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Structural and functional properties of plant mitochondrial F-ATP synthase

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ABSTRACT

The mitochondrial F-ATP synthase is responsible for coupling the transmembrane proton gradient, generated through the inner membrane by the electron transport chain, to the synthesis of ATP. This enzyme shares a basic architecture with the prokaryotic and chloroplast ones, since it is composed of a catalytic head (F_1), located in the mitochondrial matrix, a membrane-bound part (F_O), together with a central and a peripheral stalk. In this review we compare the structural and functional properties of F-ATP synthase in plant mitochondria with those of yeast and mammals. We also present the physiological impact of the alteration of F-ATP synthase in plants, with a special regard to its involvement in cytoplasmic male sterility. Furthermore, we show the involvement of this enzyme in plant stress responses. Finally, we discuss the role of F-ATP synthase in shaping the curvature of the mitochondrial inner membrane and in permeability transition pore formation.

1. Introduction

Mitochondrial oxidative phosphorylation is the energy-conserving process that represents the main source of ATP for virtually all eukaryotic cells. Plant mitochondria are essential for supporting bio-energetic demand in heterotrophic organs and in green tissues exposed to dark/low light conditions. In addition, they play a critical role during photosynthesis, since they catalyse the biosynthesis of carbon skeletons necessary for carbon fixation and for several cofactors. In C3 photosynthetic metabolism, mitochondria are involved in the photorespiration pathway improving the CO_2/O_2 ratio and act as a sink for recycling the exceeding reducing power synthetized by chloroplasts (Schwarzländer et al., 2012). Plant mitochondria are also the central processing units during programmed cell dismantling and senescence, responsible for coordination of the active and energy-requiring events leading to cell death (Pastore et al., 2007).

The mitochondrial electron transport chain (mETC, Complexes I-IV) generates a transmembrane proton gradient across the inner mitochondrial membrane (IMM), and the F_OF_1-ATP synthases (F-ATP synthase or Complex V) is able to couple the flow of protons towards the matrix to the synthesis of ATP. This review compares the subunits of the mitochondrial F-ATP synthase, which have been characterized and classified in yeast and mammals, with those identified in plants, discussing the components that have been suggested to be plant specific. Furthermore, the consequences of alterations of some F-ATP synthase subunits are discussed to evaluate the involvement of this enzyme in some physiological features, including cytoplasmic male sterility and stress responses in vascular plants. Finally, we cover the structural and functional roles of F-ATP synthase in its dimeric form.

2. Overall subunit organization and catalytic mechanism of F-ATP synthase

The complex structure of the F-ATP synthase is at the basis of its unique functional mechanism, which has been extensively studied in some prokaryotes and eukaryotes, but not yet sufficiently in plants. For this reason, hereafter we describe the known structures and mechanisms so far proposed in bacteria, *Saccharomyces cerevisiae* and mammals that, considering the conserved structure, would be shared by plant F-ATP synthase.

In all energy-converting membranes, the F-ATP synthase complex consists of a water-soluble, catalytic F_1 head and a membrane-embedded F_O sub-complex. The latter is connected by a peripheral stalk, which is structurally part of the F_O moiety, while the central stalk is related to the F_1 sector (Fig. 1).

In its simplest form, in the bacterial plasma membrane and

Abbreviations: CMS, cytoplasmic male sterility; cryo-EM, cryo-electron microscopy; CsA, cyclosporin A; CyPD, cyclophilin D; mETC, mitochondrial electron transport chain; F-ATP synthase, mitochondrial F_1F_O-ATP synthase; IF1, inhibitory factor; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; OSCP, oligomycin sensitivity conferring protein; PPR, pentatricopeptide repeat; PT, permeability transition; PTP, permeability transition pore; STF, F_1 inhibitor stabilizing factor; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane

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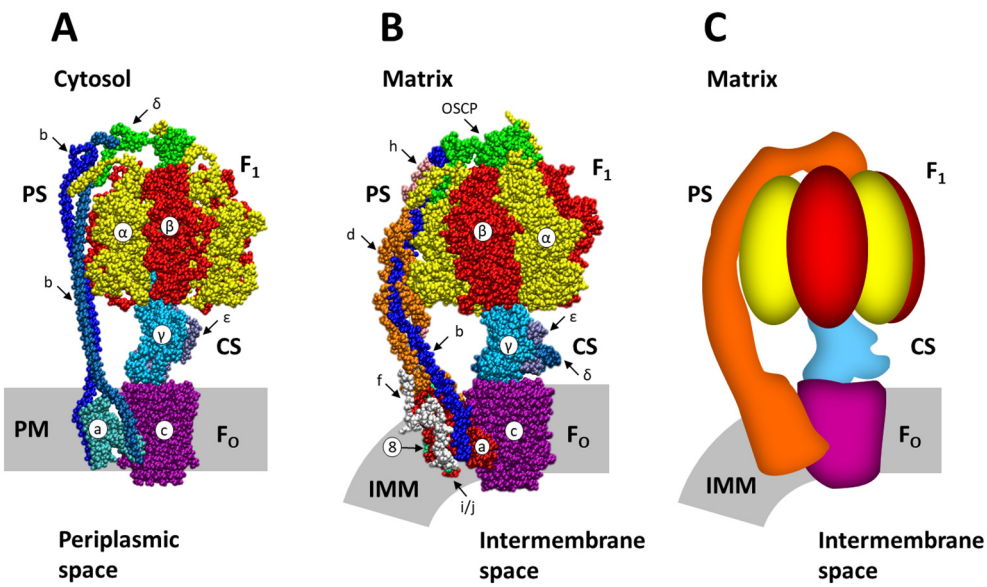

Fig. 1. Structures of the monomeric F-ATP synthase. The molecular structure of the complexes from *Escherichia coli* (A) and *Saccharomyces cerevisiae* (B) are built from available structures provided by (Sobti et al., 2016) and (Srivastava et al., 2018), respectively. In the upper part, F_1 is always shown with the alternating subunits α (yellow) and β (red), and the central stalk (CS) connecting subunits α/β to the c-ring in the membrane, which includes subunits γ (cyan) and ε (ice blue) in *E. coli* and also subunit δ (blue) in *S. cerevisiae*. The membrane-embedded c-ring is always composed of identical subunits c (purple) and is in contact with subunit a (light blue in *E. coli* and light red in *S. cerevisiae*). In both complexes the peripheral stalk (PS) includes subunit b (blue) and, located on top of F_1 in green, subunit δ in *E. coli* or OSCP in *S. cerevisiae*, respectively. PS includes subunits h (pink) and d (orange) and, in the lower part within the IMM, subunits f (light grey), i/j (red) and 8 (light green), mostly covered by other subunits. C) Schematic structure of F-ATP synthase in plants, drawn according to the localization of the homologous subunits in yeast. F_1 is composed by 3 copies of subunits α and β; CS comprises subunits γ, δ and ε; F_O possesses subunits a, b, 8, f and g; subunits i/j and k (yeast specific) are not present in plants; the actual number of subunit c copies in plants is still unknown. PS consists of subunits b, d, and OSCP; subunit h has not been identified in plants and the presence of subunit e has still to be confirmed. The location of subunits F_Ad and 6 kDa, which have been reported to be plant specific, is still uncertain.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chloroplast thylakoid membrane, the F_O sector consists of a ring of 9–17 copies of subunit c, two copies of subunit b constituting the peripheral stalk, and one copy of subunit a. The latter is organized into a four-helix horizontal bundle wrapping around the c-ring, thus forming two aqueous semi-channels accessible to H^+ (Morales-Rios et al., 2015). Eukaryotic F_O sector comprises a smaller ring of 8 or 10c subunits (ATP9) in metazoans and *S. cerevisiae*, respectively (Watt et al., 2010), and a sub-complex of 6 conserved subunits: a (ATP6), b (ATP4), 8 (A6L in mammals, ATP8), f (ATP17), g (ATP20) and e (ATP21). Additional subunits have been identified, namely 6.8PL (ATP5MPL) and DAPIT (ATP5MD) in vertebrates and subunits i/j (ATP18) and k (ATP19) in yeast (Kühlbrandt, 2019) (Table 1), the homology of which is still debated (Gu et al., 2019; He et al., 2018). In yeast, subunits e, g and k are defined "dimer-specific" because they are only present in the dimeric form of the complex (Paumard et al., 2002) (see below). Conversely, in mammals, subunits e and g remain associated also with the monomeric form (Zhou et al., 2015). The F_O proteins are products of either nuclear or mitochondrial genes. In yeast, the three F_O core proteins a, 8 and c are encoded in the mitochondrial DNA, while in mammals only subunits a and A6L are encoded in the mitochondrial genome (Kühlbrandt, 2019).

As for F_O sector, the eukaryotic peripheral stalk is more complex. Its membrane distal part is constituted by one copy of subunits b (ATP4), h (ATP14, F6 in mammals), d (ATP7) and OSCP (oligomycin sensitivity conferring protein, ATP5) (Rees et al., 2009), while its base also comprises the C-terminal region of subunit 8, the N-terminal domain of subunit f (Guo et al., 2017) and the subunit i/j in yeast (Srivastava et al., 2018).

The F_1 sector is always composed of three αβ dimers (ATP1 and ATP2) and the central stalk. Each αβ dimer contains a catalytic nucleotide binding site on the subunit β and surrounds the central stalk comprising the subunit γ (ATP3) (Abrahams et al., 1994), which is associated at the foot with subunits δ (ATP16) and ε (ATP15) in all eukaryotes (Bason et al., 2015). All the F_1 and peripheral stalk subunits are nuclear gene products in yeast and mammals, implying a complex assembly process that involves coordinated expression of the nuclear and mitochondrial genomes, as well as protein import into mitochondria. The enzyme assembly proceeds via intermediates, which appear to be slightly variable in different organisms (He et al., 2018), and requires accessory factors that have been well defined in yeast (Artika, 2019).

The F_O and F_1 parts form two nanomotors linked by a rotor. Proton translocation through the two half-channels of F_O powers the rotation of the c-ring that is firmly attached to the central stalk (Kühlbrandt, 2019). The rotation of subunit γ within the $\alpha_3\beta_3$ sub-complex is not continuous, but rather proceeds in 120° steps, comprising sub-steps that are different depending on the organism. Rotation forces each of the three catalytic sites into three major functional conformations. Such configurations are denoted βE (empty), βDP (bound to ADP) and βTP (bound to ATP), and account for the synthesis of three Mg^{2+}-ATP molecules during each 360° rotation (Futai et al., 2012; Noji et al., 2017). The peripheral stalk acts as a stator to counter the tendency of the static parts in F_O and F_1 to follow the rotation of the central stalk (Rees et al., 2009). The synthetic motor can work in reverse, driving the rotor backwards with energy from ATP hydrolysis and generating a membrane potential. In both directions, a metal cofactor is essential for catalysis, which requires the nucleotide to be complexed with either the most abundant Mg^{2+} or with metal ions, such as Mn^{2+} or Ca^{2+} (Nesci et al., 2017; Papageorgiou et al., 1998). However, unlike other metal ions, Ca^{2+} only sustains ATP hydrolysis not coupled to generation of a proton gradient (De Col et al., 2018; Papageorgiou et al., 1998), in spite of its ability to induce the rotation of subunit γ (Tucker et al., 2004).

Measurement of the H^+/ATP ratio in a bacterial complex excluded "slip" of the rotor, i.e. rotation without carrying a proton, revealing that the F-ATP synthase exerts a "perfect chemo-mechanical coupling" between proton translocation, rotary motion, and ATP synthesis/hydrolysis (Soga et al., 2017). On the other hand, the existence of two stepping motors (F_O and F_1), which differ in the number of steps during the catalytic cycle, poses a challenge to efficient energy conversion (Kühlbrandt, 2019). While it is widely accepted that cooperation between the F_O and F_1 motors is smoothed by elastic power transmission (Junge et al., 2009), it is still debated which sub-complex is most flexible. In *Escherichia coli* it has been proposed that the elastic buffer is located in the rotor, namely where the globular portions of subunits γ and ε contact the c-ring (Sielaff et al., 2008), or in the coiling of the two helix bundle in subunit γ (Martin et al., 2018). However, the cryo-

Table 1
Composition of ATP synthase subunits in *Saccharomyces cerevisiae*, *Homo sapiens* and *Arabidopsis thaliana*. * from (Kühlbrandt, 2019) and (Cabezón et al., 2002). STF1 and STF2 are in brackets because they are yeast-specific proteins. ** for *Homo sapiens*, new symbols, according to Human Gene Nomenclature Database (HGNC), have been used. In bold, the subunits encoded by mitochondrial DNA. [1] "A stretch of 270 kb of the mitochondrial genome is duplicated within the centromere of chromosome 2 resulting in the duplication of the gene. The expression of this duplicated gene (AT2G07698) has not been demonstrated. It is also probably not RNA edited and therefore differs in all the positions known to be edited." The same for AT2G07741 (from the National Center for Biotechnology Information, NCBI, https://www.ncbi.nlm.nih.gov/). [2] "The atp6 gene is located on the border of one of the mitochondrial DNA repeats resulting in two identical copies of the mature protein with different propeptide extensions." (from Uniprot, https://www.uniprot.org/). [3] To be confirmed. **a**, (Kruft et al., 2001); **b**, (Werhahn and Braun, 2002); **c**, (Heazlewood et al., 2004); **d**, (Meyer et al., 2008); **e**, (Taylor et al., 2011); **f**, (Klodmann et al., 2011); **g**, (Brugière et al., 2004); **h**, (Heazlewood et al., 2003b); **i**, (Senkler et al., 2017); **j**, (Eubel et al., 2003); **k**, (Sabar et al., 2003); **l**, (Nakazono et al., 2000). n.d.: not detected in plants.

*S. cerevisiae**	*H. sapiens***		*A. thaliana*		
		Primary function	Symbols	AGI	References
F_1 head					
α (ATP1)	ATP5F1A	Structural, catalytic	**α (ATP1)**	**ATMG01190**	**a, b, c, d, e, h, i**
				AT2G07698[1]	**f, i**
β (ATP2)	ATP5F1B	Catalytic	β (ATP2)	AT5G08670	**a, b, c, d, e, f, g, h, i**
				AT5G08680	**c, d, f, h**
				AT5G08690	**a, b, c, d, f, h, i**
Central stalk					
γ (ATP3)	ATP5F1C	Torque transmission	γ (ATP3)	AT2G33040	**c, d, e, f, g, h, i**
δ (ATP16)	ATP5F1D	Connection to c-ring	δ (ATP16)	AT5G47030	**a, b, c, d, e, f, g, h, i**
ε (ATP15)	ATP5F1E	Connection to c-ring	ε (ATP15)	AT1G51650	**c, d, f, h, i**
Peripheral stalk					
b (ATP4)	ATP5PB	Stator, F_1-F_0 link	**b (ORF25, ATP4)**	**ATMG00640**	**c, d, f, h, i**
OSCP (ATP5)	ATP5PO	Flexible hinge	OSCP (ATP5)	AT5G13450	**a, b, c, d, e, f, g, h, i**
d (ATP7)	ATP5PD		d (ATPQ, ATP7)	AT3G52300	**a, b, c, d, e, f, g, h, i**
h (ATP14)	ATP5PF (F6)			n.d.	
F_O motor					
a (ATP6)	**MT-ATP6**		**a-1 (ATP6-1)**	**ATMG00410**[2]	**d, f**
			a-2 (ATP6-2)	**ATMG01170**[2]	**d, f, i**
				AT2G07741[1]	
c (ATP9)	ATP5MC1	c-ring in F_O	**c (ATP9)**	**ATMG01080**	**d, g, h**
	ATP5MC2				
	ATP5MC3				
e (TIM11, ATP21)	ATP5ME		e (ATP21)	AT5G15320[3]	**i**
g (ATP20)	ATP5MG		g (ATP20)	AT2G19680	**f, i**
				AT4G26210	**f, i**
				AT4G29480	**e, f, j, i**
f (ATP17)	ATP5MF		f (ATP17)	AT4G30010	**d, f, i**
j (I, ATP18)				n.d.	
k (ATP19)				n.d.	
8 (ATP8)	**MT-ATP8 (A6L)**		**8 (ORFB, ATP8)**	**ATMG00480**	**c, d, h, k, f, i**
	ATP5MD (DAPIT)			n.d.	
	ATP5MPL (6.8PL)			n.d.	
"Plant specific"					
			6 kDa	AT3G46430	**d, f, g**
				AT5G59613	**d, f, i**
			F_Ad (24 kDa)	AT2G21870	**a, b, c, d, e, f, g, h, i**
Inhibitory factor					
IF1	ATP5IF1		IF1-1	AT5G04750	**b, l, h, i**
			IF1-2	AT2G27730	**a, c, l, i**
(STF1)				n.d.	
(STF2)				n.d.	

electron microscopy (cryo-EM) structures of the F-ATP synthase from mammals (Zhou et al., 2015), *E. coli* (Sobti et al., 2016) and the green alga *Polytomella* spp. (Murphy et al., 2019), which have greatly contributed to clarify the architecture of the F_O sector and thus the mechanisms of proton translocation, have also revealed that the central stalk rotates as a rigid body. The flexible coupling between F_1 and F_O sub-complexes appears therefore to be primarily mediated by the inter-domain hinge of the conserved subunit OSCP (Murphy et al., 2019), a well-established target of physiologically important F-ATP synthase inhibitors, including cyclophilin D (CyPD), a mitochondrial immunophilin possessing peptidyl-prolyl *cis–trans* isomerase activity (Giorgio et al., 2019).

The catalytic activity of F-ATP synthase is modulated by numerous effectors, among which the inhibitory factor 1 (IF1) plays a fundamental role (Esparza-Moltó et al., 2017). This protein has been initially described in bovine heart mitochondria in the 1960s (Pullman and Monroy, 1963) and then in other mammals (Cintrón and Pedersen, 1979; Di Pancrazio et al., 2004; Rouslin and Pullman, 1987), *Caenorhabditis elegans* (Ichikawa et al., 2006), yeast (Cabezón et al., 2002) and plants (Norling et al., 1990). Site-directed mutagenesis and crystal structure studies have established that the IF1 N-terminal domain interacts with five of the nine F_1 subunits, fully inhibiting ATP hydrolysis (Bason et al., 2011). Its binding is essential to prevent ATP waste when mitochondria face a decrease in transmembrane potential, such as under hypoxic and ischemic conditions (Di Pancrazio et al., 2004). At low pH, in both mammals and yeast, the active form of IF1 is dimeric, whereas at higher pH only the bovine IF1 oligomerizes (Cabezón et al., 2002). In bovine IF1, His49 is responsible for its tetramerization and inactivation at high pH (Cabezón et al., 2001). In yeast, two additional regulators are present, i.e. the F1 inhibitor stabilizing factor1 (STF1), which tends to form dimers and inhibit F_1 at higher pH; and STF2, which interacts with F_O, to facilitate binding of IF1 and STF1 to F_1 (Cabezón et al., 2002). A central role of IF1 in cellular homeostasis has been highlighted by the finding that IF1 is overexpressed in human

carcinomas, where it exerts a metabolic rewiring to an enhanced glycolysis by activating ROS-dependent signaling pathways and, possibly, by inhibiting ATP synthesis by the F_OF_1 complex (Esparza-Moltó et al., 2017). Whether IF1 could also inhibit ATP synthesis in mammals is still matter of debate (Boreikaite et al., 2019).

A remarkable feature of mitochondrial ATP synthases is that they all form dimers in the membrane, as initially detected by mild detergent extraction followed by Blue-Native PAGE (Paumard et al., 2002). The first structural characterization by electron microscopy and single particle analysis was performed on a stable ATP synthase dimer purified from the alga *Polytomella* that revealed the existence of specific interaction of the F_O sectors (Dudkina et al., 2005). Later analyses by cryo-EM showed that in animals and yeast the dimers are V shaped (type I) with an angle of ~86° between the two central stalks (Davies et al., 2011; Hahn et al., 2016; Kühlbrandt, 2019) and the peripheral stalks turning away from one another. The dimers self-assemble into long rows of oligomers localized at the cristae edges to maintain the typical IMM morphology (Paumard et al., 2002). While the dimerization interface is made up of several F_O subunits (Guo et al., 2017), the interactions between the dimers are less constrained. Interestingly, using a single-particle cryo–EM method, the porcine tetrameric ATP synthase structure was shown to consist of the two antiparallel dimers linked by two IF1 dimers that induce an inhibited state (Gu et al., 2019) (Fig. 2). In each dimer the two monomers are in two states, E and DP, differing in the subunit γ direction, $\alpha_3\beta_3$ conformation and peripheral stalk position, while in the tetramer the diagonal protomers adopt similar conformations thus forming an H-shaped F-ATP synthase tetramer, as viewed from the matrix.

3. Structural properties of F-ATP synthase in plant mitochondria

Plant F-ATP synthase shares the basic structure of the enzyme complexes described above. Nevertheless, the composition of F_O in plant mitochondria has not been completely defined, but homologues for all subunits except ATP18 and ATP19 have been found (Table 1). The identity of subunit e (ATP21) in Arabidopsis has still to be confirmed because the protein At5g15320 identified by mass spectrometry in Arabidopsis mitochondria does not cluster with F-ATP synthase subunits even if it represents a suitable candidate (Senkler et al., 2017).

Proteomic analyses identified two proteins associated with F_O, subunits F_Ad and 6 kDa, which have been tentatively described as "plant specific", since there are no corresponding proteins in mammals or yeast (Table 1). In soybean, the subunit F_Ad gene has been initially characterized and the corresponding 179-aa protein possesses a cleavable N-terminal sequence representing its mitochondrial targeting sequence (Smith et al., 1994), which contains hydrophobic residues critical for the correct import process (Lee and Whelan, 2004). This subunit has been later identified in mitochondria from Arabidopsis (Kruft et al., 2001; Millar et al., 2001), pea (Bardel et al., 2002), rice (Heazlewood et al., 2003a) and potato (Salvato et al., 2014). The lack of direct biochemical and structural evidence still poses some doubts about the actual involvement of F_Ad in F-ATP synthase assembly and/or catalysis.

Subunit 6 kDa has been proposed as an F_O component in potato (Jänsch et al., 1996; Salvato et al., 2014), rice (Heazlewood et al., 2003a) and Arabidopsis (Brugière et al., 2004; Klodmann et al., 2011; Meyer et al., 2008). In rice, subunit 6 kDa consists of 58 aa, possesses a mitochondrial targeting sequence and a single transmembrane region (Zhang et al., 2006). This subunit represents a further source of uncertainty about F-ATP synthase composition in higher plants, since it has sometimes ambiguously been reported as MtATP6 (Li et al., 2013; Moghadam et al., 2013; Zhang et al., 2006). To avoid confusion, we suggest that this subunit should be clearly distinguished from subunit a, which is actually classified as ATP6 in yeast and consistently also in plants (Table 1). Thus we propose to name it unequivocally as "subunit 6 kDa". As for F_Ad, direct evidence about structure and function of subunit 6 kDa and its actual presence in F-ATP synthase is still scarce.

In higher plants, each subunit of the peripheral stalk has a homologue, except for subunit h, ATP14 (Table 1). We suspect that the F_Ad subunit represents the equivalent of the peripheral stalk subunit h, even if alignment of subunit h sequences from mammals or yeast with F_Ad does not give significant results. Further structural and functional studies are needed to confirm this hypothesis.

Regarding the plant F_1 sector, in contrast to yeast and mammals, subunit α is encoded in the mitochondrial genome (Clifton et al., 2004; Dubinin et al., 2011; Heazlewood et al., 2003a; Rao et al., 2017) (Table 1). In Arabidopsis, three highly-conserved isoforms for subunit β encoded in a small multigene family (β1-3, ATP2.1–3) are present (Table 1). In *Nicotiana sylvestris* and *Petunia hybrida*, ATP2.1 and ATP2.2 are expressed in all vegetative tissues, whereas ATP2.3 has been found only in pollen (Lalanne et al., 1998; Paepe et al., 1993). The putative mature sequences of ATP2.1–3 share a very high similarity with the corresponding bovine subunit (approx. 91.1%), but a low similarity in the putative mitochondrial targeting signal (between 57.1 and 59.3%). It has been proposed that, since the three β1-3 precursors differ mainly in their signal peptide amino acid sequence (Fig. 3) and possibly in their expression levels, the mature complexes might be either homogeneous (i.e. $\alpha_3\beta1_3$, $\alpha_3\beta2_3$, or $\alpha_3\beta3_3$) or heterogeneous (i.e. $\alpha_3\beta1\beta2\beta3$ or other combinations), probably affecting their activity or stability (Lalanne et al., 1998).

As shown above, subunit α is the only component of F_1 encoded in the plant mitochondrial genome, raising a question about the coordinate expression with the nuclear-encoded subunit β for the correct assembly of this sub-complex. Indeed, cell cultures of Arabidopsis subjected to sucrose starvation exhibited changes in nuclear gene expression, but no significant change in mitochondrial gene expression (Giegé et al., 2005). This resulted in decrease of about 40% for subunit β, whereas no change was observed for subunit α. This coordination might be post-translational, occurring at the assembly level among proteins, maintaining the stoichiometry of the complexes and leading to an excess of unassembled subunits encoded by the mitochondrial genome (Giegé et al., 2005).

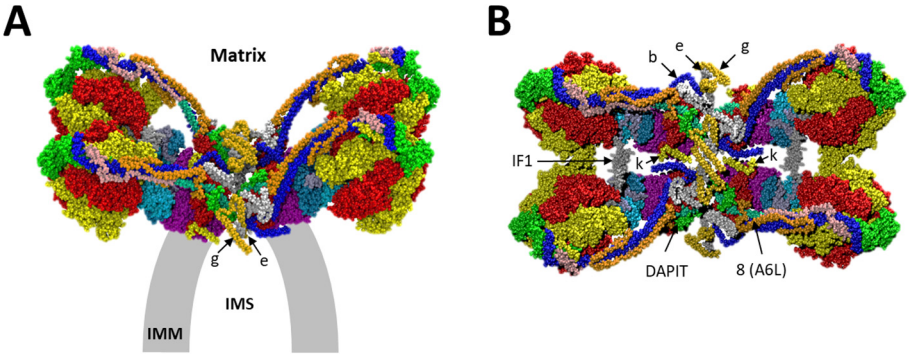

Fig. 2. F-ATP synthase supercomplexes shaping the mitochondrial cristae. Views from side (A) and from matrix (B) of pig heart F-ATP synthase tetramer built from available cryo-EM structure (Gu et al., 2019). Two ATP synthase dimers are linked by two IF1 dimers (dark silver) and form an H-shaped tetramer. Subunit e is in silver, subunit f in light gray, subunit g in light orange, DAPIT in green and subunit 8 (A6L) in dark green. The other subunit color codes are as in Fig. 1. IMS, intermembrane space.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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AT5G08670 ATP2.1    MASRRVLSSL LRSSSGRSAA ---KLGNRNP RLPSPSPARH AAPCSYLLGR VAEYATSSPA SSAAPSSAPA 67
AT5G08680 ATP2.2    MASRRILSSL LRSSSSRSTS KSSLIGSRNP RLLSPGPAHG AAPCGTLLGR VAEYSTSSPA NSAAPSSAPA 70
AT5G08690 ATP2.3    MASRRVLSSL LRSSSGRSAA ---KLVNRNP RLPSPSPARH AAPCSYLLGR VAEYATSSPA SSAAPSSAPA 67
                    *****:**** *****.**::     : .*** ** **.**:  ****. **** ****:***** .*********
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Fig. 3. Multiple alignment of the first 67–70 amino acids of the three isoforms of ATP2 (subunit β) in Arabidopsis. The multiple alignment was performed using the program Clustal Omega at UniProt (https://www.uniprot.org/align/). *, identity; :, residues with strongly similar properties; ., residues with weakly similar properties. The arrow indicates the putative cleavage site of the mitochondrial target peptide, predicted by the program TargetP-2.0 (http://www.cbs.dtu.dk/services/TargetP/). The following amino acids in the three sequences are identical.

In Arabidopsis, similarly to what described in yeast (Rak et al., 2011; Rühle and Leister, 2015), the turnover of soluble F_1 in the mitochondrial matrix is higher than F_O, supporting a modular model of complex assembly (Li et al., 2012; Meyer et al., 2019). This model indicates that the F_1 and F_O complexes are independently assembled, the former in the matrix and formed by complexation of subunits α, β, γ and δ. Then, the F_1 associates to the IMM with F_O, to be later stabilized by the addition of the subunits of the peripheral stalk (Li et al., 2012).

In plants, IF1 has been initially described in potato mitochondria as an 8.3 kDa protein that is heat-stable, trypsin-sensitive and able to stoichiometrically inhibit ATPase activity of the F-ATP synthase complex (Norling et al., 1990). Later, the actual molecular mass of potato IF1 was determined to be about 6.7 kDa by protein sequencing (Polgreen et al., 1995). IF1 is poorly conserved between mammals, yeast and plants, but these proteins share the "minimal inhibitory sequence" that has been identified in residues 14–47 of bovine IF1 (van Raaij et al., 1996). This sequence corresponds to a region that shows high similarity between the two plant IF1 isoforms, namely IF1-1 from potato, rice and Arabidopsis, and IF1-2 from rice and Arabidopsis (Table 1) (Nakazono et al., 2000).

F-ATP synthase complex in plants represents a significant amount of total mitochondrial proteins, estimated in Arabidopsis to be present in about 8.4% of the area of the IMM, a value similar to the one shown for the area occupied by complexes I–IV (9.7%) (Fuchs et al., 2020). A single mitochondrion contains an average of 6426F-ATP synthase particles, which could produce a remarkable 1 million molecules of ATP per second, corresponding to about 150 ATP molecules synthesized per second by each F-ATP synthase complex (Fuchs et al., 2020), a value consistent with the ATP synthesis rate estimated in non-plant mitochondria (Gu et al., 2019). Like the other eukaryotic enzymes, plant F-ATP synthase might be organized in super-complex structures. Single-particle electron microscopy has revealed that F-ATP synthase from potato mitochondria (Bultema et al., 2009) exhibits a small angle between the monomers. Later, cryo-EM of F-ATP synthase dimers from potato mitochondria showed a broad angle between monomers, around 108° (Davies et al., 2011), but new evidence has confirmed that the plant dimers have a small angle, comparable to animal and fungi complexes (K.M. Davies, personal communication).

The detection of the dimeric form of plant F-ATP synthase after Blue-Native PAGE was achieved only in the presence of low concentrations of detergent, such as Triton X-100 (Eubel et al., 2003, 2004) or digitonin (De Col et al., 2018; Krause et al., 2004), a problem that may depend either on low stability of the dimers in the presence of high concentration of detergents or on their low density. Based on the number of F-ATP synthase present in a single Arabidopsis organelle proteome, it is estimated that Complex V might form about 3000 dimers, involved in the formation of up to 18 cristae sheets in each mitochondrion (Fuchs et al., 2020). Nevertheless, plant IMM might contain fewer cristae and therefore a higher number of monomers or more complex structures, possibly composed of F-ATP tetramers (Fuchs et al, 2020).

4. Physiological impact of F-ATP synthase alterations in plants

More than fifty years have passed since Lynn Margulis Sagan proposed that mitochondria and plastids were products of the symbiosis of living prokaryotes, an α proteobacterium and a cyanobacterium (Margulis Sagan, 1967; for a recent overview see Gray, 2017) with a proto-eukaryotic host cell. A revised phylogenomic analysis suggests that such proto-eukaryotic cell would be evolved from an *Archaea*, which initially engulfed a bacterial endosymbiont (Williams et al., 2020). After the broad acceptance of Lynn Margulis' theory, it was clear that the symbiosis was based on a coordinate cross-talk and interactions between the genomes of organelle and nucleus. During evolution, this has led to the progressive transfer of mitochondrial genes to the nucleus and to the development of complex systems in the outer mitochondrial membrane (OMM) and IMM, respectively named translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM), to facilitate the acquisition of cytosol-encoded proteins into the mitochondrion (Wiedemann and Pfanner, 2017). This has happened also for some F-ATP synthase subunits, which are mostly encoded by nuclear genes and possess N-terminal targeting signal for the correct mitochondrial localization. It is noteworthy that the subunits encoded in the mitochondrial genome are just two in mammals (a and A6L), three in *S. cerevisiae* (a, c and 8-A6L) (Kühlbrandt, 2019) and five in most land plants (α, ORF25 equivalent to b, a, c, and ORFB equivalent to 8/A6L, Table 1). This feature is not unexpected, since the number of proteins encoded by mitochondrial DNA in mammals is smaller than in land plants (Chase, 2007; Gray, 2015).

Complex maturation steps have been described for all the five subunits of F-ATP synthase encoded by plant mitochondrial genomes, in particular RNA editing (Chase, 2007). This is an interesting feature of plant mitochondria, which consists of post-transcriptional conversion, typically of cytosine to uracil, insertion and/or deletion of nucleotides in mitochondrial mRNA. This results in mature mRNAs that could be modified in codons for some amino acids or for the presence of new initiation or termination codons (Chateigner-Boutin and Small, 2011; Takenaka et al., 2008; and in this Special Issue, Takenaka et al., 2020).

Modifications of RNA editing have consequences for several physiological processes, including the development of cytoplasmic male sterility (CMS), a phenomenon in which the male reproductive structures do not develop correctly, leading to the production of little or no pollen (Carlsson et al., 2008; Chase, 2007; Hanson and Bentolila, 2004; Horn et al., 2014; Yang et al., 2009). CMS has been utilized extensively in agriculture as a selective approach to produce hybrid lines, since it represents a useful tool to eliminate the need of hand or mechanical emasculation (Kaul, 1988). Besides alteration of mRNA editing, two more routes to CMS have been proposed: i) mitochondrial DNA recombination and nuclear interaction, which could generate new chimeric sequences; ii) production of specific proteins, which could be toxic through interference with the mitochondrial membrane structure and its selective permeability (Chen et al., 2017).

Dysfunction of ATP synthesis is a feature often observed in CMS plants. Although this might be tolerated in many vegetative organs, it leads to failure of pollen production because of the high-energy requirements during the development process (Carlsson et al., 2008; Chase, 2007; Hanson and Bentolila, 2004). Nevertheless, the comparison between respiratory mutants and CMS lines have raised some questions about the actual role of decrease in ATP synthesis as the main cause for CMS (Touzet and Meyer, 2014). An intriguing alternative hypothesis suggests that CMS might be associated with the impairment of oxidative phosphorylation, which would lead to programmed cell death during the development of anthers, with the involvement of F-ATP synthase through a still unknown mechanism (Balk and Leaver, 2001; Sabar et al., 2003).

Hereafter we focus on the involvement of the F-ATP synthase

Table 2
Involvement of plant mitochondrial F-ATP synthase subunits in physiological processes and stress responses. CMS, cytoplasmic male sterility.

Subunit	Species	Modification / Treatment	Effect / Response	Reference
α (ATP1)	Sunflower[1], radish-rapeseed cybrids[2], sugar beet[3,4], stem mustard[5,6], upland cotton[7]	Gene rearrangement	CMS	[1], Siculella and Palmer, 1988; [2], Sakai and Imamura, 1992; [3], Kubo et al., 1999; [4], Senda et al., 1993; [5], Yang et al., 2009; [6], Yu et al., 2010; [7], Wu et al., 2011
	Arabidopsis	mRNA editing	Slow growth and delayed development	Hammani et al., 2011
	Cotton	mRNA editing	Fibre cell elongation	He et al., 2018
	Wheat	Decrease in protein abundance	CMS	Wang et al., 2015
	Oat	Heat stress	Decreased expression	Chen et al., 2016
	Pea, Arabidopsis, soybean, *Vigna radiata*, peach, *Zoysia japonica*, rice	Low temperature stress	Change in expression	Heidarvand et al., 2017
	Arabidopsis	Oxidative stress	Decrease in abundance	Tan et al., 2012
β (ATP2)	Wheat	Decrease in protein abundance	CMS	Wang et al., 2015
	Rice	Low temperature stress	Enhanced expression	[1], Gammulla et al., 2011; [2], Neilson et al., 2011
	Arabidopsis	Oxidative stress	Increase in protein abundance[1] Decrease in protein abundance[2]	[1], Obata et al., 2011; [2], Tan et al., 2012
	Arabidopsis	*ftsh4* mutant	Decrease in protein abundance	Kolodziejczak et al., 2007
γ (ATP3)	Arabidopsis	Decrease of expression (anti-*atp*3)	Seedling death, slow growth	Robison et al., 2009
	Cauliflower	Heat stress	Decrease in abundance	Rurek et al., 2015
	Sunflower	Low temperature stress	Increase in protein abundance in chilling-sensitive cv.Decrease in protein abundance in chilling-tolerant cv.	Balbuena et al., 2011
δ (ATP16)	Wheat	Decrease in abundance	CMS	Wang et al., 2015
	Rice	Osmotic and salt stresses	Increase in abundance (tolerance to stress)	Zhang et al., 2006
a (ATP6)	Sugar beet	Gene rearrangement	CMS	Satoh et al., 2004
	Sunflower	Chimeric form orf1287	CMS	Makarenko et al., 2019
	Pepper	Gene rearrangement (*Ψatp6-2*)	CMS	Kim and Kim, 2006; Ji et al., 2013, 2014
	Sorghum[1], maize[2,3], rice[4]	mRNA editing	CMS	[1], Howad and Kempken, 1997; [2], Wang et al., 2009; [3], Li et al., 2019; [4], Hu et al., 2013
	Arabidopsis	*ftsh4* mutant	Decrease in protein abundance	Kolodziejczak et al., 2007; Gibala et al., 2009
b (ATP4)	Maize	mRNA editing	Kernel alterations	Wang et al., 2017
	Cauliflower	Heat stress	Enhance in abundance	Rurek et al., 2015
c (ATP9)	Stem mustard[1], soybean[2], ramie[3], rice[4]	mRNA editing	CMS	[1], Yang et al., 2007; [2], Jiang et al., 2011; [3], Liu et al., 2012; [4], Hu et al., 2013
	Transgenic tobacco	Unedited *atp9* from wheat	CMS	Hernould et al., 1993
		Antisense RNA (*as-atp9*)	Restoration of fertility	Zabaleta et al., 1996
	Carrot[1,2,3], cauliflower [4], sunflower[5,6]	Gene rearrangement	CMS	[1], Mandel et al., 2012; [2], Szklarczyk et al., 2000; [3], Szklarczyk et al., 2014; [4], Dieterich et al., 2003; [5], Makarenko et al., 2018; [6], Reddemann and Horn, 2018
d (ATP7)	Rice	Osmotic and salt stresses	Increase in abundance	Zhang et al., 2006
	Arabidopsis	Oxidative stress	Decrease in abundance	Tan et al., 2012
F_Ad (24 kDa)	Wheat	Repression of gene expression	CMS	Xu et al., 2008
	Arabidopsis	Gene mutation	CMS	Li et al., 2010
		T-DNA insertion -*Phosphite-insensitive* (*phi1*) mutant	Decrease in expression	Leong et al., 2018
OSCP (ATP5)	Arabidopsis	T-DNA insertion	Gametophyte lethality	Moore et al., 2003
		Decrease of expression (anti-*atp5*)	Seedling death, slow growth	Robison et al., 2009
ORFB (8, ATP8)	Sunflower[1], rice[2,3]	mRNA editing	CMS	[1], Sabar et al., 2003; [2], Das et al., 2010; [3], Chakraborty et al., 2015
6 kDa	Pepper	Interactions with Orf507	CMS	Li et al., 2013
	Rice[1], Arabidopsis[2]	Salt stress	Increase in abundance	[1], Zhang et al., 2006; [2], Zhang et al., 2008
	Arabidopsis	Oxidative stress	Decrease in expression	Zhang et al., 2008

subunits in some crucial physiological processes in plants, with special regard to CMS (Table 2).

4.1. Subunit α (ATP1)

In some plants, e.g. sunflower (Siculella and Palmer, 1988), cybrids between *Raphanus sativus* and *Brassica napus* (Sakai and Imamura, 1992), sugar beet (Kubo et al., 1999; Senda et al., 1993), stem mustard (*Brassica juncea*) (Yang et al., 2009; Yu et al., 2010) and upland cotton (*Gossypium harknessii*) (Wu et al., 2011), rearrangements of the gene encoding for subunit α have been linked to CMS. Nevertheless, in radish (Makaroff et al., 1990), sunflower (Köhler et al., 1991) and sugar beet (Xue et al., 1994), the involvement of this gene in CMS has been questioned. In tobacco, co-transcript of a novel reading frame *orf274* with *atp1* was found in both fertile and CMS plants (Bergman et al., 2000). A lower ATP/ADP ratio in floral buds of CMS plants was also observed, suggesting that the accumulation of the *orf274-atp1* co-transcripts might lead to CMS by interference with the expression of other mitochondrial genes (Bergman et al., 2000). A recent analysis of the wheat mitochondrial proteome revealed that a complex protein network is involved in the manifestation of CMS in wheat (Wang et al., 2015). Indeed, in such plants, a high proportion of proteins involved in mETC and in ATP synthesis is downregulated. In particular, the abundance of subunits β and δ, as well as subunit α is lowered, leading to impaired F-ATP synthase assembly and catalysis (Wang et al., 2015).

The importance of RNA editing of *atp1* was demonstrated in Arabidopsis, where the mutant for *OTP87* gene, which encodes for an editing factor belonging to the pentatricopeptide repeat (PPR) protein family, shows slow growth and delayed development (Hammani et al., 2011). This PPR protein is involved in the recognition and editing of at least two sites, namely *nad7-C24*, resulting in an unaltered protein sequence for subunit NAD7 in Complex I, and *atp1-C1178*. The editing of the latter site converts a Ser to Leu in subunit α and the loss-of-function of *OTP87* in mutated plants is related to an altered phenotype with drastic reduction in F-ATP synthase assembly (Hammani et al., 2011). In cotton (*Gossypium hirsutum*), RNA editing of *atp1* mRNA is also crucial for the energy requirements during fiber cell elongation: the absence of editing at C1292 and C1415 in *Ghatp1* is related to a decrease in F-ATP synthase activity and ATP content (He et al., 2018). This effect has been ascribed to the presence in the unedited subunit α of a Pro rather than a Leu, which is located in a conserved α helix. Such a non–conservative mutation has a dramatic effect on the subunit structure, probably because it destabilizes the α helix, leading to alteration of protein stability and assembly (He et al., 2018). A similar scenario was recently described in maize, where a novel PPR protein EMP21 was reported to be involved in mitochondrial RNA editing (Wang et al., 2019). The loss-of-function mutant *Emp21* is impaired in C-to-U conversion at five sites, among which are *atp1-1292* and *atp8-437*. Whereas the change seems not to be relevant in the latter for the synthesis and stability of subunit 8, in the former it leads to the same Leu/Pro substitution described above for the cotton subunit α, with consequent severe alteration of embryogenesis and endosperm development (Wang et al., 2019).

4.2. Subunit b (ORF25, ATP4)

In Arabidopsis, the *atp4* transcript is edited at site 89 by the RNA editing factor MEF3, which belongs to a subgroup of PPR proteins (Verbitskiy et al., 2012). In maize, the defective kernel mutant *dek36* is characterized by small and collapsed kernels, associated with alterations of embryo and seedling (Wang et al., 2017). It has been shown that *DEK36* encodes a PPR protein that is necessary for the correct editing, besides *nad7* (Complex I) and $ccmF_N$ (Complex III), of *atp4* at position 59, leading to substitution of Ser with Phe. Surprisingly, the lack of editing in the mutant *dek36* affects only slightly ATP4 function, since the abundance of F-ATP synthase resembles that of the wild type (Wang et al., 2017). However, these authors did not measure F-ATP synthase activity in either mutant or wild type.

4.3. Subunit a (ATP6)

The *atp6* gene is one of the most rearranged genes in the plant mitochondrial genome. CMS has been linked to rearrangements in mitochondria in sugar beet, where four new transcribed ORFs, which are absent in normal mtDNA and include *Satp6*, have been characterized (Satoh et al., 2004). Recently, a new open reading frame *orf1287*, a chimeric form of *atp6* gene, was shown to be associated with CMS in sunflower (Makarenko et al., 2019). Furthermore, the *atp6* gene has been proposed to be responsible for CMS in pepper: in male fertile plants, two copies of *atp6* are present, while in sterile plants, *Ψatp6-2* is a pseudogene resulting from mitochondrial genome rearrangements (Kim and Kim, 2006). In CMS pepper, *Ψatp6-2* is highly expressed in the anthers and correlates with enhanced hydrolysis of ATP (Ji et al., 2013). When the *Ψatp6-2* gene is silenced, fertility is restored and ATP hydrolysis is reduced (Ji et al., 2014). These authors speculated that the pseudogene could either have no protein function or encode a novel altered ATP6, which would promote ATP hydrolysis. Alternatively, we propose that, as subunit a is involved in the structure of F_O, its alteration might have significant consequences for F-ATP synthase, probably resulting in the increase of free F_1 sub-complex in the matrix, similar to that observed in maize (Li et al., 2019, see below). Free F_1, once not connected to the membrane sub-complex, might be responsible for the enhancement of ATP hydrolysis and therefore for the low energy production in CMS pepper.

Altered RNA editing of subunit a has been linked to CMS also in sorghum (Howad and Kempken, 1997), maize (Wang et al., 2009) and rice (Hu et al., 2013). Recently, it was shown in maize that EMP18, a mitochondrial DYW-PPR (a subclass of PPR), is crucial for RNA editing of the subunit a. In the *emp18* mutant, a change of amino acid in ATP6 from Leu212 to Pro causes the disruption of an αhelix and induces a strong decrease of F-ATP synthase assembly and activity, with a consequent accumulation of F_1 in the matrix. This results in altered seed formation, due to inhibition of development in the embryo and endosperm (Li et al., 2019).

In Arabidopsis, the mitochondrial ATP-dependent metalloproteases AtFtsH3 and AtFtsH4, present on the IMM and facing the matrix and the intermembrane space, respectively, are necessary for the correct assembly and stability of F-ATP synthase (Kolodziejczak et al., 2007). Accordingly, the mutant *ftsh4* contains a reduced amount of ATP6, probably because this unassembled subunit is degraded by matrix proteases, and exhibits impairment of mitochondria and chloroplasts, as well as alteration of leaf morphology (Gibala et al., 2009).

4.4. Subunit c (ATP9)

The mRNA for this subunit is extensively edited, causing amino acid changes and, in presence of a new termination codon, a shortened mature protein in *Oenothera* (Schuster and Brennicke, 1990), wheat (Bégu et al., 1990), potato (Dell'Orto et al., 1993) and in the moss *Physcomitrella patens* (Ichinose et al., 2013). The alteration of mRNA editing in *atp9* leads to CMS manifestation in stem mustard (*Brassica juncea* var. *tumida*) (Yang et al., 2007), soybean (Jiang et al., 2011), ramie (*Boehmeria nivea*) (Liu et al., 2012) and rice (Hu et al., 2013). Furthermore, when transgenic plants of tobacco are transformed with *atp9* from wheat, the presence of the unedited version of such a gene induces a significant number of plants showing CMS, while the plants possessing the edited *atp9* are fertile (Hernould et al., 1993). Restoration of fertility of CMS transgenic tobacco was obtained by antisense RNA (*as-atp9*), which inhibits unedited *atp9* gene expression (Zabaleta et al., 1996). Alterations of *atp9* due to gene rearrangements have also been associated with CMS in carrot (Mandel et al., 2012; Szklarczyk et al., 2000, 2014), *Brassica napus* (Dieterich et al., 2003) and sunflower

(Makarenko et al., 2018; Reddemann and Horn, 2018).

4.5. Subunit 8 (ORFB, ATP8)

In sunflower, where ORFB is the equivalent of subunit 8 (ATP8), the editing of *orfB* transcript converts cytosine to uracil at nucleotide positions 47, 58 and 452, leading to changes in amino acid residues Ser16 to Leu, Leu21 to Phe, and Phe151 to Leu, respectively (Sabar et al., 2003). In CMS sunflower, the aberrant chimeric ORF522 protein, which shares similarity with ORFB/ATP8 in the N-terminus, competes with ORFB inducing an impairment in the composition or assembly of F-ATP synthase (Balk and Leaver, 2001), as confirmed by the decrease in ATPase activity in sterile lines (Sabar et al., 2003). In rice, the unedited *orfB* gene transcript is responsible for male sterility and is associated with the decrease in ATP synthase activity (Das et al., 2010). The unedited rice ORFB has a Leu instead of a Phe at position 58, which alters the hydrophobicity of the protein and thus its correct position in the F_O complex in the IMM (Chakraborty et al., 2015).

4.6. Subunit δ (ATP16)

In cotton, ATP production by mitochondria is essential for the elongation process of the fibre cells and positively correlates with an upregulation of *GhATPδ1* (Pang et al., 2010). The expression of functional *GhATPδ1* in *S. cerevisiae atp*16Δ mutant, which lacks ATP16 and is impaired in ATP production, restores ATP synthesis to levels comparable to the wild type (Pang et al., 2010). Consistently, in Arabidopsis the subunit δ gene is highly expressed in pollen, ovules and floral primordia, all tissues characterized by a high-energy requirement (Geisler et al., 2012). Arabidopsis *atpδ-1* mutant, obtained by T-DNA insert in the intron of such a gene, shows a reduced production of pollen, with severe alteration in germination capacity. Furthermore, downregulation of subunit δ gene by RNA interference (δRNAi) induces a decrease in the amount of F-ATP synthase and the modified plants exhibit stunted growth and male sterility. Unexpectedly, the levels of ATP in δRNAi lines are comparable to wild type, suggesting a possible compensation of ATP production by glycolysis. The retarded growth in δRNAi lines has been attributed to metabolic adjustments rather than energy deficiency in vegetative tissues, but flower and pollen development are compromised because of their high-energy requirements (Geisler et al., 2012).

4.7. OSCP (ATP5)

The insertion of T-DNA in the ATP5 gene in Arabidopsis is gametophyte lethal (Moore et al., 2003) and transgenic Arabidopsis plants, in which the expression of either OSCP (ATP5) or subunit γ (ATP3) has been lowered, show similar altered phenotype (Robison et al., 2009). In particular, during germination in the light, anti-*atp3* or anti-*atp5* induction by a dexamethasone-inducible promoter causes the death of the seedlings soon after emergence. When the induction is provided after germination, the plants show slow growth, alterations in development, as well as in leaf and inflorescence morphology. Similarly to the *atpδ-1* mutant, ATP content increases in induced transgenic etiolated seedlings grown with sucrose or in soil-grown transgenic plants in the light. This observation suggests that, similarly to what described above for subunit δ, when mitochondria are defective, other sources for ATP production, such as photophosphorylation or increase in glycolysis, might at least partially support the cellular energy requirements (Robison et al., 2009).

4.8. Subunit F_Ad

The role of subunit F_Ad has been linked to the correct development of anther in wheat, where the expression of *TaF_Ad* is repressed in sterile plants (Xu et al., 2008). In Arabidopsis, the *MALE GAMETOPHYTE DEFECTIVE 1 (MGP1)* gene, which encodes subunit F_Ad, is highly expressed in pollen during the late developmental stages. The mutant *mgp1/+* shows altered mitochondrial morphology during the dehydration phase of pollen, causing their degeneration (Li et al., 2010).

4.9. Subunit 6 kDa

In chili pepper, the mitochondrial protein Orf507 has been proposed as a candidate for CMS (Li et al., 2013). These authors suggested that the mechanism leading to CMS is due to the interaction of the N-terminus and middle regions of Orf507 with the subunit 6 kDa, causing the decrease in F-ATP synthase activity and the subsequent decrease in ATP content observed in defective pollen grains (Li et al., 2013).

5. How environmental stress affects F-ATP synthase

Mitochondria are recognized as one of the central units for the reception of stress signals and the arrangement of the immediate responses (Rasmusson and Møller, 2011), especially in the case of tolerance development in plants (Pastore et al., 2007). The high energetic demand required by each strategy to counteract the stress is further evidence for the pivotal role of mitochondria and particularly of the F-ATP synthase (Manatt and Chandra, 2011). ATP availability is essential to support the synthesis and translocation of osmolytes to be used during several types of stress that ultimately result in an imbalance of the osmotic potential. This is observed under conditions of salt excess, drought and low temperatures. The contribution of F-ATP synthase to ATP production, therefore, is crucial not only in the dark, but even in the light, when energetic support for *ex novo* biosynthetic activities, transport and creation of electrochemical gradients are required for an adequate response to stresses (Jacoby et al., 2018). Since plants are sessile organisms, they must withstand changing environmental conditions, and the analysis of plant responses to temperature stress represents a good model to study these events (Kerbler et al., 2019).

5.1. Temperature stress

Temperature variations do not only concern day/night and seasonal alternations, but also affect the above-ground organs and the roots differently, as the latter benefit from more constant temperature conditions. Low, but not freezing, temperatures induce a decrease in F-ATP synthase activity, lowering of the ADP/O ratio and lowering of the amount of ATP. This enzyme is more markedly inhibited than the other components of the mETC (Rurek et al., 2018). A specific physiological response of plants to cold consists of induction and activation of alternative oxidase (Rurek et al., 2018; Vanlerberghe, 2013, 2020, in this Special Issue), but a minimum synthesis of ATP is maintained, thanks to the proton transport still exerted by Complex I (Vianello et al., 1997). In contrast, at high temperatures (e.g. above 35 °C for mesothermal plants), adenylate restriction and changes in substrate supply become the limiting factors (Kerbler et al., 2019).

The effects due to cold and warm environmental conditions are different also regarding the proteomic and transcriptomic profiles, as verified in a study carried out on cauliflower (*Brassica oleracea* var. *botrytis*) curds. In particular, the proteomic analysis revealed significant quantitative effects at high temperatures only, during which the expression of F-ATP synthase subunit b was enhanced and subunit γ was decreased, showing a completely distinct pattern if compared to cold stress. When normal conditions were restored, during the recovery phase following heat stress, the subunit γ was still underexpressed, decreasing the stability of F-ATP synthase and impairing its assembly. Consequently, the enzymatic activity decreased both during the heat treatments and during the recovery phase (Rurek et al., 2015). In a subsequent study, these authors showed that during heat stress the abundance of subunits α and d increased to a different extent, while during the recovery phase both subunits decreased. It was therefore

suggested that the enhanced ATP requirement during heat stress induces the overexpression of selected subunits, favoring assembly of F-ATP synthase complexes, which nevertheless are labile during the following recovery phase (Rurek et al., 2018, 2015).

Heat stress affects germination of oat seeds, previously treated during storage period by high temperature and two different moisture levels, and modulates the expression of the F-ATP synthase subunits α and δ (Chen et al., 2016). Subunit α is progressively down-regulated when the storage temperature increases up to 50 °C, while subunit δ exhibits a different pattern with a peak of expression at 45 °C. Notably, in this experiment the abundance of the F-ATP synthase showed a clear direct correlation with germination and thermo-tolerance of oat seed, which was suggested to depend on the decrease in the supply of ATP (Chen et al., 2016).

Heidarvand et al. (2017) published a comprehensive review on the variations of the F-ATP synthase subunits induced by low temperature stress in plants. Such a response is not easily attributable to a definite behavior, since both increasing and decreasing effects on the abundance of some F-ATP synthase subunits have been reported. This complex response might depend on several factors such as species, duration and severity of the stress. Accordingly, in the case of subunit α, there are several reports of its increased abundance at low temperature, and several others that find a decreased or even no change in abundance (Heidarvand et al., 2017). More consistent data are available for subunit β, which becomes more abundant at temperatures lower than 5 °C, while temperatures ≥ 15 °C induce a decrease in abundance (Gammulla et al., 2011; Neilson et al., 2011). Three different papers on soybean seeds, wheat and rice leaves, report that over a wide range of low temperatures, the abundance of subunit F_Ad is lowered (Gammulla et al., 2011; Rinalducci et al., 2011; Yin et al., 2009). A further peculiar feature concerns subunit γ, the abundance of which in sunflower is either increased or decreased in chilling-sensitive and in chilling-tolerant cultivars, respectively (Balbuena et al., 2011).

5.2. Salt stress

Experimental analyses performed on *Mesembryanthemum crystallinum* have shown that the responses of such a halophytic plant to saline stress have a dual nature, since both osmotic alteration and an ionic unbalance can occur (Tran et al., 2019). Increase in ATP synthesis was demonstrated to be dependent on the ionic effect, and this feature is specific for halophytic plants. They exhibit an increase of ATP content in the presence of NaCl up to 300 mM, probably because such highly specialized plants show high fitness in saline environments and even a biomass increase with NaCl concentrations up to 100 mM. Similar results have been found in the case of NaCl treatments in wheat, where a high salt concentration increased F-ATP synthase activity, even if there are several overlapping factors able to enhance respiratory rates during stress (Jacoby et al., 2016). On the other hand, this stimulation is hardly explained by increased abundance of F-ATP synthase subunits, since contrasting effects of salinity have been shown on the various isoforms (Jacoby et al., 2016).

Jacoby and coworkers studied the salt tolerance in wheat by comparing a salt-sensitive cultivar with a salt-tolerant amphiploid, obtained by crossing bread wheat with the wild wheatgrass *Lophopyrum elongatum*, which is adapted to salt marshes (Jacoby et al., 2013). Besides other biochemical and physiological traits, they focused on a detailed analysis of the genotypic differences in the mitochondrial proteome. Although salt treatment induced a decrease of the respiratory parameters in isolated mitochondria, the composition of the main respiratory complexes was only slightly modified, except for a significant induction of the alternative oxidase. Modifications of protein patterns, and in particular of antioxidant enzymes, is a peculiar feature of the hybrid wheat. Nevertheless, the genetic basis for salt tolerance did not appear to be associated with F-ATP synthase, since only subunits β and F_Ad changed in abundance compared to the sensitive variety. In this case, the modulation of the protein content cannot explain the contribution of F-ATP synthase catalytic activity to the metabolic adjustments during stress.

The pattern of F-ATP synthase subunits during abiotic stress responses has been analyzed with the aim to dissect the contribution of the different components. This experimental approach has frequently demonstrated the involvement of subunit 6 kDa, one of the putative components of the F_O sub-complex, during salt stress (Zhang et al., 2008). In rice, osmotic and saline stresses, beyond the enhancement of subunits *δ*1 (homologous to Arabidopsis subunit δ/ATP16) and *δ*2 (homologous to Arabidopsis subunit d/ATP7) in leaves, caused the overexpression of the subunit 6 kDa in both leaves and roots (Zhang et al., 2006). Consistently, salt resistance was induced by overexpressing subunit 6 kDa in transgenic tobacco plants at the seedling stage (Zhang et al., 2006). In addition, subunit 6 kDa gene expression was induced during salt excess, drought and low temperatures in Arabidopsis (Zhang et al., 2008). This effect was confirmed in transgenic Arabidopsis plants, where subunit 6 kDa gene overexpression induced a significant increase in resistance against the aforementioned abiotic stresses (Zhang et al., 2008). There is also evidence for the participation of subunit 6 kDa in metabolic adaptations during the early phases of abiotic stress in wheat (Moghadam et al., 2012). The gene shows *cis*-acting elements able to respond to ABA, suggesting a potential role of this subunit in the modulation of the signaling stress pathway.

Even if subunit 6 kDa has not been unambiguously associated with F_O (Brugière et al., 2004), it has been suggested that its binding to the F_O portion could help to activate the phosphorylating activity to meet the high energetic needs required by the plant cell under stress. The function proposed for this small protein would be to modulate the mitochondrial activity. This hypothesis suggests that during stress subunit 6 kDa might induce an early and quick response, increasing the amount of ATP provided by F-ATP synthase (Zhang et al., 2006, 2008).

5.3. Other environmental stresses

Some interesting studies have been published on the effects of stress caused by either Al toxicity or phosphate starvation on F-ATP synthase. As demonstrated for Al toxicity in wheat (Hamilton et al., 2001), the level of transcripts coding for subunits α and β was unchanged, suggesting that the stimulation of the F-ATP synthase activity might be due to post-translational modifications. In Al-resistant wheat cultivars, this mechanism would increase ATP production to maintain energy balance in plants under metal pollution stress (Hamilton et al., 2001).

Along with ADP, Pi represents the substrate for ATP synthesis and it is a limiting factor for plant growth. Pi starvation induces complex responses that are suppressed by phosphite (HPO_3^{2-}), a non-metabolizable Pi analog. Arabidopsis *phi1* mutants, which are impaired in the gene encoding subunit F_Ad, retained the activation of Pi starvation responses even in presence of HPO_3^{2-}, showing a decrease in ATP content in roots, together with a more pronounced effect of oligomycin on growth, and a larger membrane potential in the mitochondria (Leong et al., 2018). These *phy1* mutants are therefore a powerful tool to study the signaling pathway involved in Pi starvation, suggesting the involvement of F-ATP synthase in the modulation of plant responses to Pi starvation (Leong et al., 2018).

5.4. Oxidative stress

Several kinds of stress affecting plant mitochondria lead to an increase in oxidative metabolism, due to both sudden increase in respiration, known as oxidative burst, and alteration of electron flow in the mETC. The accumulation of reduced intermediates with unpaired electrons causes the production of reactive oxygen species, mainly at Complex I and III (Braidot et al., 1999; Casolo et al., 2000; Møller, 2001; Jacoby et al., 2018). In Arabidopsis, these events have a negative impact on F-ATP synthase, in which the degradation of subunits α, β

and d has been linked to the induction of protease activity (Sweetlove et al., 2002). Among F-ATP synthase subunits, subunit β seems to be particularly sensitive to different oxidative agents, as demonstrated by the presence of its degradation products caused by ATP-dependent protease activity. On the other hand, degradation of subunit β still maintains a residual activity of F-ATP synthase, able to sustain the metabolism responses during oxidative stress. This hypothesis is supported by the presence of a partial mitochondrial respiratory activity, despite the addition of H_2O_2 (Sweetlove et al., 2002).

Tan and coworkers (Tan et al., 2012) applied a quantitative proteomic approach to investigate how oxidative stress affects the abundance of the components of the mitochondrial oxidative phosphorylation in Arabidopsis. Consistent with previous results, oxidative stress induced by antimycin, $CuCl_2$ or H_2O_2 treatments lowered the abundance of F-ATP synthase subunits α, β and d. In contrast, an increase in subunit c abundance was detectable when mitochondria were exposed to menadione, a redox-active quinone that stimulates the production of superoxide anion. These results show the dynamic responses of the phosphorylating machinery during environmental abiotic stresses (Tan et al., 2012).

Furthermore, experiments carried out on Arabidopsis by microarray techniques have excluded inhibitory effects of oxidative stress on the expression of nuclear genes that code for the F-ATP synthase (Yu et al., 2001). However, treatments with the herbicide Paraquat or with H_2O_2 caused inhibitory effects limited to the expression of the gene coding for subunit 6 kDa in Arabidopsis suspension cell culture (Zhang et al., 2008).

A multifactorial approach using proteomic and metabolomic analyses was applied in Arabidopsis suspension cell cultures to disentangle the mechanisms underlying the modifications in mitochondrial supercomplex composition, in particular during oxidative stress (Obata et al., 2011). In agreement with previous studies, the relationship between the transcription and the abundance of components of the F-ATP synthase and its enzymatic activity was not tight. The immunochemical detection of the subunit β showed a significant increase after treatments with menadione, while its transcript level was unchanged. Strikingly, subunit 8 showed the opposite trend, exhibiting only a stimulation of its transcript level. Therefore, the modulation of F-ATP synthase during oxidative stress still demonstrates a separation between the effects on proteome profile and the metabolic changes due to stress response. Especially during early events of the stress response, it is conceivable that post-transcriptional (Koussevitzky et al., 2008) or post-translational (Morgenthal et al., 2007; Møller et al., 2020) modifications, e.g. phosphorylation (Struglics et al., 1998; Havelund et al., 2013), might prevail, because this strategy allows a fine tuning of the biochemical pathways to maintain homeostasis.

Finally, a comprehensive model for the involvement of F-ATP synthase in stress responses is still lacking, owing to the heterogeneity of the stress duration and magnitude applied in the different experiments. In addition, the number of mitochondrial proteins affected by stress is still largely underestimated (Rurek et al., 2018). Poor coordination of mitochondrial gene transcriptional machinery to stress and the possible interactions with import mechanisms should be also considered (Rurek et al., 2018, 2015). Furthermore, the occurrence of post-translational modifications, able to stimulate enzymatic F-ATP synthase activity, cannot be excluded (Hamilton et al., 2001). All these features make it difficult to establish clear relationships between transcript levels, subunit expression, assembly status and phosphorylation activity of F-ATP synthase (Meyer et al., 2019).

6. F-ATP synthase beyond ATP synthesis

More than 60 years ago it was observed that mitochondria could undergo a sudden permeability increase of the IMM that leads to mitochondrial swelling (Raaflaub, 1953a, b). This feature was later defined permeability transition (PT) (Haworth and Hunter, 1979; Hunter and Haworth, 1979a, b) and the putative channel involved in the PT was named the Permeability Transition Pore (PTP). From the 1970s, the chemiosmotic theory proposed by Peter Mitchell (Mitchell, 1961) has been largely accepted and therefore the PT has been considered as an artifact, since its occurrence leads to the collapse of the proton gradient, with the consequent decrease in ATP synthesis. However, the PT was reconsidered when cyclosporin A (CsA), an immunosuppressive agent interacting with CyPD, was discovered to be a potent PT inhibitor (Broekemeier et al., 1989; Broekemeier and Pfeiffer, 1989; Crompton et al., 1988; Davidson and Halestrap, 1990; Fournier et al., 1987) and a useful tool to demonstrate the occurrence of the PTP opening in cells and living organisms (Bernardi et al., 2015). The discovery that PTP is involved in the release of cytochrome *c* and in the activation of the intrinsic pathway to apoptosis rapidly made the PT very popular in mitochondrial research (Bernardi et al., 2015). A common feature shown by PTP in the species so far examined is the dependence on matrix Ca^{2+}. The molecular structure of the PTP was long elusive and many potential proteins were proposed to be component of the pore, such as the voltage-dependent anion channel (VDAC), the benzodiazepine receptor, the adenine nucleotide translocase (ANT) and the phosphate carrier. However, PT is still observed in isolated mitochondria when the expression of each of these proteins has been suppressed (Baines et al., 2007; Gutiérrez-Aguilar et al., 2014; Kamei et al., 2018; Kokoszka et al., 2004; Krauskopf et al., 2006; Šileikytė et al., 2014). Recently, in mammalian (Alavian et al., 2014; Giorgio et al., 2013), *S. cerevisiae* (Carraro et al., 2014; Kamei et al., 2018) and *Drosophila melanogaster* (von Stockum et al., 2015) mitochondria, the F-ATP synthase, in its dimeric form, was shown to be an essential component responsible for PT (Fig. 4). In mammals, CyPD interacts with subunit OSCP, and modulates the F-ATP synthase activity as well, in a CsA-sensitive way as CsA displaces CyPD from OSCP (Giorgio et al., 2013). This finding and the results obtained by site-directed mutagenesis of mammalian and yeast F-ATP synthase (Antoniel et al., 2018; Giorgio et al., 2017; Guo et al., 2018, 2019) led to the proposal that F-ATP synthase undergoes a Ca^{2+}-dependent conformational change. Such a modification is favored by CyPD binding and propagates from the catalytic site through OSCP and the peripheral stalk to the inner membrane, where the PTP forms (Fig. 4B). Highly purified F-ATP synthase dimers inserted into liposomes form Ca^{2+}-activated channels with properties matching those of the PTP, further demonstrating that

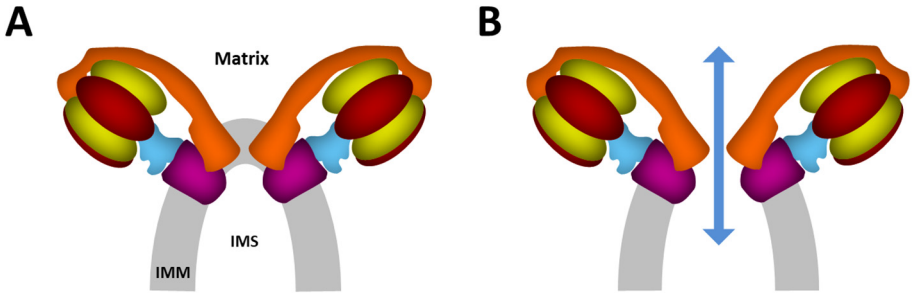

Fig. 4. F-ATP synthase dimers forming the PTP. A) The dimers are formed by the interactions between F_O components. This structure is stabilized by ATP, ADP, Mg^{2+}, or by CsA in the matrix, which displaces CyPD (not shown), in a «desensitized» conformation. B) When the matrix Ca^{2+} concentration is raised and/or in the presence of oxidizing conditions («sensitized» conformation), the dimers would dissociate, leading to the opening of PTP and leakage of solutes from the matrix.

this complex represents a strong candidate for PTP formation (Mnatsakanyan et al., 2019; Urbani et al., 2019). This hypothesis has been questioned by the finding that in HAP1 cells the PT still occurs after genetic ablation of subunit c (He et al., 2017b), or peripheral subunits b and OSCP (He et al., 2017a). However, as discussed by Bernardi (Bernardi, 2020), a careful analysis of those results highlights that mitochondria lacking an assembled F–ATP synthase displayed bongkrekate (a specific inhibitor of ANT) -sensitive Ca^{2+}-activated channels. Such a finding suggests that the PT pathway could also be provided by ANT, which forms smaller CsA/bongkrekate-sensitive channels (Brustovetsky et al., 2002). Thus, mitochondria appear to have at least two pathways for permeabilization, mediated by F-ATP synthase and by the ANT.

In plants, PT has been observed in several species. In mitochondria from etiolated pea stems, Ca^{2+} induced a collapse of the transmembrane electrical potential ($\Delta\psi$), which was delayed by CsA (Vianello et al., 1995). In mitochondria from potato tuber, Ca^{2+} induced PT that was reported to be either CsA-sensitive (Arpagaus et al., 2002) or -insensitive (Fortes et al., 2001). In mitochondria from oat leaves (Curtis and Wolpert, 2002) and wheat roots (Virolainen et al., 2002), a CsA-insensitive $\Delta\psi$ collapse was induced by Ca^{2+} and Pi, leading to matrix swelling. In Arabidopsis, the opening of PTP induced by Ca^{2+} was shown to play a fundamental role in salt stress response (Zhao et al., 2013). Even if plant mitochondria show diverse PTP phenomenology, their PTP opening shows remarkable similarities with mammalian mitochondria, such as induction by Ca^{2+} and release of cytochrome *c* in the cytosol as consequences of matrix swelling (Vianello et al., 2012). This would lead to the onset of programmed cell death as a common characteristic shared between yeast, insects, mammals, and plants (Arama et al., 2006; Balk et al., 1999; Giannattasio et al., 2008; Robertson and Orrenius, 2002). Our group has recently examined some functional features of the PT in pea stem mitochondria, in the light of recent advances in other species described above. Pea stem mitochondrial PTP is characterized by Ca^{2+} induction and inhibition by CsA similarly to PTP in mammals, Drosophila and yeast, yet it possesses some peculiar features, such as inhibition by Pi, lack of swelling and activation by oligomycin (De Col et al., 2018). The latter characteristic could be related to the observation that oligomycin is also a strong inhibitor of Ca-ATPase activity of F-ATP synthase, as expected if ATP hydrolysis is coupled to proton translocation. In the presence of Ca^{2+}, proton backflow would be the possible result of the ATPase activity, which cannot generate (or maintain) a proton gradient across the IMM. This supports the proposal that also in pea stem mitochondria the PTP may originate from a Ca^{2+}-dependent conformational transition of F-ATP synthase (De Col et al., 2018). Unfortunately, it was not possible to unequivocally assign the current elicited by Ca^{2+} to F-ATP synthase dimers incorporated into lipid bilayer by electrophysiology experiments, nor was it possible to identify the matrix protein responsible for CsA sensitivity. Therefore, even if some results suggest the involvement of F-ATP synthase in PT manifestation in pea stem mitochondria, it has not yet been possible to draw a conclusive picture of the molecular identity of PTP in plants.

7. Conclusions

As documented in this review, due to the limited knowledge of structural and functional properties of F-ATP synthase in plants, many interesting questions are still open. We are looking forward to a better characterization of all the subunits comprised by this complex, especially those that have been classified as plant specific and which would be responsible for the unique features of this enzyme in plants. Furthermore, the picture would be clearer if the presence, alteration, or absence of the F-ATP synthase subunits could be related to the actual assembly state and/or to the enzymatic activity, evaluated directly either as ATP synthesis or hydrolysis.

Another issue that still requires deeper investigations is the analysis of post-translational modifications of F-ATP synthase subunits, which would represent a key strategy for fine enzymatic regulation. In our opinion, future work should also examine how F-ATP synthase, beyond providing energy for most of the cytosolic requirements, is involved in the regulation of the cell energetic status. This is a particularly crucial aspect related to the plant responses to stress and to the initial stages of programmed cell death. In this scenario, it would be interesting to verify if this complex represents one of the structural equivalents of the PTP. It is remarkable that F-ATP synthase might be an auto-regulative system, able to switch from energy production to energy dissipation triggering cell death, the latter being a crucial process for initiating the pro-apoptotic pathway activated under many physiological (e.g. xylem differentiation) or stress conditions (e.g. salt stress).

The extraordinary conservation shown by many subunits of F-ATP synthase suggests that comparison between different *phyla*, could reveal both common and unique features linked to diverse adaptive strategies. In particular, this enzyme would represent a key element for a systemic and conservative view of the energetic metabolism in different organisms.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2020.06.001.

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