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
ABA	Abscisic acid
AREB/ABF	ABA-responsive element binding/ABA-responsive element binding factor
ABRE	Abscisic acid response element
MYC	Myelocytomatosis oncogene cellular homolog
MYB	Myeloblastosis viral oncogene homolog
W-boxes	WRKY-binding sites
C_T	Cycle threshold
HKT	High affinity potassium transporter
SOS	Salt overly sensitive
DRE	Dehydration-responsive element
GTLs	GT-element-binding proteins
CBF/DREB	C-repeat binding factor/Dehydration-responsive element binding protein
AP2	Apetala 2
ROS	Reactive oxygen species
AOX	Alternative oxidase
ABI4	Abscisic acid insensitive 4

Introduction

Abiotic stresses can decrease the yield potential to less than 50 % of that possible under typical growing conditions [4]. One of the most yield-threatening important stresses is salt stress [10]. About 20 % of the all cultivated land and nearly half of the irrigated land are affected by soil salinity [13]. Soil salinity is increasing, especially in arid and semiarid climates as example Iran, where 12.5 % of the agricultural lands are affected by soil salinity [2].

Wheat is the major crop among all grains in the world and is well adapted to the Mediterranean environments, where it often suffers salt stress due to increasing soil salinity [28]. Iran is one of the main regions where wild relatives of wheat evolved [35]. These species are valuable recourses for wheat improvement, distributed in various regions of central Asia [49], especially in arid and semiarid regions of Iran [2].

Extensive genetic studies of wild germplasm have indicated considerable variation in biotic and abiotic stress tolerance, but this valuable variation has been difficult to exploit due to the relatively poor understanding of the underlying molecular basis of stress tolerance in wild wheat species [26]. Another limitation is that their genomes have not been completely sequenced. Many stress tolerance mechanisms of the wild genotypes have not been transferred to the current cultivated genotypes. For example, Gorham et al. [20] tested several accessions of *Ae. crassa* and showed they have much lower Na^+ concentrations and higher K^+/Na^+ ratios in leaves than durum wheat, similar to bread wheat. They suggested that genome D imparts the Na^+ 'exclusion' and enhances the K^+/Na^+ discrimination in bread wheat.

Another stress tolerance mechanism in plants is the synthesis of compatible solutes such as sucrose, proline, and glycine betaine [22]. These compounds play vital roles in higher stress tolerance of the wild genotypes than cultivated genotypes. Compatible solutes synthesis mechanisms are associated with energy cost and therefore involve a potential growth penalty. For example, the ATP requirement for the synthesis or accumulation of solutes has been estimated as 3.5 for Na^+, 34 for mannitol, 41 for proline, 50 for glycine betaine, and 52 for sucrose [41].

Mitochondria are the unique and important organelles in terms of ATP (energy) production for the eukaryotic cell [6]. The mitochondrion is often one of the first recognition sites of stress within the cell, as its activities and responses are crucial in maintaining cell viability during these conditions [32]. The central role of the mitochondria in stress tolerance has been well-documented [37]. ATP, the universal biological energy currency, is synthesized by the mitochondrial F_1F_0-ATP synthase complex that is final complex of the electron transport chain [6]. This complex generally provides the required ATP for osmoticum synthesis under stress. Communications among the nucleus, mitochondria, and chloroplasts are essential in stress tolerance. In eukaryotic organisms, all of the subunits of the F_1 ($\alpha 3\beta 3\gamma\delta\varepsilon$) and a subset of the F_0 part are encoded by the nucleus, and only two or three of the hydrophobic components of the F_0 part are encoded by mitochondrial DNA [1].

It seems that MtATP6 subunit (mitochondrial F_1F_0-ATP synthase 6-kDa subunit) as a anterograde signal protein, encoding by nucleus is a subunit of the F_0 part of the plant mitochondrial F_1F_0-ATP synthase complex but also may be a subunit of the chloroplastic F_1F_0-ATP synthase complex, probably playing the same function in activating the complex in both organelles. In other words, 6-kDa subunit is a communicating protein between the nucleus and these two organelles in the regulation of energy metabolism of cell under stress.

Some evidences come from expression and transformation experiments. Hamilton et al. [21] reported that exposure of wheat (*T. aestivum*) to aluminum increases the mitochondrial F_1F_0-ATP synthase complex activity only in aluminum-tolerant genotype. Zhang and Liu [59] identified a salt-responsive gene (*RMtATP6*, NCBI accession no. AB05576) from rice that is a subunit of F_0F_1-ATP synthase. *MtATP6* from Arabidopsis (*AtMtATP6*, AK117680 and NM_148152), rice (*RMtATP6*, HM173320), wheat (*WMtATP6*, GQ503255), and *Ae. crassa* (*AcMtATP6*, GU183146) and *T. monococcum* ssp. *aegilopoides* (*T. boeoticum*) (*TmMtATP6*, GU183145) have been isolated [31, 33]. Overexpression of *AtMtATP6* and *RMtATP6* significantly enhanced the tolerance of transgenic Arabidopsis, transgenic tobacco and yeast under salt, cold, oxidative, and drought stresses [60, 61]. For example, functional evaluation of the mitochondrial F_1F_0-ATP synthase 6-kDa subunit (MtATP6) in salt-hypersensitive mutant yeast showed that it confers NaCl tolerance up to 1,000 mM NaCl [12]. The expression of the *ApNa*$^+$*-atp* operon (a putative F_1F_0-type Na^+-ATP synthase) of *Aphanothece halophytica* in a heterologous cyanobacterium *Synechococcus* sp. PCC_{7942} increases salt stress tolerance [46]. These findings demonstrate that 6-kDa subunit plays a vital role in energy homeostasis in response to wide range of environmental stresses.

In this study, a particular attention was paid to 6-kDa subunit, a nucleus-encoded subunit of the F_0 part of the plant mitochondrial F_1F_0-ATP synthase complex, whose role is not clearly understood in abiotic stress tolerance mechanisms [1]. The *MtATP6* expression in Mahuti (salt-tolerant) and Alamut (salt-sensitive) cultivated wheat genotypes, ($2n = 6x =$ AABBDD), in the wild wheat plants *T. boeoticum* ($2n = 2x =$ AA), and *Ae. crassa* ($2n = 4x =$ MMDD) was analyzed by real-time PCR under salt stress to investigate its possible biological role in salt tolerance in wheat. Another reason for using *Ae. crassa* and *T. boeoticum* was to examine and compare the activity of 6-kDa subunit in the A and D wheat genomes.

In addition, regarding the prominent role of promoter analysis in identifying the unknown function and underlying regulatory mechanism in silico promoter analysis carried out on *MtATP6* orthologous genes. The promoter of a drought-, high salinity-, and cold-inducible gene contains the major *cis*-acting elements, Abscisic acid response element (ABRE); Myelocytomatosis oncogene cellular homolog/Myeloblastosis viral oncogene homolog (MYB/MYC), and dehydration-responsive element (DRE), which are involved in stress-inducible gene response [55]. The existence of these elements can explain how *MtATP6* respond to several types of stresses, especially abiotic stresses, via an intricate mechanism. In addition, protein structure analysis is another strategy that can help to identify functions of 6-kDa subunit. In fact, another our hypothesis is that 6-kDa subunit may be a nuclear regulatory factor transferring to mitochondria.

With regards to the recent findings indicating a prominent role for 6-kDa subunit, the data presented here will provide further evidences of 6-kDa subunit contribution to environmental stress tolerance based on nuclear and mitochondrial communications.

Materials and Methods

Plant Materials and Growth Conditions

In this experiment, Mahuti (salt-tolerant) and Alamut (salt-sensitive) genotypes were used as cultivated wheat ($2n = 6x = 42$, AABBDD), and *T. boeoticum* ($2n = 2x = 14$, AA) and *Ae. crassa* ($2n = 4x = 28$, MMDD) were used as wild wheat relatives. Imbibed seeds were kept in the dark for 24 h at 4 °C and germinated for 3 days (d) at 25 °C. Then, germinated seeds were uniformly selected and transferred to the hydroponic culture medium. Seedlings were irrigated with a modified Hoagland solution with pH 6.8 [22]. A 16 h light/8 h photoperiod and 25 °C temperature were used in a stabilized greenhouse.

Stress Conditions

After plants reached to the "Zadoks code" 13 (3 leaves stage) (http://www.lethamshank.co.uk/gs.htm), salinity treatments, including control 0, 50, 100, and 200 mM NaCl, were used in combination with Hoagland solution. All solutions contained $CaCl_2$ to maintain the Na^+:Ca^{2+} ratio below 10:1. Sampling was carried out after 0, 3, 6, 10, 24, and 72 h from leaves of all treatments.

RNA Extraction

Total RNA was extracted from leaf samples using RNX-Plus buffer (Cinnagen, Tehran, Iran). Briefly, about 100 mg of leaf was grounded in liquid nitrogen. The ground powder was transferred to 1 ml of RNX-Plus buffer in an RNase-free microtube, mixed thoroughly and left at room temperature for 5 min. Chloroform (0.2 ml) was added to the slurry, and mixed gently. The mixture was centrifuged at 10,000×*g* at 4 °C for 15 min, and the supernatant was transferred to a new tube and precipitated with an equal volume of isopropanol for 15 min on ice. The RNA pellet was washed using 75 % ethanol, briefly dried, and resuspended in 50 µl of RNase-free water. The integrity and quantity of RNA was checked by visual observation of 28S and 18S rRNA bands on a 1.2 % agarose gel. Then, the quantity and concentration of RNA was

measured using a NanoDrop ND 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Prior to use, RNA samples were stored at −80 °C.

DNase Treatment and cDNA Synthesis

DNase treatment was carried out using a Fermentas DNase Kit (Fermentas, Leon-Rot, Germany) according to the manufacturer's instructions. Again, the integrity of RNA was checked by visual observation of 28S and 18S rRNA bands on a 1.2 % agarose gel. Then, 1 μg of DNase-treated RNA was used for first-strand cDNA synthesis using 100 pmol oligo-dT (18-mer), 15 pmol dNTPs, 20 U RNase inhibitor and 200 U M-Mulv reverse transcriptase (all from Fermentas) in a 20 μl final volume. The cDNA samples were stored at −20 °C prior to use.

Primer Design

Primers were designed using Allele ID 7 and Vector NTI 10 software for the reference genes and *MtATP6* (Table 1). *MtATP6* primers were designed based on the aligned nucleotide file, including AB055076 [60], HM173320, GQ503255, GU183145, and GU183146 [33]. The wheat elongation factor-1α (*ef1α*) (M90077) and beta tubulin (*β-tubulin*) (U76745) genes were used as the reference genes for data normalization because salt stress does not influence their expression [24]. In this project in order to higher precise, we quantified the final expressions based on the means of two reference genes of ef1α and β-tubulin.

qRT-PCR Analysis

First, primer specificity was confirmed by PCR and sequence analysis. To minimize pipetting error, the cDNA samples were diluted 1:10 by using nuclease-free water, and 5 μl cDNA was used for qRT-PCR.

Relative qRT-PCR was performed in a 20 μl volume containing 5 μl cDNA (diluted), 10 μl 2× Sybr Green buffer, 0.5 μl of 10 μM primers (forward and reverse), and 0.12 U of *Taq* DNA polymerase (5 U/μl) (all from Bioer, China). The amplification reactions were carried out in a Line-gene K thermal cycler (Bioer, China) under the following conditions: 2 min at 94 °C, 40 cycles of 94 °C for 10 s, 57 °C for 15 s, and 72 °C for 30 s. After 40 cycles, the specificity of the amplifications was tested by melting curve analysis by heating from 50 to 95 °C. All amplification reactions were repeated two times under identical conditions and included a negative control and 5 standard samples. To ensure that the PCR products were generated from cDNA and not genomic DNA, proper control reactions were carried out without reverse transcriptase treatment.

Statistical Data Analysis

For qRT-PCR data, the relative expression of *MtATP6* was calculated based on the threshold cycle (C_T) method. The C_T for each sample was calculated using the Line-gene K software and the Larionov et al. [27] method. When replicate PCRs are run on the same sample, it is more appropriate to average C_T data before performing the $2_T^{-\Delta\Delta C}$ calculation [24].

The determined mean C_T values for both the target (*MtATP6*) and internal control (elongation factor-1α and β-tubulin) genes were used in equation $2_T^{-\Delta\Delta C} = (C_{T,\ MtATP6} - C_{T,\ \text{housekeeping genes}})_{\text{Time } x} - (C_{T,\ MtATP6} - C_{T,\ \text{housekeeping genes}})_{\text{Time } 0}$. Time *x* represents the expression of the *MtATP6* any time point after salt stress and Time 0 represents the expression of the *MtATP6* before salt stress. The fold change ratios of *MtATP6* were normalized to internal control genes and were calculated relative to the expression at time zero.

Finally, statistical analysis was carried out from the replicate samples at each time point using MINITAB 14 (Minitab, Inc., Pennsylvania, USA) and SAS6.12 (SAS institute Inc., Cary, NC, USA). Analysis of variance was employed to investigate significant differences in the *MtATP6* expression profile under different NaCl concentrations (0, 50, 100, and 200 mM) and different times (0, 3, 6, 10, 24, 72 h) after NaCl treatment. Mean comparisons were carried out by Duncan's multiple range test in SAS.

Table 1 Sequences of primers used for qRT-PCR amplification and the resulting product sizes

Primer (bp)	Sequence	Amplicon length (bp)	T_a (°C)
MtATP6-F	CCGTGGCCGGTGTTCTTCC	145	59
MtATP6-R	TGTGCGCCTGGACGAACTTG		
Ef1α-F1	TTTCACTCTTGGAGTGAAGCAGAT	103	59
Ef1α-R1	GACCTCCTTGACAATTTCTTCATAA		
*Ef1α-F*2	GGTTAAGATGATTCCCACCAAGCC	112	59
*Ef1α-R*2	GACAACACCAACAGCAACAGTCTG		
β-Tubulin-F	TGTGGCAACCAGATCGGTGC	211	60
β-Tubulin-R	CATAAGGCCCAGTGCGGACAC		

The wheat elongation factor-1α (ef1α) (M90077) and beta tubulin (β-tubulin) (U76745) genes were used as the reference genes for data normalization

T_a temperature annealing, *F* Forward, *R* Reverse

Pearson's correlation coefficient was used to quantify the relationship between gene expression levels under different NaCl concentrations and different times after NaCl treatment. A paired *t* test was used to determine the significance of differences in the gene expression profiles between wheat genotypes.

Promoter Identification and Promoter Analysis

To identify the promoter region of the 6-kDa subunit gene in rice (*RMtATP6*, AB05576) and Arabidopsis (*AtMtATP6,* AK1176809) and its paralogous genes (*AtMtATP6*, AK117680, and NM_148152), CDS (coding sequence) of *MtATP6* were blasted in the Phytozome database (http://www.phytozome.net/). Then, the 2000 bp upstream of the transcriptional start point of rice (*RMtATP6*) and Arabidopsis (*AtMtATP6* homologous genes) 6-kDa subunit were considered as promoters [33] (Supplementary data 2).

The obtained 2000 bp upstream region of the *MtATP6* CDS was analyzed using the PlantCARE database [29]. This database focuses on *cis*-acting elements, aiming to identify the regulatory mechanism that determines the expression profiles [29].

Results

The Quantitative Expression Patterns of *MtATP6* in Wheat Genotypes Under Different Salt Concentrations

The differences in the *MtATP6* expression profile at different times after NaCl treatment in each wheat genotype were investigated (Fig. 1). A sudden significant increase ($P \leq 0.05$) in *MtATP6* transcript 3 h after NaCl treatment happened in all genotypes except salt-sensitive genotype Alamut (Fig. 1). Seventy-two hours (72 h) after NaCl treatment, *MtATP6* expression in *Ae. crassa* was significantly higher compared to Mahuti and Alamut (Table 2 part A), but *T. boeoticum* was not different from Mahuti or Alamut (Table 2 part A). There was a sudden decrease in *MtATP6* 72 h after NaCl treatment in all of the genotypes (Table 2 part A; Fig. 1). Expression analysis showed significant differences between all four genotypes at each of the 50, 100, and 200 mM NaCl concentrations (Table 2 part B; Fig. 1). There was a significant difference in *MtATP6* level between 72 h and every other time point at 200 mM (Table 2 part B). Finally, there was a significant difference between the *MtATP6* expression pattern at 10 and 24 h compared to 72 h at 50 mM (Table 2 part B; Fig. 1).

Investigation of Differences in *MtATP6* Expression Profile Under NaCl Concentrations in Each Wheat Genotype

Analysis of variance showed significant differences between *MtATP6* expression in *Ae. crassa* and Alamut under different NaCl concentrations. *Ae. crassa* had significantly higher expression of *MtATP6* in 100 and 200 mM NaCl compared to 50 mM, while *T. boeoticum* and Mahuti did not have significantly higher *MtATP6* expression in 100 mM NaCl compared to 50 or 200 mM (Table 3). In contrast, Alamut had significantly higher expression of *MtATP6* in 50 mM NaCl compared to 100 and 200 mM. In all NaCl concentrations, in general, wild genotypes had greater *MtATP6* expression than cultivated genotypes. In all the NaCl concentrations, *Ae. crassa* had

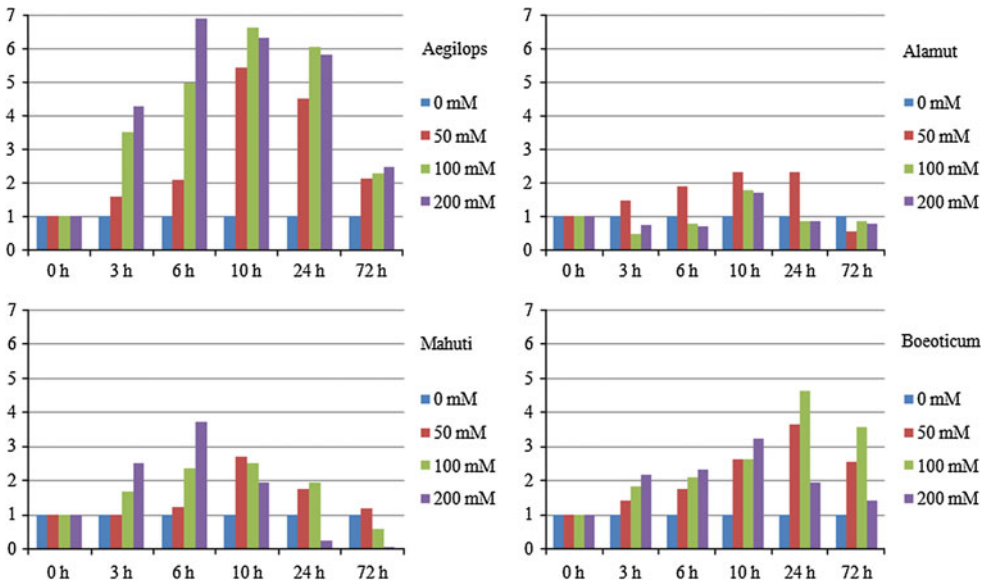

Fig. 1 *MtATP6* expression pattern under different NaCl concentrations (control, 50, 100, and 200 mM NaCl) and different times after NaCl treatment (0, 3, 6, 10, 24, 72 h) in Mahuti (salt-tolerant) and Alamut (salt-sensitive) genotypes as cultivated wheat ($2n = 6x = 42$, AABBDD), and *T. boeoticum* ($2n = 2x = 14$, AA) and *Ae. crassa* ($2n = 4x = 28$, MMDD) as wild wheat relatives. The *x*-axis shows time after NaCl treatment and *y*-axis shows the *MtATP6* relative expression

Table 2 Duncan's Multiple Range Test ($P \leq 0.05$) for investigation of differences in *MtATP6* expression profile under different times after NaCl treatments in Mahuti (salt-tolerant) and Alamut (salt-sensitive) genotypes as cultivated wheat ($2n = 6x = 42$, AABBDD), and *T. boeoticum* ($2n = 2x = 14$, AA) and *Ae. crassa* ($2n = 4x = 28$, MMDD) as wild wheat relatives (Part A) and under different NaCl concentration (Part B)

Part A				
Time (h) after NaCl treatment	Genotypes			
	Mahuti	Alamut	*T. boeoticum*	*Ae. crassa*
3	1.712^{ab}	0.882^{b}	1.319^{a}	3.112^{bc}
6	2.416^{a}	1.111^{b}	1.508^{a}	4.637^{ab}
10	2.372^{a}	1.920^{a}	2.299^{a}	6.127^{a}
24	1.309^{ab}	1.334^{ab}	3.038^{a}	5.445^{a}
72	0.583^{b}	0.720^{b}	2.297^{a}	2.290^{c}

Part B			
Time (h) after NaCl treatment	Different NaCl concentration (mM)		
	50	100	200
3	1.346^{b}	1.856^{ab}	2.068^{a}
6	1.726^{b}	2.539^{ab}	2.985^{a}
10	3.254^{a}	3.371^{a}	2.913^{a}
24	3.042^{a}	3.361^{a}	1.941^{a}
72	1.595^{b}	1.808^{b}	1.010^{b}

Means with the same letter are not significantly different. Significance levels are presented vertically

Table 3 Duncan's Multiple Range Test for investigation of differences in *MtATP6* expression profile under different NaCl concentration in each wheat cultivar ($P \leq 0.05$)

NaCl (mM)	Mahuti	Alamut	*T. boeoticum*	*Ae. crassa*
50	1.55^{a}	1.695^{a}	2.383^{a}	3.143^{b}
100	1.796^{a}	0.938^{b}	2.939^{a}	4.675^{a}
200	1.690^{a}	0.946^{b}	2.194^{a}	5.150^{a}

Means with the same letter are not significantly different. Significance levels are presented vertically

the highest *MtATP6* expression and Alamut the lowest (Table 3; Fig. 1).

Analysis of Variance for the Differences in *MtATP6* Expression in Wheat Genotypes Under NaCl Concentrations

We observed significant differences ($P \leq 0.05$) in *MtATP6* expression level between all four genotypes at 50, 100, and 200 mM NaCl. The *MtATP6* expression level of *Ae. crassa* was significantly different from every other genotype at 100 and 200 mM. There was no significant difference between Mahuti and Alamut at any NaCl concentration.

Table 4 Duncan's Multiple Range Test for investigation of differences in *MtATP6* expression profile between different genotypes under different NaCl concentration ($P \leq 0.05$)

Genotypes	NaCl (mM)		
	50	100	200
Ae. crassa	3.141^{a}	4.675^{a}	5.150^{a}
T. boeoticum	2.383^{ab}	2.939^{b}	1.194^{b}
Mahuti	1.695^{b}	1.796^{bc}	1.690^{b}
Alamut	1.550^{b}	0.938^{c}	0.946^{b}

Means with the same letter are not significantly different. Significance levels are vertically

Nor was there a significant difference between *Ae. crassa* and *T. boeoticum* at 50 mM (Table 4; Fig. 1). Thus, it seems that *Ae. crassa*, due to the presence of genome D, shows a different expression pattern of *MtATP6* than other genotypes.

Comparison of the *MtATP6* Expression Profile in Different Wheat Genotypes

The comparison of the *MtATP6* expression profiles in different wheat genotypes using the paired *t* test showed significant ($P \leq 0.05$) differences between *T. boeoticum* and Mahuti, *Ae. crassa* and Mahuti, *T. boeoticum* and Alamut, *Ae. crassa* and Alamut, and *Ae. crassa* and *T. boeoticum* (Table 5). The two cultivated genotypes (Alamut and Mahuti) were not significantly different. *MtATP6* showed the greatest expression fold change (3.67, $P \leq 0.01$) between *Ae. crassa* and Alamut (salt-sensitive cultivar). *MtATP6* also showed a greater expression fold change in *T. boeoticum* than the cultivated genotypes (Table 5; Fig. 1). All together, it seems that longer-term *MtATP6* expression is one of the reasons for the superiority of wild relatives. It is possible that the mechanism of energy management of wild genotypes have not been fully transferred to cultivated genotypes.

Table 5 Paired *t* test compares *MtATP6* expression mean between different wheat genotypes

Paired *t* test	Mean	Fold change
Alamut:Mahuti	1.7:1.2	1.41 (0.05)
T. boeoticum:Mahuti	2.5:1.7	1.47 (0.05)
Ae. crassa:Mahuti	4.4:1.7	2.59 (0.001)
Ae. crassa:Alamut	4.4:1.2	3.67 (0.001)
T. boeoticum:Alamut	2.5:1.2	2.08 (0.001)
T. boeoticum:*Ae. crassa*	2.5:4.4	1.76 (0.001)

The *P* values reported in the parenthesis represents the statistically significance ($P \leq 0.05$)

Relationships Between *MtATP6* Expression in Different NaCl Concentrations

The *MtATP6* expression levels in different NaCl concentrations were compared using Pearson's correlation coefficient. There was a significant ($P \leq 0.05$) positive correlation in gene expression profile between 50 and 100 mM in *T. boeoticum* ($P = 0.01$) and between 100 and 200 mM NaCl in Alamut ($P \leq 0.01$). In contrast, there was no significant relationship between gene the expression profiles of Mahuti and *Ae. crassa* at any NaCl concentration.

Correlations Between the Expression Levels of *MtATP6* at Different Times After NaCl Treatment

In Alamut, significant correlations were observed between *MtATP6* expression at 3 and 72 h ($P \leq 0.01$), 6 and 10 h ($P \leq 0.01$), and 6 and 24 h after NaCl treatment ($P \leq 0.05$). Similar results were obtained between 3 and 72 h ($P \leq 0.05$) and 6 and 24 h ($P \leq 0.05$) in *T. boeoticum*. However, there were no temporal correlations in Mahuti or *Ae. crassa* (Fig. 1). It seems that *MtATP6* expression profiles of Mahuti and *Ae. crassa* (salt-tolerant genotypes) are similar over time (Fig. 1).

Comparison of the *MtATP6* Expression Fold Changes Before and After Salt Stress in Each Genotype

The *MtATP6* expression fold changes before and after salt stress in each genotype was compared by using the paired *t* test. There were significant ($P \leq 0.05$) differences between 0 and 100 mM and between 0 and 200 mM in *Ae. crassa* only; *MtATP6* expression did not change significantly in Mahuti, Alamut or *T. boeoticum* after salt stress compared to before. Finally, the wild genotypes had greater expression of *MtATP6* than the cultivated genotypes after stress (Table 6), while the expression of *MtATP6* in wild genotypes (especially *Ae. crassa*) was lower than in the cultivated genotypes before stress (Table 6).

The *MtATP6* expression profile at different times before NaCl treatment in each wheat genotype showed significant ($P \leq 0.05$) differences between the four genotypes (Table 6). Additionally, there was a significant difference between the *MtATP6* levels of *T. boeoticum* and those of other genotypes before NaCl treatment.

In Silico Promoter Analysis and Protein Structure Prediction

The *MtATP6* promoter analysis showed the existence of the elements MYB/MYC, W-boxes, ABRE, and GTLs (Fig. 2). This suggested that the 6-kDa subunit might induce under different stress conditions (Fig. 2) (Supplementary data 2) (Table 7). A mitochondrial signal peptide, an N-terminal cleavage site, phosphorylation sites, and a transmembrane domain were founded in *RMtATP6* [33].

Discussion

The central role of mitochondria in the adaptation to environmental stresses at the subcellular level has become evident [28]. The electron transport chain in the plant mitochondrion is one of the aspects underlying the so-called energetic flexibility [9]. The mitochondrial F_1F_0-ATP synthase is the terminal complex of the electron transport chain that catalyzes the final step of ATP production in the cell [6]. One of the newly found subunits of the mitochondrial F_1F_0-ATP synthase is *MtATP6*, whose overexpression in transgenic plants and yeast confers a considerable resistance to various abiotic stresses [12, 60, 61]. However, its role in response to environmental stresses is still unclear. More importantly, it is unknown which role the nucleus plays in the energy production that occurs through the F_1F_0-ATP synthase complex in mitochondria. Additionally, it will be interesting to know whether this salt tolerance pathway based on energy management confers a selective advantage to wild genotypes.

Table 6 Two sample *t* test compares *MtATP6* expression fold change before stress and after stress

Genotypes	Before stress (mean)	Fold change before stress and after stress		
		50 mM:0 mM	100 mM:0 mM	200 mM:0 mM
Mahuti	1.47097[a]	1.25 (0.567)	1.2 (0.377)	0.98 (0.966)
Alamut	1.40017[ab]	2.75 (0.185)	1.07 (0.889)	1.2 (0.720)
Ae. crassa	1.33853[b]	2.97 (0.059)	4.42 (0.008)	4.86 (0.004)
T. boeoticum	1.02187[c]	1.73 (0.311)	2.03 (0.214)	1.62 (0.414)

Means with the same letter are not significantly different. Significance levels are vertically. Duncan's Multiple Range Test for investigation of differences in *MtATP6* expression profile under different times before NaCl treatment in each wheat cultivar ($P \leq 0.05$). The *P* values reported in the parenthesis represents the statistically significance

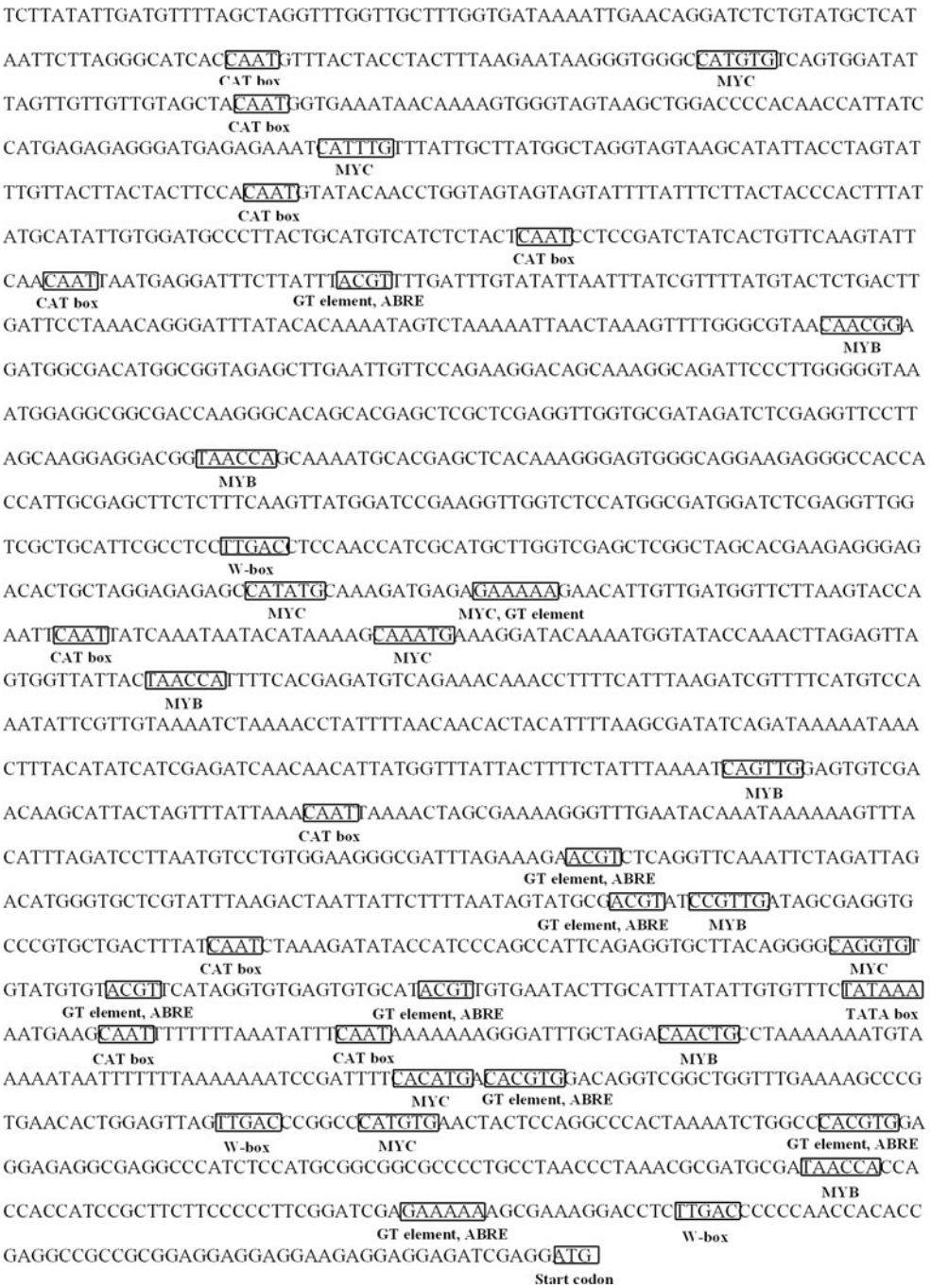

Fig. 2 The sequence (2000 bp) of the putative promoter region of the *RMtATP6* (HM173320) in rice; the ATG in the translation initiation codon (Adenine) was regarded as +1. The putative CAAT and TATA boxes are *shaded* and *underlined*. The ABRE, MYB, MYC, GTLs, and W-boxes as early responsive elements that are potentially involved in stress responsiveness are shown, *shaded*, and *boxed*. Based our hypothesis, the ABA hormone correlates the mitochondria and nucleus communications based on activation of ABA responsive elements of *RMtATP6* promoter

We presumed that *MtATP6*, as a nuclear regulatory element, regulates mitochondrial F_1F_0-ATP synthase activity to produce more ATP, especially in stress-tolerant wheat genotypes and in particular in *Aegilops* genotype DD, when the cell needs additional energy to deal with an abiotic stress. Stress conditions have a great impact on the cellular ATP levels. Ensuing energy deficiency signal triggers downstream transcriptional responses that are shared by different types of stresses [3]. In fact, energy support can be seen as a crosstalk between different stresses system responses this can happen because of activation of other resistance mechanisms in wheat to deal with salinity or because sudden salt stress shock increases in the expression of other salt-responsive genes, for example, metallothionein-like protein [8, 41], *HKT* (high affinity potassium transporter) [58], *SOS1* (salt overly sensitive), and *SOS4* [40] are observed after salt treatment; therefore, the first response (increased ATP production) seems to be stronger. At first it may be argued that *Ae. crassa*, *T. boeoticum*, and *T. aestivum* have different-sized

Table 7 Summary of key *cis*-acting elements, transcription factors (TF), target genes, and functions in stress-responsive promoters

Cis-acting	Sequence	TF	Target genes	Function	References
MYB	(C/T)AAC(C/T)(G/A)	MYB2-4	P5CS1, ADH1, RD22	Regulating of drought inducible gene expression	[14, 45]
MYC	CATGTG; CACATG; CANNTG	MYC2	P5CS1, ADH1, RD22, WRKYs	Involving in early response to drought and ABA induction	[14, 36, 55]
GT	GGTAATT	GT-1-3	PhyA, CHS, ATP synthase, Rubisco, DREB2A, ABI4	Requiring for rapid response to pathogen attack, salinity and salicylic acid inducible gene expression	[14, 33, 36, 54–56]
ABRE	(T/C)ACGTG(T/G)	ABF1-4	RD29A, RD29B, KAT1, KAT2, CHS, RBCS	Involving in ABA responsiveness	[14, 55]
W-box	TTGAC	WRKYs	PR1, ABF2, ABF4, ABI4, ABI5, MYB2, DREB1a, DREB2a and RD29A and COR47, ABI5, ABI3, ABA2 and ABA3	Involving in pathogen, salt ABA-responsive signaling pathways	[14, 36, 48, 55]

ADH alcohol dehydrogenase; *P5CS* pyrroline-5-carboxylate synthetase; *RD* related to dehydration; *WRKYs* tryptophan, arginine, lysine, tyrosine; *PhyA* phytochrome A; *CHS* chalcone synthase; *DREB* dehydration-responsive element binding; *KAT* 3-ketoacyl-CoA thiolase; *PR1* pathogen related; *ABF* ABA-responsive element binding factor; *COR* cold regulated

genomes and that the absence of 66 % of the genome of *T. aestivum* in *Ae. crassa* and *T. boeoticum* would predict a lower level of tissue expression of *MtATP6*. Our findings of *MtATP6* expression in wild genotypes contradict this expectation. The high expression level of *MtATP6* in wild genotypes may indicate that *MtATP6* is an important mechanism for salt tolerance in the leaves of this wild wheat.

In this study, qRT-PCR confirmed that *MtATP6* in the leaves of all four genotypes suddenly increased around 3 h after the salt stress and peaked at 6–10 h (Fig. 1; Table 2). Possibly, *MtATP6*, an early responsive gene, may play a key role in the early stages of the response to salt stress (Fig. 1). Similar to our results, Zhang et al. [60] showed that *AtMtATP6* peaks shortly after the introduction of different $NaHCO_3$, drought, H_2O_2 and low temperature stresses.

Our result indicated that *MtATP6* behaves as an early stress-response gene (Fig. 1).

In agreement with this result, promoter analysis demonstrated that *RMtATP6* and *AtMtATP6* include early stress-responsive *cis*-acting elements such as ABRE, MYB/MYC, GTLs, and W-boxes in the upstream region (Fig. 2). The existence of these elements (Table 7) in the promoter (Fig. 2) candidate *MtATP6* as an early stress-related energy-regulating gene (Fig. 2), a similar result was reported by Heise et al. [23]. The promoter of a drought-, high salinity-, and cold-inducible gene contains major *cis*-acting elements, ABRE, MYB/MYC, and DRE, which are involved in stress-inducible gene expression [55].

ABRE is a major *cis*-acting element in ABA-responsive gene expression [44] because ABA plays a pivotal role in adaptation to drought, high salinity, and cold (Table 7) [55]. MYB/MYC regulatory element involves in early response to osmotic stress and ABA induction (Table 7) [45]. WRKY (tryptophan, arginine, lysine, tyrosine domain) transcription factors act as major regulatory proteins by binding to the W-box [30, 53]. The WRKYs has proven significant induction under salt and drought stresses, and pathogens, implying that those response to various biotic and abiotic stresses [30]. Other important elements on the *MtATP6* promoters are GT-element-binding proteins (GTLs) [48]. It has been documented that promoters of genes carrying GTL rapidly induce their target genes after encountering pathogen attack or NaCl stress [36]. Presence of GTLs and rapid response to NaCl are evidences showing that *MtATP6* is a novel early stress responsive gene (Figs. 1, 2). For example, *GTL1* overexpression enhances drought stress tolerance in plants [56]. Moreover, these elements are involved in repression or activation of different plant photosynthetic genes [54]. Furthermore, their known downstream target genes encode proteins functioning in the mitochondria [11] and chloroplast, such as Rubisco, ATP synthase, and ribosomal proteins [62], as well as other non-chloroplastic genes, and consequently are involved in the regulation of carbon metabolism and energy balance [14]. It seems that 6-kDa subunit not only has important roles in mitochondria but also has unknown roles (or similar roles) in photosynthetic pathways in chloroplasts because the F_1F_0-ATP synthase complex exists in both of these organelles. It is possible GTLs in *MtATP6* promoter are other related factors of organelle communications that act in ABA-dependent pathways [39] in abiotic or biotic stress conditions. All of these results

showed that possibly the *MtATP6* is an early ABA-responsive gene under stress conditions.

Interestingly, these results can explain why the overexpression of *AtMtATP6* and *RMtATP6* in transgenic yeast and Arabidopsis plants [60, 61] or *JcMtATP6* in the salt-hypersensitive mutant of yeast [12] increases the tolerance to various stresses such as: salt, drought, oxidative, and cold. For example, under salt stress to reduce Na^+ toxicity and maintain ion homeostasis, plants need extra energy, mainly in the form of ATP, whose production depends on phosphorylation efficiency [52].

The pattern of *MtATP6* transcript accumulation differed in Mahuti and Alamut (Fig. 1; Table 2 part A). We observed more *MtATP6* upregulation, especially in salt-tolerant Mahuti and the wild genotypes, than in salt-sensitive Alamut (Fig. 1; Table 2 part A). Alamut did not adapt to high NaCl concentrations (Fig. 1). The increased level of *MtATP6* transcripts at 10 h (especially in 100 and 200 mM NaCl) in Alamut may be viewed as a final attempt to avoid death (Fig. 1; Table 2 part A).

In Mahuti, after an increase in the level of *MtATP6* from 3 to 10 h in response to salinity, there was an ascending trend of transcript accumulation from 24 to 72 h in all NaCl concentrations, while in Alamut this expression pattern was seen only in 50 mM NaCl. In Alamut, *MtATP6* expression generally decreased in 100 and 200 mM NaCl and only slightly increased at 10 h after the beginning of the stress. Apparently, Alamut is unable to cope with the toxicity of high concentrations of sodium (see 100 and 200 mM in Fig. 1; Table 2 part A) because salt-sensitive genotypes have lower ATP production than salt-tolerant genotypes [21, 25]. Alamut was able to manage only the low NaCl concentrations, so *MtATP6* expression was induced only at 50 mM NaCl in this genotype (Fig. 1).

However, Mahuti (salt-tolerant wheat) and the wild wheat genotypes coped with the toxicity of accumulated sodium in the cytosol of the high-NaCl treatments because salt-tolerant genotypes had higher ATP production than salt-sensitive genotypes (Fig. 1; Table 2 part A). Ghavami et al. [16] reported that Mahuti uses different mechanisms comparing with the other Iranian wheats under high salt stress and therefore performs better than other salt-tolerant genotypes. This difference between Mahuti and Alamut could also be due to the different efficiency of Na^+ efflux and vacuolar compartmentation of Na^+ (for more information, see results of Hamilton et al. [21] and Kong et al. [25]). For these reasons, Mahuti leaves accumulate *MtATP6* transcript over time to upregulate ATP production whenever the Na^+ and Cl^- concentrations increase, especially at 100 and 200 mM NaCl (Fig. 1; Table 2 part A).

Hamilton et al. [21] suggested that the differential salt sensitivity between contrasting wheat genotypes is in part conferred by higher F_1F_0-ATP synthase activity (possibly from upregulated *MtATP6* expression, according to our hypothesis) in salt-tolerant genotypes. Based on these results, we propose that *MtATP6* is an important regulator of ATP production by the mitochondrial F_1F_0-ATP synthase, especially under stress conditions (Fig. 1; Table 2 part A). The higher *MtATP6* expression under salt stress induces higher F_1F_0-ATP synthase activity to produce ATP needed for energy balance in both tolerant and sensitive genotypes [60, 61]. According to our results and the results of Kong et al. [25] and Hamilton et al. [21], more sustained *MtATP6* expression is induced under high NaCl concentrations, especially in salt-tolerant genotypes.

Ae. crassa (genome D) had the highest *MtATP6* expression in this study. Apparently, our expression results are related to the type of genome and polyploidy level. In wheat (hexaploid), the 4D chromosome, derived from the wild grass *Aegilops*, is responsible for salt tolerance and K^+/Na^+ discrimination [43]. Gorham et al. [19] tested several accessions of *Ae. crassa* and showed they have much lower Na^+ concentrations and higher K^+/Na^+ ratios in leaves than durum wheat. They suggested that genome D imparts the Na^+ 'exclusion' and enhances the K^+/Na^+ discrimination to bread wheat. *T. urartu* (AA), the species that probably gave rise to the genome As of durum and bread wheat, shows greater Na^+ 'exclusion' and K^+/Na^+ discrimination than durum wheat (AABB), as do the closely related A-genome species *T. monococcum* ssp. *monococcum* and *T. monococcum* ssp. *aegilopoides* (syn. *T. boeoticum*) [19].

In this work, cultivated genotypes had lower *MtATP6* expression than wild genotypes (Fig. 1; Tables 2 part A, 3, 4, 5, 6). This might have been due to the traits conferred by genome A [7], whereas the wild genotypes of *Ae. crassa*, which lacks the A and B genomes, and *T. boeoticum*, which lacks the B genome (some of the salt tolerance genes are located on genomes A and B) showed more extensive *MtATP6* upregulation after salt treatment (Fig. 1).

The cultivated genotypes significantly consume more *MtATP6* than the wild genotypes before NaCl treatment (Table 6). This result suggests that energy management in the wild genotypes is more effective. As shown in Table 6, *T. boeoticum* showed similar *MtATP6* expression profiles as cultivated genotypes. These results confirm that *T. boeoticum* has a closer affinity with cultivated genotypes, and consequently, genome D in *Aegilops* can be considered a better genomic source transferring these energy-based abiotic tolerance genes (Tables 5, 6). The wild genotypes showed greater *MtATP6* expression fold changes than cultivated genotypes after stress. *T. boeoticum* (AA) showed similar *MtATP6* expression fold changes to the cultivated genotypes at all NaCl concentrations, but it was significantly different from *Ae. crassa* at 100 and 200 mM after salt stress compared to before salt stress (Table 6). In

other words, *Ae. crassa* has an efficient regulation of F_1F_0-ATP synthase complex via *MtATP6* and activates the pump when it is really require fold change in *Ae. crassa* (genome D) was significantly larger under high NaCl concentrations than before stress (Table 6). Many salt tolerant genes are on genomes A and D and *Aegilops*, the source of genome D in bread wheat, exhibits high discrimination between K^+ and Na^+ due to traits conferred by genome D [19, 20, 50]. Many resistance genes from wild ancestors of wheat are inherited [34], but it seems that energy management mechanisms have not been transferred to the cultivated genotypes. Resistance to fungal pathogens that cause severe crop damage by infecting the spikes, leaves, and roots is another important trait that is not inherited in wheat [38]. Resistance to fungal pathogens is closely associated with genome A, and wild species are valuable sources of resistance to the main fungal diseases and resistance to root rot [57]. Another overlooked but important trait is energy homeostasis under stress, possibly related to genome D, which has not transferred to cultivated wheat. In fact, one under-studied genetic pathway in plants is the electron transport chain in mitochondria, which is central to the energy metabolism [9]. The encoding genes of this pathway play important roles in plant acclimation and response to stress. These genes are novel, important targets for genetic engineering to enhance stress tolerance in crop plants [9]. It seems that the higher stress tolerance of the wild genotypes include unknown mechanism that has not transferred to cultivated genotypes and better energy management is one mechanism by which wild wheat relatives can overcome stresses.

Wild genotypes had lower *MtATP6* expression before stress and had higher *MtATP6* expression after stress (Table 6). Therefore, wild genotypes can produce more ATP in stressed conditions, while they save energy when this is not required (Table 6). This trait enables higher K^+ maintenance and lower Na^+ accumulation and explains the NaCl tolerance in tolerant wheat genotypes as compared to NaCl-sensitive ones [25].

The wild-genotype *T. boeoticum* (harboring A genome), based on the results presented in this investigate, has lower *MtATP6* expression than *Ae. crassa* (harboring D genome). Wild genotypes, especially *Ae. crassa*, manage energy better than cultivated genotypes, a characteristic that is possibly related to genome D and has not been transferred to cultivated genotypes, opening a new avenues for plant breeding.

All together, under stress conditions, energy management and cell homeostasis need close communications between the nucleus and mitochondria [42]. These processes require the expression of a specific collection of nuclear genes which their products need to be targeted to the organelle, a process which involves sensing of disorders in mitochondrial functions and retrograde signaling to the nucleus [42]. In fact, mitochondrial response to nuclear signaling is a key component of the overall plant responses [51]. It has become evident that a variety of nuclear genes that code for mitochondrial proteins are responsive to a wide range of stress conditions which confirms the role of mitochondria as a significant target and regulator of stress responses [51]. For example, oxygen species (ROS) are common productions of many stress responses in mitochondria [15] causing oxidative damage to proteins, lipids, and nucleic acids. Alternative oxidase (AOX) has the ability to suppress ROS production which is a component of the alternative mitochondrial electron transport chain [51]. Almost in all plants, AOX is encoded by a small gene family [51]. These observations make AOX a one of the best-described target genes for mitochondrial retrograde signaling in plants [51]. Therefore, AOX lack of activity or activity leads to a radical alteration of the defence equilibrium at cellular level playing a key role in programming the stress response [51]. Another example in nuclear-organelle communications is ABI4 (abscisic acid insensitive 4) which regulates mitochondrial retrogrades signaling and provides point of convergence in the plastid and mitochondrial retrograde signaling pathways [18]. In addition ABI4, as an AP2 (Apetala 2)-type transcription factor is a component of the plastid retrograde signaling pathway [18]. ACGTL (GT element) significantly over-represented in the promoter of *ABI4* [39]. Indeed, in retrograde signaling, ACGT is the core sequence of ABA response elements, implicating components of the ABA response pathway [55]. The *MtATP6* promoter analysis interestingly demonstrated that *MtATP6* also contains GTLs (Fig. 2) that possibly responds to the stress hormone ABA and similar to *ABI4*, plays roles in ABA-mediated signaling and the associated sugar, energy metabolism, and organelle communications under stress. The identification of 6-kDa subunit, ABI4, and AOX as components of the mitochondria or chloroplasts providing an additional way in which internal hormonal and developmental signals can be integrated with stress signal (through ABA) to affect a high-order regulation of organelle function [18]. More importantly, crosstalk between chloroplast and mitochondria seems to contribute to chloroplast-to-nucleus retrograde signaling [17]. We suggest 6-kDa subunit is another communicating protein between the nucleus and these two organelles in regulation of cell energy under stress and roles in ABA-mediated signaling (Supplementary data 1, Fig. 1). Although ABA is not synthesized in plastids or mitochondria, it may represent an important intermediate or signaling molecules (such as 6-kDa subunit, ABI4, and AOX) to mediate and integrate plastid and mitochondrial retrograde signals [17].

It seems that *MtATP6* as an early stress-ABA-energy responsive gene involves in the similar communication

mechanisms (Supplementary data 1, Fig. 1). Dual targeting of plastids and mitochondria is an interesting phenomenon [5] so a specific targeting signal peptide was found in chloroplastic and mitochondrial proteins [47]. The reason for dual targeting of 6-kDa subunit is that may comprise as a mean of inter-organelle communication [51] and can save energy for the cell. Sending the same proteins to both organelles at the same time ensures that they are both at least capable of carrying out these functions in a coordinated manner [51].

In conclusion, our data and previous data indicate that *MtATP6* behaves as an early stress-response gene, suggesting multiple roles for *MtATP6*. Although MtATP6 is a subunit of F_0, it is also a nuclear protein with alpha-helix structures and phosphorylation sites, suggesting its role as a regulatory factor activating the expression of F_1F_0-ATP synthase subunits.

Further studies are required to shed light on this interesting hypothesis. Apparently, *MtATP6* suddenly accumulates in early hours after stress in ABA interacting manner, and it induces or completes the expression of other subunits of F_1F_0-ATP synthase. Therefore, in response to certain stresses, F_1F_0-ATP synthase will be more active, producing more ATP for ion balance and energy management in cells. Soontharapirakkul et al. [46] postulated that this complex maintains the ATP levels of cells by electron-transfer chain in the stress conditions. All together, these results suggest that the induction of F_1F_0-ATP synthase probably plays critical roles in stress tolerance.

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