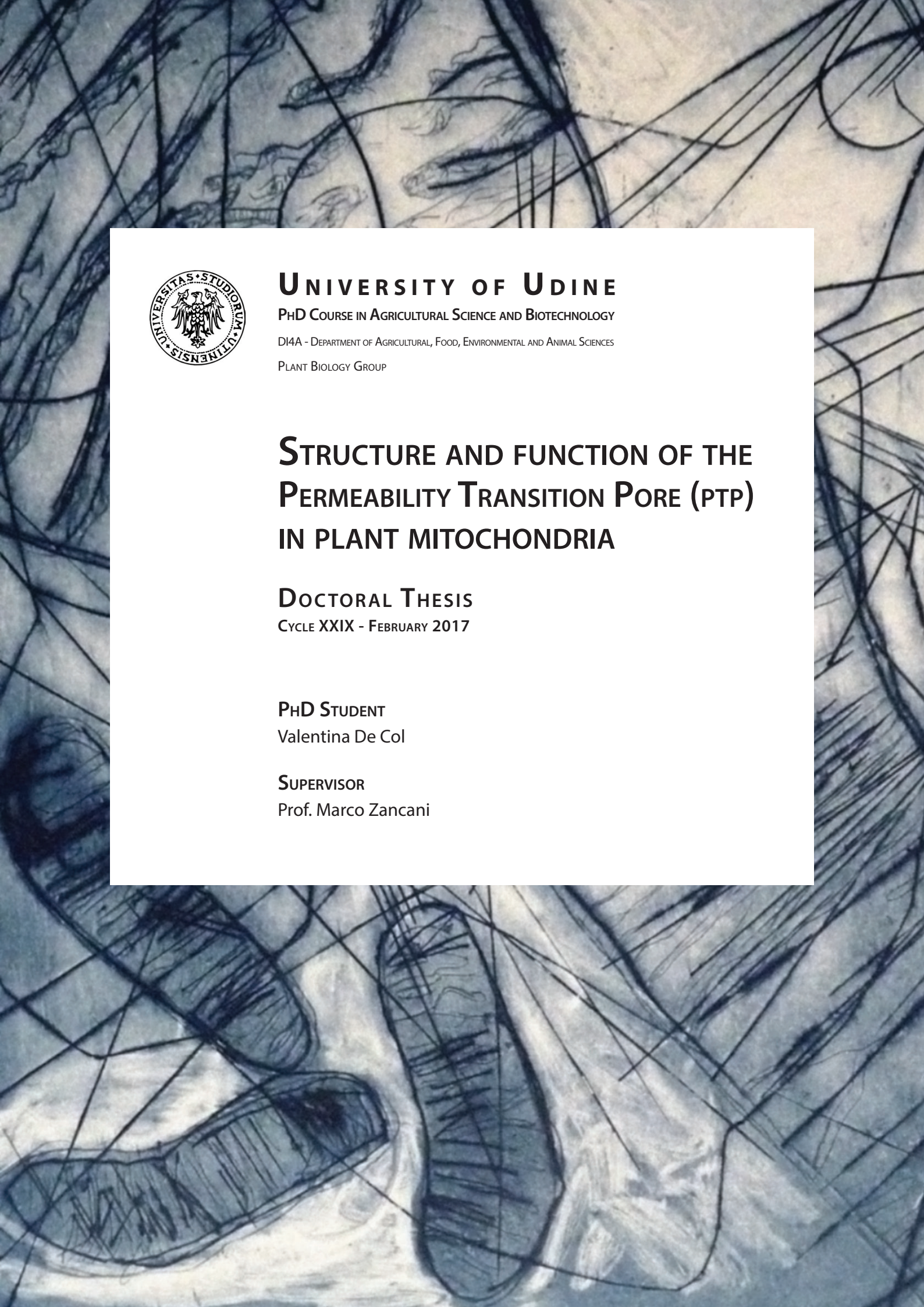

University of Udine

PhD Course in Agricultural Science and Biotechnology

DI4A - Department of Agricultural, Food, Environmental and Animal Sciences

Plant Biology Group

Structure and function of the Permeability Transition Pore (PTP) in plant mitochondria

Doctoral Thesis

Cycle XXIX - February 2017

PhD Student

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Supervisor

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Cover painting *Mitochondial lines*
Courtesy of the artist Melissa Belli

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Table of content

Abstract

Mitochondria are multifunctional organelles with a crucial role in bioenergetics, being the major generators of ATP in the cell. The energetic machinery is responsible for a tightly controlled process, but in some cases it can encounter a sudden disruption through the opening of the mitochondrial permeability transition pore (PTP).

The identity of PTP has not been completely unravelled yet, and it represents one of the most elusive topics in cell biology. The current idea is that PTP coincides with mitochondrial proteins of major importance, preserved through evolution.

In the controversial scenario of molecular attributions for PTP, F-ATP synthase dimers were recently suggested and proved to be one strong candidate for the PTP in mammals, yeast and flies. This work aims to investigate if this model could also be applied to plant mitochondria, where no evidence has been collected so far. In this scenario, F-ATP synthase might represent an enzyme with multiple functions: shaping mitochondrial cristae architecture, sustaining life through ATP production and becoming also a permeability system, hence controlling life and death processes.

In this 'three-paper dissertation' we reviewed the literature on PTP in plants, collected new evidence on permeability transition (PT) and PTP in isolated pea mitochondria and established a novel methodology for monitoring ATP levels and dynamics with the fluorescent protein sensor ATeam.

Thesis Structure

This thesis consists of one published review and two manuscripts to be submitted. It is organized in one introduction on the general topic (chapter 1), the presented papers (chapter 2), each preceded by a short preface, and a conclusion with ideas for further research (chapter 3). References for chapters 1 and 3 are at the end (cited literature). The appendix presents a list of different contributions (posters, abstracts and participation as speaker in a conference) as additional activities performed during the PhD course.

The introduction is divided into different sections that cover various aspects of the main topic. This will give a general background, using selected literature related to the main topic. It aims also to highlight how the knowledge of some bioenergetic aspects in plants is still limited, compared to other organisms.

All the work presented is the result of my three year of research during the PhD program. Two papers presented in this thesis are centred on the phenomenon of the permeability transition in plants: one in the form of a review (chapter 2.A) and one is an experimental work (chapter 2.B). Both were pursued with the support and the experience of the Plant Biology Group of the University of Udine. The third paper (chapter 2.C) presents the scientific outcome achieved at the Plant Energy Biology Group, University of Bonn (Germany), during two different internships. The result of the latter work is a methodologic paper that will be included in a manuscript of wider application.

List of abbreviations

Abbreviations	Explanation
AA	antimycin
AK	adenylate kinase
ANT	adenine nucleotide translocatoe
AO	acridine orange
Ap5A	P^1, P^5-di(adenosine-5')pentaphosphate
ATP	adenosine-5'-triphosphate
BN-PAGE	blue native PAGE
Bz-423	benzodiazepine-423
cATR	carboxyatractyloside
CM	crude pea stem mitochondria
CRC	calcium retention capacity
CsA	cyclosporin A
CyP-D	cyclophilin D
CyPs	cyclophilins
Cyt *c*	cytochrome *c*
HK	hexokinase
HR	hypersensitive response
IEF	isoelectric focusing
KCN	potassium cyanide
MCU	mitochondrial Ca^{2+} uniporter
MMC	mitochondrial megachannel
mPT	mitochondrial permeability transition
OMM	outer mitochondrial membrane
OSCP	oligomycin sensitivity-conferring protein
PBR	peripheral benzodiazepine receptor
PCD	programmed cell death
PheAsO	phenylarsine oxide
P_i	inorganic phosphate
PiC	phosphate carrier
PM	Percoll-purified mitochondria
PT	permeability transition
PTP	permeability transition pore
ROS	reactive oxygen species
SMP	sub-mitochondrial particles
SPG7	spastic paraplegia protein 7
TSPO	benzodiazepine receptor
VDAC	voltage-dependent anion channels
$\Delta\Psi$	transmembrane electrical potential difference

1. The mitochondrial Permeability Transition (PT) and the Permeability Transition Pore (PT)

1.1 Definitions, occurrence and consequences

It is well established that inner mitochondrial membrane needs to be an almost sealed and selective barrier to molecules and ions in order to maintain the electrochemical gradient across the inner membrane. The energy of the gradient built up by the respiratory H^+ pumps (complexes I, III, IV) is then used to drive ATP synthesis by F-ATP synthase (complex V), as postulated by the chemiosmotic theory proposed by Mitchell in 1961 (Mitchell, 1961; Brookes et al., 2004).

From the late 1970s, a peculiar phenomenon was noticed in mammalian mitochondria. They could experience, in fact, a sudden and non-selective increase in the permeability of the inner membrane to low and medium molecular weight compounds up to 1.5 kDa (Hunter and Haworth, 1979). The process was then named as 'mitochondrial permeability transition' (mPT) (Hunter and Haworth, 1979) and it was found to occur via the opening of the pore, a channel for the passage of solutes through the inner membrane.

This membrane permeabilization causes severe bioenergetic dysfunctions in the mitochondrion. The opening of the permeability transition pore (PTP) triggers a cascade of events, generally summarised in membrane potential collapse (depolarisation), uncoupled oxidative phosphorylation, depletion of ions and metabolites, swelling, disruption of the outer mitochondrial membrane and release of intermembrane compounds, among which pro-apoptotic factors, crucial for the activation of different death pathways (Bernardi, 1999; Arpagaus et al., 2002; Brookes et al., 2004). Swelling and outer membrane rupture are not, however, inevitable consequences of PTP opening: swelling apparently does not take place for very short opening and/or for lower conductance states of PTP (Bernardi, 1999). *Cristae* remodelling can also occur in the absence of outer membrane disruption (Bernardi and von Stockum, 2012).

The role of PTP in pathological cell injury and death has been established by the discovery that the opening of the pore is mechanistically linked to cytochrome *c* (Cyt *c*) release (Brookes et al., 2004). Cyt *c* is the only water-soluble cytochrome and mobile electron carrier (Crompton, 1999) and its release from the intermembrane space into the cytosol proved to trigger apoptosis, one of the most important programmed cell death in animals (Jones, 2000). PTP occurrence was found to be crucially related to several human pathologies as myocardial ischemia-reperfusion injury, stroke, cancer, hepatocarcinogenesis, neurodegenerative disease and muscular dystrophies (Bernardi et al., 2006; Biasutto et al., 2016; Šileikyte and Forte, 2016). Despite the fact that the manifestation of PT can decide the entire cell destiny, the complexity of the mechanism and the players involved is still far from being completely resolved.

1.2 Modulation of PTP

The primary requirement for PTP opening is the matrix accumulation of Ca^{2+} (Hunter and Haworth, 1979; Halestrap et al., 1993; Bernardi et al., 2016). Ca^{2+} uptake is a finely regulated mechanism under physiological conditions, but it can turn into a potentially harmful process leading to cell death (Duchen, 2000). The concentration of Ca^{2+} necessary for PTP opening depends, beside species and tissues, on inducers with a synergic effect (Arpagaus et al., 2002). The list includes: inorganic phosphate (P_i), inside-positive membrane potential, thiol-oxidizing reagents, low ATP levels, fatty acids and anoxia. In contrast, thiol-reducing agents, low pH, inside-negative membrane potential and divalent cations other than Ca^{2+} (e.g. Mg^{2+}) counteract the opening (Halestrap et al., 1993; Bernardi, 1999; Kushnareva and Sokolove, 2000; Arpagaus et al., 2002; Bernardi et al., 2015; Biasutto et al., 2016). Only the immunosuppressive agent cyclosporin A (CsA) was found to modulate effectively the PTP, binding cyclophilin D (CyP-D), its matrix receptor (Bernardi et al., 2015). CsA can increase or even double the Ca^{2+} threshold required to open PTP (Bernardi and von Stockum, 2012) and its effect is best described as 'desensitization', more than an inhibition, meaning that PTP becomes more resistant to opening after the uptake of Ca^{2+} and P_i (Azzolin et al., 2010). The use of submicromolar concentration of CsA during *in vitro* and *in vivo* experiments is still used today as the primary and sharpest diagnostic trait of PT (Arpagaus et al., 2002; Bernardi et al., 2015). Recent pharmacological strategies are now searching for potent and selective PTP inhibitors, in addition to CsA and its derivatives (Šileikyte and Forte, 2016). This, in combination with advances in genetics, will hopefully provide novel clues about PTP and a tool for understanding and treating human diseases.

1.3 What is the molecular identity of the pore?

From early studies, a multi-state channel defined as 'mitochondrial megachannel' (MMC) was characterised in rat liver mitochondria (Szabó and Zoratti, 1992). Later on, it was noticed that the properties and the modulation of this channel corresponded to those of PTP, leaving little doubt that MMC represents the electrophysiological equivalent of PTP, particularly for the specific effect of CsA (Bernardi, 1999; Bernardi et al., 2006).

When PTP became a topic of interest, no single candidate protein was believed to account for the complexity of PT modulation, therefore the tendency was to increase the number of putative components. Among them were the peripheral benzodiazepine receptor (PBR or TSPO), the adenine nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC), the phosphate carrier (P_iC), CyP-D as a detachable regulatory protein (Bernardi et al., 2006; Biasutto et al., 2016), the anti-apoptotic protein Bcl-2, hexokinase (HK), the intermembrane creatine kinase and nucleoside diphosphate (Bernardi et al., 2015).

A long-standing hypothesis suggested that PTP was formed at a contact sites between the inner and the outer mitochondrial membranes (Bernardi et al., 2006; Šileikyte and Forte, 2016), particularly involving VDAC (outer membrane), ANT (inner membrane) and CyP-D (Crompton, 1999; Bernardi et al., 2015). For many years, VDAC and ANT were considered the main candidates for the PTP, until genetic deletion studies had unequivocally shown that

neither of these proteins are involved in PT manifestation (Azzolin et al., 2010). Mitochondria lacking ANT and/or VDAC isoforms revealed that a Ca^{2+}-dependent PT was still occurring (Bernardi et al., 2015).

An alternative model proposed that the pore is the result of the aggregation of misfolded integral membrane proteins damaged by oxidant and other stresses (He and Lemasters, 2002), but in this hypothesis PTP regulation by the voltage and matrix pH were both not considered (Bernardi et al., 2006).

In the rather heated debate on PTP, CyP-D is still agreed to be an important regulator of the pore opening (Biasutto et al., 2016) and, although it does not represent a structural pore component, it is considered for its pivotal role in the interaction with CsA and, more generally, for PTP modulation (Bernardi et al., 2006). For this reason, many attempts to identify the channel-forming components of the PTP have relied also on the identification of CyP-D interacting proteins (Biasutto et al., 2016).

1.4 Three recent molecular models

To date, the molecular identity of PTP is still a mystery. Only recently, three hypotheses have been proposed, of which two suggested that PTP involves F-ATP synthase. PTP might form at the interface between F-ATP synthase dimers (Giorgio et al., 2013) or arise from the membrane-embedded ring of c subunits (c-ring) of the F_O-ATP synthase sub-complex (Bonora et al., 2013; Alavian et al., 2014). The third hypothesis pointed to another protein complex, the spastic paraplegia protein 7 (SPG7) (Shanmughapriya et al., 2015).

1.4.1 Dimers of F-ATP synthase

This model proposes that PTP could form at the membrane interface between two adjacent F_O sub-complexes of F-ATP synthase (Giorgio et al., 2013). In a previous finding, CyP-D was demonstrated to interact with the lateral stalk of F-ATP synthase, in an interaction favoured by P_i and counteracted by CsA (Giorgio et al., 2009). Later, it has been clarified that CyP-D binds specifically to the oligomycin-sensitivity conferring protein (OSCP), a specific subunit of F-ATP synthase lateral stalk (Giorgio et al., 2013). The binding site of CyP-D also corresponds with that of benzodiazepine-423 (Bz-423), a compound that inhibits F-ATP synthase and induces PTP opening (Giorgio et al., 2013). The interaction CyP-D/OSCP was found to be electrostatic and CsA was proved to displace CyP-D from OSCP (Bernardi, 2013; Giorgio et al., 2013).

Direct evidence on F-ATP synthase dimers forming the PTP were obtained by electrophysiological experiments, incorporating purified dimers from BN-PAGE into artificial bilayers and by measuring the passage of current after the application of a voltage difference. Dimers treated with Ca^{2+} generated currents and the opening of high-conductance channels was enhanced by Bz-423 and thiol oxidants, while inhibited by Mg^{2+}/ADP and AMP-PNP (a non-hydrolysable ATP analog). Despite the same overall subunit composition as the dimers, monomers of F-ATP synthase did not show channel activity (Bernardi, 2013).

In addition to this evidence on mammalian mitochondria, studies on two model organisms, the fruit fly *Drosophila melanogaster* and the yeast *Saccharomyces cerevisiae*, further verify the

molecular identity of PTP. Drosophila mitochondria PTP revealed sensitivity to Ca^{2+}, Bz-423 and thiol oxidants (i.e. diamide) and inhibition by P_i and Mg^{2+}/ADP. Other particular features are the insensitivity to CsA, that seems to be a consequence of the lack of mitochondrial CyPs, and a low conductance that is consistent with the absence of matrix swelling (von Stockum et al., 2011; von Stockum et al., 2015). The channels formed by purified F-ATP synthase dimers were relatively small (53 picosiemens, pS), not permeable to sucrose, with a conductance lower than that observed in mammals (500 pS) and yeast (250-300 pS) (von Stockum et al., 2015). In yeast mitochondria, purified F-ATP synthase dimers exhibited a Ca^{2+}-dependent channel activity as well (Carraro et al., 2014). Nevertheless, Ca^{2+} uptake into isolated mitochondria required the ionophore ETH129 and PTP showed inhibition by P_i and Mg^{2+}/ADP, insensitivity to CsA and sensitivity towards thiol oxidants (i.e. phenylarsine oxide and diamide) (Carraro et al., 2014). Interestingly, mitochondria from yeast mutants lacking some specific subunits involved in the F-ATP synthase dimerization (subunits e and g) turned out to be resistant to PT, confirming that dimer formation is crucial for the PTP (Carraro et al., 2014).

The model of F-ATP synthase as PTP was substantiated by pharmacological data and by key pathophysiological effectors (Bernardi, 2013). In fact, Ca^{2+}, Mg^{2+}, adenine nucleotides and P_i interact with F-ATP synthase by binding to the catalytic core at the F_1 sub-complex, while membrane potential and matrix pH are both PTP modulators and key regulators of F-ATP synthase. Recently it has been speculated that PTP might be formed by F-ATP synthase tetramers, but further evidence is needed to support this hypothesis (Bernardi et al., 2015).

1.4.2 c-ring of F-ATP synthase

The embedded c-ring (F_O sub-complex) of F-ATP synthase represents a membrane-spanning channel (Biasutto et al., 2016). Two recent theories proposed by Bonora et al. (2013) and Alavian et al. (2014) suggested the c-ring as candidate for the pore. In particular, Alavian and coworkers (2014) observed that, upon purification and reconstitution into an artificial bilayer, assembled c subunits formed a large, unselective, voltage-dependent channel, Ca^{2+} insensitive and inhibited by ADP and CsA, thus responding to the classical characteristics of PTP. However, the main regulatory sites for PTP, which confers sensitivity to Ca^{2+}, CsA and ADP, resides in the F_1 moiety and in the peripheral stalk of F-ATP synthase. High Ca^{2+} concentrations enlarged the c-ring and dissociated it from the F_1 sector, providing the putative mechanism for the opening (Alavian et al., 2014).

1.4.3 Spastic paraplegia protein 7 (SPG7)

Recently SPG7, an AAA-type protease located on the outer-inner membrane contact site has been proposed as core component of PTP on the inner mitochondrial membrane (Shanmughapriya et al., 2015). SPG7 emerged from a screening as candidate for PTP opening mediated by Ca^{2+} and ROS. This protein was also found to be CsA-sensitive and to co-immunoprecipitate with CyP-D, in an interaction generally considered crucial for the PTP.

This hypothesis pointed to PTP as a hetero-oligomeric complex composed by SPG7, VDAC and CyP-D through the multiple interactions of SPG7/CyP-D and SPG7/VDAC (Shanmughapriya et al., 2015). This update reconsiders the formerly and popular model envisioning PTP formation

by a protein complex spanning between inner and outer mitochondrial membrane, interacting with the regulatory matrix protein CyP-D (Biasutto et al., 2016).

1.5 Is PT and PTP conserved among different organisms?

Most of the studies on PTP were carried out in mitochondria obtained from mammals and only lately extended to yeast (Azzolin et al., 2010). In recent years, the growing interest on PT and its involvement in cell death has promoted researches on mitochondria from other organisms including plants, fish and amphibians.

Mammalian PT model seems to be the largely shared among different *phyla*. Thus appears that mammals have inherited a 'perfect' function from more ancient vertebrates, fulfilling a vital role (Vianello et al., 2012). PTP may have arisen as a new function linked to a multiple molecular exaptation of an assembly of different, pre-existing and functional proteins, which still perform their original role. This might have happened in a very flexible way, in order to cope with both internal (cell) and external (environmental) factors (Vianello et al., 2012). PTP could have appeared during the establishment of endosymbiosis in eukaryotic cells and its characteristics should therefore be highly conserved in evolutionarily divergent organisms (Azzolin et al., 2010; Vianello et al., 2012). A unifying factor of PT among yeast and higher eukaryotes and the minimum requirements for its definition is the regulation by Ca^{2+}, the modulation by P_i, in the absence or presence of CsA, and the sensitivity to redox effectors. Inorganic P_i is known as a fine regulator of mammalian PTP, since it forms amorphous matrix precipitate with Ca^{2+}, lowering in this manner the probability of PTP opening. In other contexts, it may be also considered a stimulator, being the chemical buffer able to maintain pH at optimal levels for PTP opening (Vianello et al., 2012).

Nevertheless, one of the major discrepancies between different organisms seems to be the sensitivity to CsA: PTP in *Saccharomyces cerevisiae* mitochondria is CsA-insensitive, in spite of the presence of mitochondrial CyPs, and inhibited by P_i (Azzolin et al., 2010; Carraro et al., 2014). In this case it was explained that the lack of sensitivity to CsA, which is a 'desensitizer' but not a PTP blocker, do not exclude that PTP could be involved in the event (Bernardi et al., 2015). Other characteristics, like swelling and size of the pore, seem to be specific for each organism. Swelling was not observed in Drosophila and this was thought to be correlated to the low conductance of the channel (von Stockum et al., 2011; von Stockum et al., 2015).

With the wide extent of PT conservation, it is an intriguing question why mitochondria possess the potential for self-destruction through PTP. A possible reason is that the pore could open occasionally and briefly under normal physiological conditions, providing a pathway for the entry and the exit of mitochondrial metabolites for which no other transport pathways exist (Halestrap et al., 1993) or for those metabolites and ions in excess that need to be removed (Crompton, 1999). Another explanation could be that the opening of the pore under condition of oxidative stress and Ca^{2+} overload might ensure the demise of damaged tissue, allowing new one to regenerate (Halestrap et al., 1993).

1.6 PT and PTP in plants

The interest in plant PT is quite recent and it dates back to the observation made in 1995 by Vianello and his group. They observed a Ca^{2+}-induced and CsA-sensitive PT in pea (*Pisum sativum* L.) stem mitochondria. The low-amplitude swelling suggested the presence of a pore permeable only for ions or small molecules and it appeared a specific feature of pea mitochondria (Vianello et al., 1995). A few years later, Fortes et al. (2001) and Arpagaus et al. (2002) collected different and contrasting results on PT from potato (*Solanum tuberosum* L.) tuber mitochondria. Fortes suggested that only high extramitochondrial Ca^{2+} (> 0.2 mM) was required to induce a PT, which occurrence was also enhanced by pro-oxidants (e.g. diamide), thiol reagents, P_i and uncouplers. Swelling was only partially inhibited by Mg^{2+} and acidic pH. Additionally, PT was insensitive to CsA and not inhibited by ADP (Fortes et al., 2002). Arpagaus et al. (2002), in contrast, demonstrated that uniquely matrix Ca^{2+} (0.5-5 mM) was needed for inducing a P_i-dependent swelling, while CsA acted as an inhibitor. When Mg^{2+} was replaced by Ca^{2+}, no swelling occurred (Fortes et al., 2002). A delayed Ca^{2+}-induced swelling was also observed in acidic environment. At pH < 7.0, PT was inhibited, whereas it was induced by pro-oxidants and by an increase of fatty acid concentration (Arpagaus et al., 2002). Other two substantial observations on plant PT were collected by Curtis and Wolpert (2002) and Virolainen and colleagues (2002) in oat (*Avena sativa* L.) leaf and wheat (*Triticum aestivum* L.) root mitochondria, respectively. Curtis and Wolpert in 2002 described a PT and a high-amplitude swelling when mitochondria were treated with Ca^{2+} (0.5 mM), but only in the presence of the Ca^{2+}-ionophore A23187. The swelling assay demonstrated a size exclusion range of 0.9-1.7 kDa, similar to animal mitochondria, but was CsA-insensitive. Differently, the spontaneous Ca^{2+} uptake (0.5-2.5 mM Ca^{2+}) by oat mitochondria was P_i-dependent and able to cause a high amplitude swelling, it was CsA-insensitive and unaffected by Mg^{2+} (Virolainen et al., 2002). The release of Cyt *c* was proportional to the P_i in the incubation medium and positively correlated with the Ca^{2+} concentration (Virolainen et al., 2002).

The situation in higher plants, as reported, is quite complicated and it is difficult to define a common pattern among all the different species and tissues considered. In this overview on PT in plants, the observations on purified potato mitochondria by Arpagaus et al. (2002) seem the most complete so far and also the one that showed more similarities with animals towards the main effectors of PT, such as the classical biochemical and physiological modulators: Ca^{2+}, thiol-oxidizing reagents, pH, free fatty acid and anoxia. The last one is a condition that flooded plants can experience in underground organs, for instance, and it has many parallels with ischemia in animal tissues (Arpagaus et al., 2002).

Plant mitochondria from different species possess also K^+ channels that display peculiar characteristics (Pastore, 2013; Trono et al., 2015). Petrussa and colleagues (2004) found that CsA had some additional effect on pea mitochondrial K^+ channel, whose opening was inhibited by ATP and stimulated by CsA. The aperture of this channel was associated with the inner membrane depolarisation, swelling and release of Cyt *c*. It appears, therefore, that swelling and Cyt *c* release could be the result of both activities of PTP and K^+ channel, which are modulated, in some cases, by CsA in an opposite manner. From this evidence, a low-amplitude PT, mediated by the K^+_{ATP} channel, might also be involved in plant PCD (Petrussa et al., 2004; Casolo et al.

2005), representing another pathway for cell death in plants.

Evidence for PTP in plants is still limited and circumstantial, therefore is difficult to claim if its occurrence is the functioning of other channels that could even mask PTP activity (Vianello et al., 2012).

1.7 Plant mitochondrial ATP synthase: a splendid (but still mysterious) molecular machine

Studies on the composition of the F-ATP synthase complex are nowadays particularly detailed in animals and fungi, but not completely unravelled in either algae or plants. Moreover, plant cells contain an ATP-producing machinery both in the mitochondrial inner membrane and in the thylakoid membrane.

The general structure of mitochondrial ATP synthase consists of the hydrophilic F_1, which contains the nucleotide-binding site with the five subunit structure (α, β, γ, δ, ε), the hydrophobic F_O that conducts protons through the membrane, and the lateral and central stalks (Hamasur and Glaser, 1990; 1992; Heazlewood et al., 2003). In particular, the proteins of the F_1 sub-complex show a high degree of similarity in the amino acidic sequences in all eukaryotic organisms (Hamasur and Glaser, 1992; Antoniel et al., 2014; Jiko et al., 2015), while the subunit composition of the F_O is so far relatively unexplored (Hamasur and Glaser, 1992; Jänsch et al., 1996; Heazlewood et al., 2003; Meyer et al., 2008; Klodmann et al., 2011) but it appears to vary between different *taxa* and species (Hamasur and Glaser, 1992).

The introduction of modern techniques has allowed to examine the entire F-ATP synthase complex from different plant species (Jänsch et al., 1996; Heazlewood et al., 2003) and also from the model plant *Arabidopsis thaliana* (Werhahn and Braun, 2002; Heazlewood et al., 2003). A convenient biochemical approach is the use of blue native PAGE (BN-PAGE), a powerful technique for dissecting the mitochondrial proteome on the basis of intact protein complexes, that can be combined with conventional isoelectric focusing (IEF) and SDS-PAGE (Werhahn and Braun, 2002).

Dimeric forms of F-ATP synthase are present in animals (Jiko et al., 2015), fungi (Buzhynskyy et al., 2007; Seelert and Dencher, 2011; Davies et al., 2012; Liu et al., 2015; Hahn et al., 2016), algae (Rexroth et al., 2004; Lapaille et al., 2010) and plant mitochondria (Eubel et al., 2003; Eubel et al., 2004; Krause, 2004; Bultema, 2009; Davies et al., 2011). The relative abundance of dimers in plant mitochondria was found to be low when compared to the total amount of F-ATP synthase, and even lower if compared to other organisms (Eubel et al., 2003; Eubel et al., 2004). Electron cryo-tomography also revealed that long rows of F-ATP synthase dimers in *cristae* membrane are a universal feature of all mitochondria and they enable the formation of tightly curved ridges, shaping the mitochondrial morphology (Davies et al., 2011). The arrangement of mitochondrial F-ATP synthase dimers in potato seems, however, different from animals and fungi, with respect to the distance between the F_1 heads and to the angle established between monomers (Davies et al., 2011).

Even though F-ATP synthase represents a crucial enzyme, very little has been unravelled about its monomeric and dimeric structure, as well as the catalysis mechanism and the subunits involved in the dimerization.

1.8 PCD in plants

Programmed cell death (PCD) is an essential process in multicellular eukaryotes and it is marked by a sequence of events that leads to a localized cell killing, needed for the normal development of complex organisms (Logan, 2008). In plants, PCD is a facet of a wide range of programmes, which vary from the beginning of the plant life cycle through essential developmental processes (e.g., somatic embryogenesis, leaf morphogenesis, tracheary element formation and xylogenesis), until the end of the life cycle (senescence). It is also a consequence of biotic and abiotic stresses, like the hypersensitive response (HR) under pathogen interactions, anoxia and heat (Curtis and Wolpert, 2002; Virolainen et al., 2002; Vianello et al., 2007; Logan, 2008; Reape et al., 2008; Lord and Gunawardena, 2012).

Like many other phenomena, PCD and death morphotype are well described and thoroughly documented in animals (Jones, 2000), where PCD can be divided mainly in three categories: apoptosis, autophagic cell death and necrosis (Jones, 2000; Lord and Gunawardena, 2012). Conversely, many tenets of cell death in plants are still very confused, leading to misinterpretations among researchers by a diverse use of terminology (Logan, 2008). In fact, PCD is often used as general term to describe most instances of death manifestation, even if different modes, markers and morphologies exist (van Doorn et al., 2011). One common thread among the wide spectrum of PCD is the involvement of mitochondria as key players, cellular stress sensors and dispatchers of PCD (Jones, 2000; Virolainen et al., 2002). Changes in the mitochondrial morphology and membrane permeability have been demonstrated to be involved very early during the execution of various types of PCD (Scott and Logan, 2007; Logan, 2008; Reape et al., 2008; Lord et al., 2011). In some of these instances, CsA was found to block PCD (Yu et al., 2002; Tiwari et al., 2002) and prevent mitochondrial morphology transition (Scott and Logan, 2007; Lord et al., 2011), supporting the connection with PTP (Scott and Logan, 2007). Therefore, PT seems to have a central role in the manifestation of PCD, and PTP could be interpreted as a 'sensing' structure (Vianello et al., 2012) and a channel for releasing PCD-activating molecules from the intermembrane space (Reape et al., 2008; Lord and Gunawardena, 2012). The plant mitochondrial K^+ channel is suggested to be another mechanism to mediate the release of PCD-activating molecules (Vianello et al., 2007; Trono et al., 2015). Cyt *c* release into the cytosol has been demonstrated in different plant systems (Arpagaus et al., 2002; Virolainen et al., 2002; Lord and Gunawardena, 2012), but there is no clear connection with an active role in the execution of death pathways in plants, compared to animal apoptosis, where this link is well established (Logan, 2008; Van Aken and Van Breusegem, 2015). Also the connection between mechanisms underlying PT and Cyt *c* release is still very vague (Jones, 2000; Logan, 2008). Cyt *c* displacement from the electron transport chain is also known to generate a lethal level of reactive oxygen species (ROS). ROS formation, particularly associated with the HR (Jones, 2000), could be an important factor in PCD signalling (Van Aken and Van Breusegem, 2015) and the inducer of a positive feedback loop that amplifies the PCD-inducing stress signal (Reape and McCabe, 2010). ROS could also magnify the Cyt *c* effect, which is insufficient to trigger the PCD by itself (Yu et al., 2002; Reape and McCabe, 2010). Besides these molecules, several other small metabolites and second messengers are involved in plant PCD signalling, like Ca^{2+}, nitric oxide and intracellular ATP levels (Van Aken and Van Breusegem, 2015).

Unravelling the intricate molecular and biochemical processes involved in plant PCD may also reveal the existence of an ancestral death mechanism, universally conserved in eukaryotic cells during evolution (Jones, 2000).

1.9 A look into Ca^{2+} dynamics and regulation in plants

A wide variety of cell signals, including biotic, abiotic and developmental *stimuli*, induce specific spatial and temporal Ca^{2+} transients in animals and plants cells, when a rapid adjustment in their physiology is needed (Carraretto et al., 2016; Wagner et al., 2016).

Mitochondria, together with the endoplasmic reticulum, are the major Ca^{2+} stores, with a key role in Ca^{2+} signalling and homeostasis (Virolainen et al., 2002) due to their ability to accumulate this cation rapidly and transiently (Wagner et al., 2016). The role of mitochondria goes beyond ATP production, in fact controlling central life processes within the organelles themselves and within the entire cell (Wagner et al., 2016).

The highly negative transmembrane potential in energised mitochondria is the driving force for the uptake of Ca^{2+} and other cations, but Ca^{2+} influx and efflux need to be tightly controlled in order to avoid overload (Carraretto et al., 2016) that, as already discussed, could trigger PTP opening. In plants, the current knowledge on ion channels is still limited and the molecular identification of the pathway mediating Ca^{2+} flux is far from being completely clarified (Carraretto et al., 2016). Ca^{2+} is thought to pass freely the outer mitochondrial membrane through VDACs or porins, while it strictly requires channels/transporters for the passage through the inner mitochondrial membrane (Wagner et al., 2016). Ca^{2+} electrophoretic influx and uptake systems in plants seem to be extremely variables among species and within different organs (Carraretto et al., 2016; Wagner et al., 2016), suggesting that mitochondria possess a diverse ability for Ca^{2+} uptake (Fortes et al., 2001; Arpagaus et al., 2002; Virolainen et al., 2002; Carraretto et al., 2016) or even an apparent inability (Curtis and Wolpert, 2002). The overall picture on Ca^{2+} transport in plants is indeed very complex and contradictory, and only recently the mechanism of Ca^{2+} import into the mitochondrial matrix has been demonstrated. The protein MICU, regulator of the plant homolog of the mammalian mitochondrial Ca^{2+} uniporter (MCU), was recognized to control Ca^{2+} uptake by moderating the influx (Wagner et al., 2015).

With regards to Ca^{2+} concentrations, animal and plant cells maintain free cytosolic Ca^{2+} at lower concentrations than most of other intracellular inorganic ions (Wagner et al., 2016) and the levels of matrix free Ca^{2+} was estimated to be very different, particularly in plants (Zottini and Zannoni, 1993).

Among other different identified or hypothesised Ca^{2+} pathways, PTP was speculated to contribute to Ca^{2+} transport across the outer and inner mitochondrial membranes (Carraretto et al., 2016), where it was suggested that transient opening of PTP could provide a fast Ca^{2+} release channel, preventing an overload and mediating mitochondrial depolarisation (Bernardi and von Stockum, 2012). Contrasting opinions formulated by Crompton (1999) and Wagner et al. (2016) consider the 'Ca^{2+} export via PTP' hypothesis weak and requiring thorough and further evidence.

In summary, the overload of matrix Ca^{2+} appears to be the single most important factor required for PTP opening, but its effect alone seems still not sufficient to induce PT occurrence.

The regulation of PTP is more complex and depends on the combinations of many other physiological effectors, like P_i and oxidative stress.

1.10 Ca^{2+} and ROS interplay

It is known that the combination of oxidative stress and increased mitochondrial Ca^{2+} content has damaging consequences (Halestrap et al., 1993). Under physiological conditions, the toxic effect of ROS is mitigated by the antioxidant system, but during biotic and abiotic stresses, the concentration of ROS in the cells can significantly rise, reaching a threshold triggering PCD (Tiwari et al., 2002). As reported, transient and recurrent PTP openings are activated by a Ca^{2+} matrix overload, with the joint effect of oxidative stress. An oxidative response can be initiated by Ca^{2+} binding to the inner membrane cardiolipins that stimulates ROS production, in particular O_2^- and H_2O_2, by the respiratory chain, making mitochondria a major source of ROS (Fortes et al., 2001; Møller, 2001; Tiwari et al., 2002). Ca^{2+} influx-triggered Ca^{2+} release has also been linked to pulsing of the mitochondrial membrane potential that can lead to transient uncoupling and decrease in ROS production (Carraretto et al., 2016). ROS act in synergy with Ca^{2+} in a feedback loop: ROS increase the Ca^{2+} cell concentration that favours ATP production and, in turn, Ca^{2+} promotes ROS generation during oxidative phosphorylation (Vianello et al., 2012).

Nonetheless, the scene is more complex, where the existing interplay between PTP opening and ROS generation in response to Ca^{2+} is intricate, as well as the overall interplay among Ca^{2+}, ROS and ATP, in both life and death cell processes. This multifactorial cross-talk, called a 'love-hate triangle' (Brookes et al., 2004), is not surprisingly centered on a crucial organelle like the mitochondrion.

1.11 On the role of ATP in PT and PCD occurrence

Ca^{2+} accumulation in physiological conditions activates the mitochondrial metabolic machinery, resulting in increased ATP synthesis and, hence, ATP levels in the cytosol (Jouaville et al., 1999; Nakano et al., 2011). Nevertheless, when Ca^{2+} exceeds a certain threshold and accumulates in the mitochondrial matrix, PTP opening can occur. In this condition, mitochondria can lose their ability to synthesize ATP because of the collapse of membrane potential, as long as the pore remains open (Bernardi, 1999). Extensive uncoupling and PTP openings can be also followed by the loss of matrix pyridine nucleotides and by the switch of F-ATP synthase from synthesis to hydrolysis (Bernardi and von Stockum, 2012; Bernardi et al., 2015). In this conditions, mitochondria could become significant consumers of cytosolic ATP (Chinopoulos, 2011).

Beyond being arguably the most important molecule for all living organisms, ATP is also essential for death execution and its levels can guide the cell toward different forms of PCD (Crompton, 1999; Zamaraeva et al., 2005; Vianello et al., 2012). Severe and sudden activation of death programmes are associated with an early loss of ATP (imprecisely defined in plants as 'necrosis'), whereas in the case of milder insults ATP levels are maintained rather stable (Crompton, 1999; Van Aken and Van Breusegem, 2015).

In the animal model, the apoptotic *stimuli* induce a rise of cytosolic ATP level that remains high for some hours, meanwhile there is caspase activation and DNA laddering. Particularly in the hypersensitive cell death, ATP plays the role of maintaining the integrity of the plant cell (Hatsugai et al., 2012).

PCD represents another complex bioenergetic scenario orchestrated by mitochondria in which different molecules and interactions can lead to either cell life or death. In an evolutionary context, cell death linked to loss of ATP might have represented a form of primordial death that allowed the elimination of cells with low energetic performances (Vianello et al., 2012).

2. Papers

2.A The permeability transition in plant mitochondria: the missing link

Preface

The topic of the mitochondrial PT has been debated by the scientific community for many decades, generating deep interest, since in mammals PT is related with many human diseases. Nonetheless, it is still an obscure and unsolved issue. In plants, where PT was also observed, there is not univocal evidence about PTP molecular players, triggering mechanisms and consequent cell death pathways.

This contribution was published in "Frontiers in Plant Science" in 2015 and its aim was to review the literature on PT in plant mitochondria and put forward a proposal on PTP. The idea is based on a comparison with the recent hypothesis that pointed to dimers of F-ATP synthase as molecular entity of the pore and which is consistent with results from mammals, yeast and flies.

Outstanding questions

Do plants possess a similar and conserved structure for PTP? Could PTP evolution in different organisms be explained in term of molecular exaptation?

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The Permeability Transition in Plant Mitochondria: The Missing Link

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The synthesis of ATP in mitochondria is dependent on a low permeability of the inner membrane. Nevertheless, mitochondria can undergo an increased permeability to solutes, named permeability transition (PT) that is mediated by a permeability transition pore (PTP). PTP opening requires matrix Ca^{2+} and leads to mitochondrial swelling and release of intramembrane space proteins (e.g., cytochrome *c*). This feature has been initially observed in mammalian mitochondria and tentatively attributed to some components present either in the outer or inner membrane. Recent works on mammalian mitochondria point to mitochondrial ATP synthase dimers as physical basis for PT, a finding that has been substantiated in yeast and *Drosophila* mitochondria. In plant mitochondria, swelling and release of proteins have been linked to programmed cell death, but in isolated mitochondria PT has been observed in only a few cases and in plant cell cultures only indirect evidence is available. The possibility that mitochondrial ATP synthase dimers could function as PTP also in plants is discussed here on the basis of the current evidence. Finally, a hypothetical explanation for the origin of PTP is provided in the framework of molecular exaptation.

Keywords: permeability transition, plant mitochondria, ATP synthase, exaptation, environmental stress

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THE PERMEABILITY TRANSITION

ATP synthesis in mitochondria occurs by a chemiosmotic coupling of substrate oxidation and phosphorylation (Mitchell, 1961). This explanation is based on the highly selective permeability of the inner mitochondrial membrane (IMM) and on utilization of protonmotive force by the F_1F_O ATP synthase (F-ATPase) for the synthesis of ATP. Nevertheless, a sudden increase in permeability of the IMM has been described in the 1950s (Raaflaub, 1953a,b) and characterized in the late 1970s (Haworth and Hunter, 1979; Hunter and Haworth, 1979a,b). Initially considered an artifact, later it has been named Permeability Transition (PT) and associated to a pore, the Permeability Transition Pore (PTP). The appreciation of its relevance has increased since it has been related to many diseases in mammals, including reperfusion injury of the heart and muscular dystrophy (Bernardi, 2013a). This mitochondrial PT requires matrix Ca^{2+} and is favored by matrix P_i, as well as benzodiazepine Bz-423 and thiol oxidants, while it can be inhibited by Mg^{2+}, thiol reductants, ADP and ATP (Bernardi, 2013b). Cyclosporin A (CsA) acts as inhibitor of PT (Crompton et al., 1988) by binding with the peptidyl-prolyl isomerase Cyclophilin D (CyPD) (Halestrap and Davidson, 1990). The features of PTP (e.g., pore diameter of ~2.8 nm and size exclusion of about 1500 Da) are consistent with those described for the Mitochondrial Mega-Channel (MMC), a high-conductance channel, which is considered to be its electrophysiological equivalent (Szabó and Zoratti, 1992).

THE PT IN PLANTS

The first evidence of a Ca^{2+}-induced and CsA-delayed collapse of transmembrane electrical potential difference ($\Delta\Psi$) in pea stem mitochondria dates back to 1995 (Vianello et al., 1995). PT has been then observed in different plant species, although the features of this phenomenon cannot be summarized in a straightforward model (**Table 1**). Potato tuber mitochondria exhibit a typical Ca^{2+}/P_i-induced PT, inhibited (Arpagaus et al., 2002) or not (Fortes et al., 2001) by CsA. These mitochondria do not show any Ca^{2+} uptake, suggesting an external effect of Ca^{2+} on PT (Fortes et al., 2001), which is not consistent with the observations in mammals (Bernardi et al., 2015). The PT described in oat leaves (Curtis and Wolpert, 2002) and wheat roots (Virolainen et al., 2002) shows a Ca^{2+}/P_i -induced $\Delta\Psi$ collapse and matrix swelling, which are CsA-insensitive. Calcium uptake by isolated plant mitochondria occurs spontaneously in wheat, but requires the addition of the Ca^{2+}/H^+ ionophore A23187 in oat.

Indirect evidence of PT in plants has been also based on the CsA-induced inhibition of programmed cell death (PCD), reviewed by Vianello et al. (2007, 2012). However, the prevention of PCD might depend on CsA binding to cytosolic Cyclophilin A (a ubiquitous enzyme) that drives enzymatic cascades (Lu et al., 2007), linked to oxidative stress (Nigro et al., 2013).

THE MITOCHONDRIAL Ca^{2+} ACCUMULATION IN PLANTS

The PT requires Ca^{2+} accumulation into the mitochondrial matrix (i.e., matrix Ca^{2+} is a permissive factor, although it may not be sufficient *per se*). Calcium transport in isolated plant mitochondria exhibits distinct features. The uptake could be mediated by a low-affinity electrophoretic P_i-dependent symport, with low or no sensitivity to ruthenium red and lanthanides (Dieter and Marme, 1980; Akerman and Moore, 1983; Silva et al., 1992), but also by a uniport mechanism (Zottini and Zannoni, 1993). CsA inhibits mitochondrial Ca^{2+} transport in *Citrus* (de Oliveira et al., 2007), suggesting its synergic effect with PT. A low concentration of matrix free Ca^{2+} (~100 nM) is maintained under steady state, where influx is balanced by an efflux through a yet speculative Na^+-independent Ca^{2+}/H^+ antiport mechanism (Nomura and Shiina, 2014). The influx of Ca^{2+} in plant mitochondria is highly variable, depending on species and tissues, or might be even completely absent (Martins and Vercesi, 1985). *In vivo* Ca^{2+} dynamics have been monitored by fluorescent probes targeted to plant mitochondria (Manzoor et al., 2012; Loro and Costa, 2013). Matrix Ca^{2+} uptake can be induced by abiotic stresses such as heat, oxidative stress, or anoxia, and follows the cytosolic Ca^{2+} pattern (Subbaiah et al., 1998; Logan and Knight, 2003; Schwarzländer et al., 2012; Rikhvanov et al., 2014).

Homologue genes of mammalian mitochondrial Ca^{2+} uniporter (MCU) and its regulatory protein MICU1 have been found in plants (Bick et al., 2012; Stael et al., 2012; Rikhvanov et al., 2014). The MICU1 homologue in *Arabidopsis* (AtMICU) is a negative regulator of mitochondrial Ca^{2+} uptake in root tips, providing strong evidence for the operation of a mitochondrial Ca^{2+} uniporter in plants (Wagner et al., 2015).

THE INVOLVEMENT OF PT/PCD IN PLANT DEVELOPMENT AND STRESS RESPONSES

The physiological role of mitochondrial PT in plants is often related to developmental processes (Reape et al., 2015) and mild environmental stresses, which involve also PCD in many cases. However, the mechanistic link between PT and PCD remains still speculative.

Permeability transition/programmed cell death are fundamental in the selection of damaged cells and in sculpturing new anatomical and morphological structures (Van Hautegem et al., 2015). Morphological modifications are also needed for adaptive responses to environment (e.g., climate changes) and, more in general, for fitness increase. In particular, *Aponogeton madagascariensis* forms lacunae on its leaves by executing PCD, which is inhibited by CsA, suggesting the involvement of PT (Lord et al., 2013). In aerenchyma formation, lack of oxygen induces stress characterized by mitochondrial PT, ATP depletion, and PCD induction (Yamauchi et al., 2013). Consistently, stressed pea plants show cytochrome *c* release, followed by DNA fragmentation (Sarkar and Gladish, 2012).

Programmed cell death is a common response in plants subjected to abiotic and biotic stresses, which may be linked to the sessile lifestyle, providing a survival strategy for the whole organism. Excess of UV-C stimulates reactive oxygen species (ROS) formation and collapse of $\Delta\Psi$ in *Arabidopsis* mitochondria (Gao et al., 2008). The role of PT has also been described in case of extreme temperatures. In *Arabidopsis* protoplasts, heat stress induces mitochondrial swelling, and $\Delta\Psi$ loss, but these damages are counteracted by a heat shock

TABLE 1 | Characteristics of permeability transition (PT) in plant mitochondria.

Plant material	Ca^{2+} stimulation	CsA inhibition	Sucrose swelling	Cytochrome *c* release	Reference
Etiolated pea stem	Yes	Yes	No	Not detected	Vianello et al., 1995
Potato tuber	Yes (external)	No	Yes	Yes	Fortes et al., 2001
Potato tuber	Yes	Yes	Yes	Yes	Arpagaus et al., 2002
Oat leaves	Yes (with A23187)	No	Yes	Yes	Curtis and Wolpert, 2002
Wheat roots	Yes	No	Yes	Yes	Virolainen et al., 2002

transcription factor (Zhang et al., 2009). Similarly, ROS and mild heat shock induce mitochondrial PT and the subsequent induction of cell death in *Arabidopsis* protoplasts, which are prevented by the superoxide dismutase analog TEMPOL, by the Ca^{2+} channel-blocker lanthanum chloride, and by CsA (Scott and Logan, 2008). The role of mitochondria in PCD is confirmed in heat-stressed rice protoplasts, where mHSP70 overexpression maintains mitochondrial $\Delta\Psi$, partially inhibits cytochrome *c* release and suppresses PCD by lowering ROS formation (Qi et al., 2011). In wheat cells subjected to freezing, ROS-dependent PCD is associated to $\Delta\Psi$ collapse and cytochrome *c* release (Lyubushkina et al., 2014). In salt-stressed tobacco protoplasts, PCD is triggered by ROS produced by mitochondria, through a process controlled by a CsA-sensitive PT (Lin et al., 2006).

The response to heavy metals requires the participation of mitochondrial PT. In particular, aluminum triggers a high ROS production in peanut, by plasmalemma NADPH oxidases, which induce mitochondrial mediated-PCD (Huang et al., 2014). Consistently, metal phytotoxicity appears to be also mediated by PT in aluminum- treated *Arabidopsis* protoplasts (Li and Xing, 2011) and in cadmium-treated rice roots (Yeh et al., 2007).

Biotic stress, such as pathogen attack, may lead to protoplast shrinkage, mitochondria swelling and cytochrome *c* release. These responses appear to be associated to PCD involvement during the hypersensitive response, a strategy to counteract biotrophic pathogens. The generation of a defensive layer, promoted by PT-induced PCD, has been shown in *Arabidopsis*. In particular, PCD is mediated by a rapid decrease in mitochondrial $\Delta\Psi$, which is partially counteracted by CsA (Yao et al., 2004). Finally, there is evidence on the release of cytochrome *c* induced by elicitors such as harpin or victorin (Curtis and Wolpert, 2002; Krause and Durner, 2004).

THE MOLECULAR STRUCTURE OF PTP

The components involved in PTP formation initially included the voltage-dependent anion channel, the benzodiazepine receptor, the adenine nucleotide translocase and the phosphate carrier. This model has been questioned, since isolated mitochondria from organisms where the expression of each of these proteins has been suppressed still exhibit a PT (Kokoszka et al., 2004; Krauskopf et al., 2006; Baines et al., 2007; Gutiérrez-Aguilar et al., 2014; Šileikytė et al., 2014).

Recent evidence shows that F-ATPase is involved in PTP formation in different species and *taxa* (Bernardi, 2013b; Bonora et al., 2013; Alavian et al., 2014). This enzyme is highly conserved in both prokaryotes and eukaryotes (Hamasur and Glaser, 1992; Heazlewood et al., 2003), consisting in the hydrophilic F_1 and the hydrophobic F_O sectors, which operate in concert to carry out distinct functions (Antoniel et al., 2014).

The F_1 contains five subunits: α and β forming the catalytic region, while γ, δ, and ε are organized in the central stalk. In all eukaryotes these subunits show a high degree of similarity in the sequences (Hamasur and Glaser, 1992; Antoniel et al., 2014; Jiko et al., 2015), while the subunit composition of the F_O varies among different *taxa* and species (Hamasur and Glaser, 1992). For details about F-ATPase components in mammals, fungi and algae, see Vázquez-Acevedo et al. (2006), van Lis et al. (2007), Dabbeni-Sala et al. (2012), Antoniel et al. (2014), Lee et al. (2015) and Liu et al. (2015). Specific subunits have been characterized in plants such as sweet potato (Morikami et al., 1992), potato (Dell'Orto et al., 1993; Polgreen et al., 1995) and soybean (Smith et al., 1994).

Plant F_1 includes the classical five-subunit structure (Hamasur and Glaser, 1990, 1992), and also a 24 kDa protein (Li et al., 2012), but the picture of F_O components remains still incomplete. Several proteins belonging to F_O have been identified in spinach (Hamasur and Glaser, 1992), potato (Jänsch et al., 1996), rice (Heazlewood et al., 2003), and *Arabidopsis* (Heazlewood et al., 2003; Meyer et al., 2008; Klodmann et al., 2011). As shown by Klodmann et al. (2011) and by Li et al. (2012), F_O includes subunits a, c, d, 4 (corresponding to subunit b or orf25, Heazlewood et al., 2003), a 6 kDa protein (plant specific), subunit 8 (also called AL6 or orfB, Heazlewood et al., 2003), ATP17 (plant specific) and Oligomycin Sensitivity-Conferring Protein (OSCP), sometimes referred to as δ' in plants (Morikami et al., 1992), for some authors belonging to F_1 (Jänsch et al., 1996). Subunit g was found detached from F-ATPase monomer, suggesting that it could represent a dimer-specific protein (Meyer et al., 2008; Klodmann et al., 2011). Plant subunit e sequences have been identified so far only in protein databases for few species (e.g., rice and *Medicago truncatula*).

Multimeric structures of F-ATPase are present in animal, fungi (Davies et al., 2011; Seelert and Dencher, 2011; Liu et al., 2015) and plant mitochondria (Eubel et al., 2003, 2004; Krause et al., 2004; Bultema et al., 2009). Eubel et al. (2003) highlighted the presence of F-ATPase dimers in *Arabidopsis*, potato, bean, and barley. The relative abundance of dimers in plants is low, with respect to the total F-ATPase, and even lower when comparing different organisms (Eubel et al., 2003, 2004).

Rows of F-ATPase dimers in *cristae* seem to be a universal feature of all mitochondria (Davies et al., 2011) that enable the formation of highly curved ridges in *cristae* (Davies et al., 2012). The Inhibitory factor 1 (IF_1) that binds F-ATPase at low pH (Campanella et al., 2008) could favor dimer formation even if it is not clear how it improves dimer stability. The arrangement of F-ATPase in mammals and fungi is different from that of potato, being the angle between monomers in the latter larger (∼115°) than in the former (∼80°) (Davies et al., 2011). Interestingly, this correlates with *cristae* morphology observed for many plant mitochondria, where irregular saccular structures with a less convex curvature appear particularly prevalent (Douce, 1985). In aging *Podospora anserina* (*Ascomycetes*) mitochondria, the IMM is progressively vesiculated, the *cristae* collapse and the F-ATPase dimers are disassembled (Daum et al., 2013). The impairment of ATP synthesis, and the outer membrane rupture by swelling, lead to the release of pro-apoptotic factors and, finally, to cell death.

Animal mitochondria F-ATPase dimers have been shown to act as pores with properties of the PTP (Giorgio et al., 2013). CyPD modulates F-ATPase activity by binding OSCP (Giorgio et al., 2009) and this interaction is favored by P_i, while CsA

displaces CyPD from the enzyme. F-ATPase is inhibited by Bz-423, which binds to OSCP (Cleary et al., 2007). These features are consistent with those observed for PT regulation. Magnesium, Ca^{2+}, adenine nucleotides, membrane potential and matrix pH are also key modulators of both F-ATPase activity and PTP. Electrophysiological experiments, after isolation and insertion of F-ATPase dimers in artificial phospholipid bilayers, showed that the pore activity matches that of PTP-MMC (Giorgio et al., 2013).

The involvement of F-ATPase dimers in PTP formation has been extended and confirmed in yeast and *Drosophila*, even if these organisms show specific differences. In yeast mitochondria the ionophore ETH129 is needed for Ca^{2+} uptake in the matrix and the PT displays a low conductance (around 300 pS). Phosphate acts as an inhibitor of PT, while CsA does not interfere with PTP. Yeast mutants lacking of subunits e and g, which are involved in dimerization, display a striking resistance to PTP opening (Carraro et al., 2014). In *Drosophila* (von Stockum et al., 2015), PTP has been initially identified as mitochondrial Ca^{2+}-induced Ca^{2+} release channel (mCrC). The main differences between mCrC and mammalian PTP are: (i) absence of swelling; (ii) absence of CsA effect, since no CyPD is present in this species; (iii) sensitivity to rotenone, an inhibitor of Complex I; (iv) inhibition of mCrC by P_i; (v) low conductance (around 53 pS) of the F-ATPase dimers in artificial bilayer.

Other research groups have also suggested that F-ATPase is involved in pore formation by the channel activity within the c-ring formed by c subunits of F_O (Bonora et al., 2013; Alavian et al., 2014). Nevertheless, this hypothesis is still under debate, since it does not justify the different pore size observed in bovine, yeast, and *Drosophila*, where similar c-rings are present (Bernardi et al., 2015). Finally, the possible involvement of IF_1 in modulation of PTP through F-ATPase dimerization needs further investigations (Faccenda et al., 2013; Bernardi et al., 2015).

The presence in plants of many common components and features of F-ATPase lead us to speculate that, similarly to mammals, yeast, and *Drosophila*, PT function could be exerted by F-ATPase dimers also in such organisms.

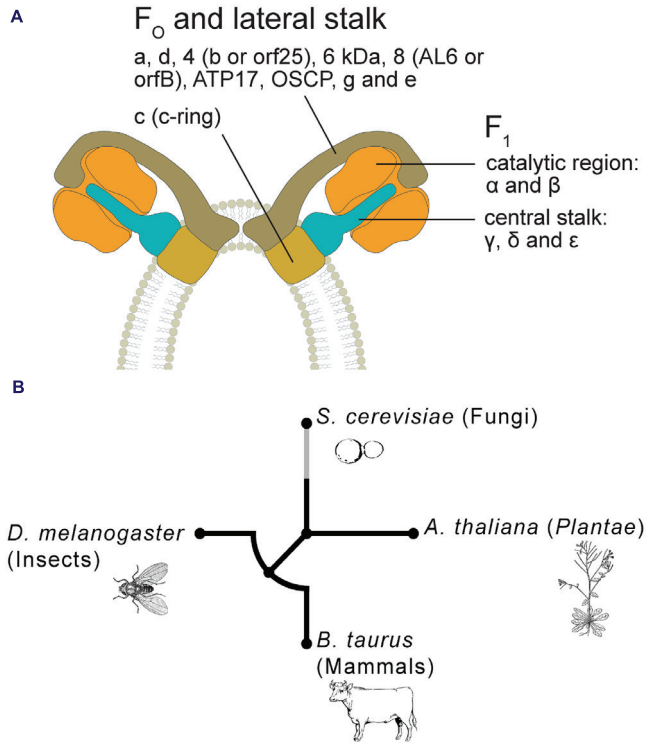

FIGURE 1 | (A) Hypothetical model of PTP in plants, based on F-ATPase dimer formation, as proposed by Bernardi (2013b), Bonora et al. (2013), and Alavian et al. (2014). Plant F-ATPase subunits are organized on the basis of their putative correspondence to the mammalian ones. **(B)** Circular phylogenetic tree of peptide sequences of homologous subunit g of mitochondrial ATP synthase in four representative *taxa* (i.e., *Bos taurus*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and *Arabidopsis thaliana*). Alignments of multiple amino acid sequences were performed using MUSCLE software (Edgar, 2004). Phylogenetic trees were obtained using phyML version 3.0 with the maximum-likelihood (ML) method (Guindon et al., 2010). The NCBI Reference Sequence accession codes for the g subunit are: *B. taurus* = NP_001019721; *D. melanogaster* = NP_609142; *S. cerevisiae* = NP_015345; *A. thaliana* = NP_179558. Where more isoforms were found in NCBI databases, we randomly selected only one of these sequences.

THE EMERGENCE OF PT DURING EVOLUTION

Evolution does not always proceed by adaptations. It may also develop a non-adaptive exaptation/cooptation (pre-adaptation), where the term exaptation/cooptation means a trait evolved to accomplish a specific function (or even no function), which may be then exapted/coopted to perform a novel function (or to acquire a function) (Gould and Vrba, 1982).

It has been suggested that the structure of PTP (as a multicomponent complex, Bernardi, 2013a) may have arisen by a mechanism of molecular exaptation, a phenomenon largely recognized at different levels of complexity (genes, proteins, organs), during evolution (Vianello et al., 2012; Barve and Wagner, 2013). The new model, involving F-ATPase dimer in PTP formation, does not contradict our previous interpretation on its origin, but rather appears to support it further. The dimer appears to be the result of a molecular exaptation/cooptation, where two monomers are assembled to perform an additional function (**Figure 1A**). In other words, F-ATPase seems to have a "Janus double face", catalyzing the synthesis of ATP, but in some circumstances preventing such a synthesis (Bernardi et al., 2015). This dimer could even possess a "triple face", because the dimerization induces also the curvature of the IMM.

The F-ATPase dimer is present in eukaryotes, but not in prokaryotes, because the F-ATPase of the latter is lacking of some crucial subunits (e and g) involved in dimer formation (Antoniel et al., 2014). It is thus reasonable to assume that the dimer/PTP may be arisen after the endosymbiosis between an alpha-proteobacterium and an archaeon (Martin and Müller, 1998). At the beginning, these dimers could have transferred ATP from the endosymbiont to the cytoplasm of the host cell, because the former presumably did not have ATP/ADP transporters. PTP was then maintained to dissipate the protonmotive force, thus regulating both ATP synthesis and exchanges of solutes between the cytoplasm and the mitochondrial matrix.

The presence of F-ATPase dimer has been assessed in different evolutionary divergent eukaryotes, some of which exhibit mitochondrial PT, such as '*Unikonts*' (*Opisthokonts*) and *Plantae* (Arpagaus et al., 2002; Giorgio et al., 2013; Carraro et al., 2014; von Stockum et al., 2015). To understand the phylogenesis of this structure/function, a cladogram has been generated by comparing the ancestral sequences of F_O subunit g from bovine and *Drosophila* (animals), yeast (fungi, *Ascomycetes*), and *Arabidopsis* (*Plantae*) (**Figure 1B**). The tree suggests an early differentiation at higher taxonomical levels (supergroups): *Plantae* show the highest phylogenetic distance and within the *Opisthokonts*, mammals, and insects exhibit similar distances, whereas yeast shows a higher distance. These phylogenetic patterns are consistent with the main evolutionary life tree (e.g., Keeling et al., 2005).

It has been suggested that F-ATPase shows a progressive differentiation along the main steps of evolution. In turn, some features of PTP seem to be occurred independently from changes in ATP synthase. As an example, swelling of mitochondria occurs only in bovine (Bernardi et al., 2015) and in some plants (see **Table 1**), suggesting that PTP has been differently shaped by exaptation during the evolution. Hence, exaptation leading to PT seems to have occurred in diverse contexts during life history, depending on the molecular characteristics of F-ATPase structure and the specific requirements of the respective *taxa*.

FUTURE DIRECTIONS

The molecular nature of PTP in plants is still elusive. Further structural and functional studies are required to verify if F-ATPase dimers represent the channel associated to PT also in plants. This is needed to understand better the relationship between mitochondrial PT and PCD in plants.

AUTHOR CONTRIBUTIONS

MZ and AV co-supervised the manuscript and co-wrote the article. VC, EP, CP, SP, AB, and EB co-wrote the article. VDC and FB performed the phylogenetic analyses and co-wrote the article.

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2.B Permeability transition and F-ATP synthase dimers as the pore in isolated pea stem mitochondria

Preface

In this work, still unpublished, we collected evidence on mitochondrial PT in isolated pea (*Pisum sativum* L.) stem mitochondria, confirming that the phenomenon occurs and is modulated by different players (e.g., Ca^{2+}, oxidants, Mg^{2+}/nucleotides). Our interest was focused also on the possibility that F-ATP synthase dimers could form the PTP. Considering the findings from mammals, yeast and flies as source of reference, we confirmed some features of plant PT and PTP and discovered peculiar ones. This model needs further and decisive validations, but its conserved key elements support the idea that PTP is a crucial mechanism for the eukaryotic cell, preserved during evolution.

Outstanding questions

Do F-ATP synthase dimers in plants possess different characteristics with respect to other eukaryotic organisms? Could alternative mechanisms explain the PT manifestation?

Permeability transition and F-ATP synthase dimers as the pore in isolated pea stem mitochondria

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Abstract

Plant mitochondria can undergo an increased permeability to solutes, named permeability transition (PT), mediated by a pore (PTP). The phenomenon was observed in etiolated pea stem mitochondria when approx. 300 μM Ca^{2+} was added, only in the presence of the Ca^{2+} ionophore ETH129. PTP opening required matrix Ca^{2+} and was sensitized by diamide, phenylarsine oxide (PheAsO) and benzodiazepine-423 (Bz-423), while it was inhibited by Mg^{2+}/ADP and Mg^{2+}/ATP. During PT, no mitochondrial swelling was observed. Since recent works on mammalian, yeast and Drosophila pointed to mitochondrial F-ATP synthase dimers as the molecular basis for PT, this work aimed to demonstrate that mitochondrial F-ATP synthase dimers may function as PTP also in plants. BN-PAGE allowed to separate multimeric and monomeric structures of F-ATP synthase, identified by in-gel activity. Bands corresponding to dimers were subjected to Western blotting, which confirmed the presence of F-ATP synthase components, such as β subunit and OSCP (oligomycin sensitivity-conferring protein). When F-ATP synthase dimers were eluted from BN-PAGE and inserted into artificial lipid bilayer, they showed a channel activity. These results suggest that PTP in plant mitochondria, like what has been demonstrated in mammals, yeast and Drosophila, could form from F-ATP synthase.

Introduction

According to Peter Mitchell (1961), the synthesis of ATP occurs in mitochondria by a chemiosmotic coupling of substrate oxidation with ADP phosphorylation. This model is based on the highly selective and regulated permeability of the inner membrane. The oxidation of reduced substrates and the transport through the electron transport chain allow the generation of a proton motive force which is then utilized by the mitochondrial ATP synthase (F-ATP synthase) for the synthesis of ATP. Way before the chemiosmotic theory, in the early 1950s, it was already described that mitochondria could undergo a sudden increase in permeability of the inner membrane (Raaflaub, 1953a, 1953b). This feature was further characterized in the late 1970s by Haworth and Hunter (1979; Hunter and Haworth, 1979a, 1979b), who named it permeability transition (PT). These authors proposed the Permeability Transition Pore (PTP) as

the channel responsible for the PT, which has gained increasing interest since it is related to many diseases in mammals, e.g. reperfusion heart injury and muscular dystrophy (Bernardi, 2013). The opening of PTP requires matrix Ca^{2+} and could be induced by matrix P_i, thiol oxidants, cyclophilin D (CyP-D), benzodiazepine-423 (Bz-423), while it can be inhibited by Mg^{2+}, thiol reductants, cyclosporin A (CsA), ADP and ATP (Bernardi et al., 2015).

In plants, around 20 years ago, a Ca^{2+}-induced and CsA-delayed transmembrane electrical potential difference ($\Delta\Psi$) collapse has been described in pea stem mitochondria (Vianello et al., 1995). Afterwards, PT has been observed in several plants, although with different features, depending on tissues and species. In potato tuber, mitochondrial PT was induced by Ca^{2+} and P_i and inhibited by CsA, possibly through interaction with CyP-D (Arpagaus et al., 2002). Fortes et al. (2001) showed, on the contrary, a CsA-insensitive PT and, since no Ca^{2+} uptake was observed, they suggested an external effect of such a cation. In mitochondria from oat leaves (Curtis and Wolpert, 2002) and wheat roots (Virolainen et al., 2002), PT triggered a CsA-insensitive $\Delta\Psi$ collapse and matrix swelling, which were induced by Ca^{2+} and P_i. Remarkably, mitochondrial calcium uptake required the addition of the Ca^{2+}/H^+ ionophore A23187 in oat, while it occured spontaneously in wheat. Plants showed therefore a different and distinctive PTP phenomenology, but with fundamental aspects shared with animals. Among them, one of the most remarkable consequences of mitochondrial swelling is the release of cytochrome *c* (Cyt *c*) in the cytosol, leading to the onset of programmed cell death (PCD), a common feature in yeast, insects, mammals and plants (Balk et al., 1999; Robertson et al., 2002; Arama et al., 2006; Giannattasio et al., 2008; Vianello et al., 2012).

PT is a highly complex phenomenon in which the entity of the PTP is, so far, obscure. An intriguing hypothesis suggests that the structure of PTP as a multicomponent complex would be the result of a molecular exaptation occurring during evolution (Vianello et al., 2012). The components of PTP that have been tentatively proposed in the past were the voltage-dependent anion channel (VDAC), the benzodiazepine receptor (TSPO), the adenine nucleotide translocase (ANT) and the phosphate carrier (P_iC). However, isolated mitochondria from different organisms, where the expression of each of these proteins has been suppressed, still showed a PT (Kokoszka et al., 2004; Krauskopf et al., 2006; Gutiérrez-Aguilar et al., 2014; Šileikytė et al., 2014), indicating that they are not *bona fide* PTP components. Recent evidence showed that, besides its enzymatic and structural roles, F-ATP synthase dimers are involved in PTP manifestation in mammal (Giorgio et al., 2013), yeast (Carraro et al., 2014) and Drosophila (von Stockum et al., 2015) mitochondria, but it is still to be proved in plants (Zancani et al., 2015).

This work was set to examine the functional and structural characteristics of PT in plant mitochondria, similarly to what observed in different organisms, where F-ATP synthase dimers are responsible for PTP manifestation. This will shed a light on plant PTP and enable a reflection on its conserved traits during evolution.

Results

Properties of the Permeability Transition in pea stem mitochondria

In the present work, the spontaneous Ca^{2+} transport into the matrix in energized pea stem mitochondria was not detectable (result not shown). Therefore, the presence of the calcium ionophore ETH129 became necessary for the matrix Ca^{2+} loading before the onset of PT. The

characterization of PT was performed by both ΔΨ (Fig. 1A) and Ca^{2+} retention capacity (CRC) (Fig. 1B) experiments. CRC allows the definition of the threshold matrix Ca^{2+} load required to trigger pore opening (Giorgio et al., 2013). Crude pea stem mitochondria (CM) were added with the dye safranin O and energized by 5 mM succinate. Pulses of 80 μM Ca^{2+} were added at regular intervals. After approximatively 4 consecutive pulses, mitochondria underwent a sudden and spontaneous ΔΨ collapse (Fig. 1A, trace a), which was unaffected by the final addition of the ion channel-forming peptide alamethicin, that have proven use on plant cell and plant mitochondria (Johansson et al., 2004; Matic et al., 2005). PT occurrence was delayed in the presence of 0.5 μM CsA, where approx. the double concentration of Ca^{2+} was required to induce ΔΨ collapse (Fig. 1A, trace b). These results were confirmed by CRC experiments (Fig. 1B), where a ETH129-mediated Ca^{2+} uptake by mitochondria was observed until the induction of PTP opening after 4 additions of 80 μM Ca^{2+} each (Fig. 1B, trace a), and again delayed by the addition of 0.5 μM CsA (Fig. 1B, trace b). Mitochondrial respiration was sustained also after ΔΨ collapse and Ca^{2+} release (not shown), confirming that, even when the ΔΨ was collapsed and followed by the Ca^{2+} release, the mitochondrial electron transport chain was still active. Percoll-purified mitochondria (PM) were used for the same experiments showing a very similar behaviour to CM (not shown).

The onset of PT was not accompanied by matrix swelling (Fig. 2A), which was instead observed in the presence of both non-ionic (Fig. 2A, trace a) and ionic osmotica (Fig. 2A, trace b), only when 5 μM alamethicin was added. Consistently, immunochemical experiments (Fig. 2B) showed that, after incubation with sufficient Ca^{2+} to induce PTP manifestation, Cyt *c* was released from mitochondria only in the presence of alamethicin.

The tuning of PT manifestation was further investigated in different experimental conditions. Increasing concentrations of Ca^{2+} were needed to induce PT opening in the presence of different respiratory substrates: succinate, succinate plus pyruvate, malate plus glutamate (results not shown). Interestingly, rotenone did not influence the PT (result not shown), that is consistent with the presence of rotenone-insensitive NAD(P)H dehydrogenases in the inner membrane of plant mitochondria (Rasmusson et al., 2008). Moreover, increasing concentrations of P_i led to PT inhibition (Fig. 3), similarly to what has been reported in Drosophila (von Stockum et al., 2011) and yeast mitochondria (Carraro et al., 2014).

The effect of other modulators of PT was assessed (Tab. 1). In particular, PT was stimulated by thiol oxidants (diamide and PheAsO) and Bz-423, but inhibited by nucleotides, such as Mg^{2+}/ADP and Mg^{2+}/ATP. The same pattern was obtained adding Mg^{2+} or Ba^{2+} after the addition of succinate (results not shown). Differently from what has been described for skeletal muscle mitochondria (Fontaine et al., 1998), in pea stem mitochondria higher Ca^{2+} concentrations were needed to induce the PTP opening in the presence of substrates of Complex I (Fig. 1S, trace c).

Characterization of F-ATP synthase activity

F-ATP synthase was characterized in sub-mitochondrial particles (SMP) through enzymatic activity as proton pumping and ATP hydrolysis. In the presence of either Mg^{2+} or Mn^{2+}, ATP induced the formation of a proton gradient (Fig. 4A, traces a and b), evaluated by acridine orange (AO) quencing, until a steady-state was reached. Subsequent addition of either oligomycin or the uncoupling agent FCCP induced the complete recovery of the fluorescence, indicating that

ATP hydrolysis was coupled to the transmembrane proton flux. When Ca^{2+} was added to the SMP in the presence of ATP (Fig. 4A, trace c), there was no change in fluorescence intensity and, therefore, no gradient was generated across the membrane.

The scalar activity of F-ATP synthase, evaluated as P_i release (Fig. 4B), was dependent on all the divalent ions tested, but the concentration-dependence of hydrolysis rate with Mg^{2+}/ATP and Mn^{2+}/ATP showed similar affinity for the enzyme (V_{max} = 3.0 and 3.4 μmol (min mg prot)$^{-1}$, respectively; K_m = 115 and 128 μM, respectively), whereas the kinetics parameters for Ca^{2+}/ATP were lower (V_{max} = 1.1 μmol (min mg prot)$^{-1}$, and K_m = 40 μM). In all these experiments, oligomycin was able to inhibit ATP hydrolysis completely (result not shown).

Characterization of F-ATP synthase

In order to analyse the structural characteristics of F-ATP synthase, we first confirmed the presence of crucial components in the isolated PM (Fig. 5A). PM were subjected to SDS-PAGE and immunoblotting with specific antibodies. The immunodetection revealed the presence of β subunit and OSCP, as crucial component of the catalytic portion and the lateral stalk, respectively, utilized as markers for F-ATP synthase. The mitochondrial isoform of the cyclophilin, CyP-D, was also present in PM, as a potential PTP regulator.

PM were also subjected to solubilisation with digitonin and, after BN-PAGE, F-ATP synthase dimers were detected through in-gel ATP synthase activity (Fig. 5B). The assay evidenced also the presence of the monomeric form and the F_1 portion of the enzyme. The active bands, corresponding to the F-ATP synthase dimers, were cut out and subjected to SDS-PAGE and immunoblotting, confirming the presence of the β subunit as a F-ATP synthase marker in both dimeric and monomeric complexes (Fig. 5C). No contamination by ANT was associated with F-ATP synthase dimers and monomers after Western blotting with anti-ANT. Very low levels of VDAC were present in F-ATP synthase dimers and monomers isolated from BN-PAGE. Western blotting after BN-PAGE revealed that VDAC migrated in the lower part of the gel, below the F-ATP synthase monomer band (result not shown).

F-ATP synthase dimers generates Ca^{2+}-induced currents

Dimers eluted from the gel were added to the *cis* side of an asolectin bilayer and current traces were recorded (Fig. 6), following the addition of 3 mM Ca^{2+} plus 100 μM PheAsO and 200 μM Bz-423, all added to the *trans* side (Figure 6A). The addition of 2.1 mM Mg^{2+} to the *trans* side caused an inhibitory effect on channel activity (Figure 6B).

Discussion

On the basis of recent evidence on the involvement of F-ATP synthase in PTP formation (Bernardi et al., 2015), a functional and structural approach was utilized in this work to define the features and the molecular components associated to PT in plants. Our results confirm that, similarly to what has been found in potato, mammals, yeast and Drosophila mitochondria (Arpagaus et al., 2001; Giorgio et al. 2013; Carraro et al., 2014; von Stockum et al., 2015), also pea stem mitochondria exhibited a PT. This observation is corroborated by the characteristics of PTP opening, in pea stem mitochondria, which was induced by Ca^{2+} and stimulated (also described as "sensitized", Bernardi et al., 2015) by diamide, PheAsO and Bz-423. The onset of

permeabilization could be delayed ("desensitized") by CsA and by F-ATP synthase substrates, such as Mg^{2+}/ADP and Mg^{2+}/ATP. PTP opening was induced by several Ca^{2+} pulses, reaching a total concentration between 240-720 μM, only in the presence of the Ca^{2+} ionophore ETH129 (Fig. 1), in contrast with what has been reported in mitochondria isolated from potato, which exhibit a spontaneous Ca^{2+} uptake, causing a PTP opening and swelling that is inhibited by CsA in the presence of antioxidants (Arpagaus et al., 2002). Nevertheless, our results are similar to what found in *Saccharomyces cerevisiae*, where mitochondria lack of a Ca^{2+} uniporter and PTP opening occurs only with the addition of ETH129 (Yamada et al., 2009; Carraro et al., 2014). In plants, Ca^{2+} transport into the mitochondrial matrix could be also mediated by a P_i symporter (Dieter and Marme, 1980; Akerman and Moore, 1983; Silva et al., 1992) or a Ca^{2+} uniporter (Zottini and Zannoni, 1993). The homologue of the mammalian mitochondrial Ca^{2+} uniporter (MCU), described in plants (Bick et al., 2012; Stael et al., 2012; Rikhvanov et al., 2014), is strictly regulated by MICU1 (Wagner et al., 2015). Therefore, it would be interesting to understand why Ca^{2+} transport is blocked in pea stem mitochondria and, possibly, find the conditions for inducing its uptake.

In the presence of Ca-ATP the F-ATP synthase activity, measured as proton pumping, was completely absent (Fig. 4A, c), while the phosphohydrolysis was low but still detectable (Fig. 4B). This behavior is similar to what has been found in SMP from beef heart (Papageorgiou et al., 1998), but in SMP from pea stem the F-ATP synthase inhibitor oligomycin was able to block the phosphohydrolytic activity in the presence of all divalent cations. These results confirm that also in plant mitochondria, similarly to mammalian ones, in the presence of Ca^{2+}, F-ATP synthase cannot couple ATP hydrolysis to proton transport (Papageorgiou et al., 1998). It has been hypothesised that when Ca^{2+} occupies the catalytic site, it could induce a different conformation of the F_1 sub-complex, which becomes unable to couple catalysis with proton translocation (Bernardi et al., 2015).

Besides the well-known and conserved components of F-ATP synthase, such as β subunit and OSCP, the presence of CyP-D was also confirmed in pea mitochondria (Fig. 5A). This protein has been so far just predicted in plant mitochondria: in Arabidopsis, two single-domain cyclophilins, AtCYP21-3 and AtCYP21-4, have been suggested as mitochondrial proteins (Romano et al., 2004); in soybean, among 62 putative cyclophilins, just four are targeted to mitochondria (GmCYP49, GmCYP51, GmCYP61, GmCYP62) and possibly a fifth (GmCYP31) (Mainali et al., 2014). Cyclophilin has also been identified in potato mitochondrial proteome (Salvato et al., 2014), but its mitochondrial localisation has no cleary predicted. To the best of our knowledge, our results for the first time directly prove by immunological detection the presence of the isoform CyP-D in isolated plant mitochondria. This evidence supports the hypotesis that this protein could be involved in the modulation of PTP, becoming the putative target for CsA. In beef heart mitochondria, CyP-D binding to OSCP is favored by P_i (Giorgio et al., 2009) and this interaction lowers the concentration of Ca^{2+} needed to open the PTP (Giorgio et al., 2013). In keeping with this, PTP of CyP-D-null mouse liver mitochondria is inhibited by matrix P_i (Basso et al., 2008). In our case, even if pea stem mitochondria possess CyP-D and PTP was modulated by CsA (Fig. 1), P_i acted as an inhibitor of PTP opening (Fig. 3), similarly to what has been described in yeast and Drosophila (Yamada et al., 2009; von Stockum et al., 2011; Carraro et al., 2014). Nevertheless, these species do not have mitochondrial cyclophilins (or at

least in yeast, the mitochondrial CyP isoform does not regulate PTP), consistently with the CsA-insensitivity of their PTP (Bernardi et al., 2015). Further experiments are needed to clarify the interactions of CyP-D with F-ATP synthase in plant mitochondria, since such a protein, even if present in PM, was not detected in purified F-ATP synthase dimers after BN-PAGE (result not shown). This could depend on the presence of the detergent digitonin or on the buffer ionic strength, both necessary to isolate functional dimers. These experimental conditions could have induced the detachment of CyP-D from the enzymatic complex, which is associated by an electrostatic interaction (Giorgio et al., 2013).

Purified mitochondria subjected to BN-PAGE evidenced the presence of dimers, monomers and the F_1 portion of F-ATP synthase by in-gel activity (Fig. 5B). The bands associated with the dimers were less evident with respect to the ones of mammals (results not shown; Giorgio et al., 2013) and yeast (Carraro et al., 2014), even if the total loaded protein was comparable, probably because of the lower density of F-ATP synthase dimers in plants, or their lower stability during the isolation and purification (Eubel et al., 2004). After the insertion of the F-ATP synthase dimers into artificial bilayer, a low conductance was observed in the presence of Ca^{2+}, PheAsO and Bz423 (Fig. 6A), which was inhibited by Mg^{2+} (Fig. 6B). F-ATP synthase dimers proved therefore to be sensitive to the same inducing compounds tested in isolated CM. It is noteworthy that immunological experiments on both F-ATP synthase monomers and dimers showed the presence of the β subunit, while no ANT was detectable and VDAC was present only as slight contamination (Fig. 5C), suggesting that the latter proteins are not involved in current formation. A further common feature between PTP of pea stem mitochondria and Ca^{2+}-induced Ca^{2+}-release channel (mCrC) of Drosophila is the lack of swelling (Fig. 2A), which is consistent with the low conductance exhibited by F-ATP synthase dimers and with the absence of Cyt *c* release by mitochondria (Fig. 2B).

In conclusion, our results show that pea mitochondria exhibited a PTP that appears to be performed by F-ATP synthase dimers, similar to what has been found in mammals (Giorgio et al., 2013), yeast (Carraro et al., 2014) and Drosophila (von Stockum et al., 2015). The evidence that F-ATP synthase dimers are capable of forming the PTP in plants extends and further confirms the suggestion that this enzyme could represent the key player in energy production for life and, at the same time, a trigger for cell signaling during stress and cell death (Bernardi et al., 2015; Zancani et al., 2015). Indeed, a common feature shown by different organisms in PTP manifestation is the requirement for Ca^{2+} in the mitochondrial matrix and oxidative conditions that match very well with the ones exhibited by F-ATP synthase in plants. This mechanism could act in plants also in synergy with K^+ channels, involved in the modulation of mitochondrial permeability during responses to environmental/oxidative stress and in the control of ROS production (Vianello et al., 2012; Trono et al., 2015). Hence, PTP could represent a common feature that, in different organisms and tissues, could be finely modulated to play specific physiological functions.

Recent evidence indicates that, starting from bacteria to humans, F-ATP synthase is one of the essential 1500 "nanomachines" that have been estimated to be crucial for life appearance and survival during evolution (Falkowski, 2015). This underlines the pivotal role of this enzymatic complex, not only in cell bioenergetics, but also during development and in pathophysiology. On the basis of these observations, we highlight also another interesting evolutionary aspect: these

structures, highly conserved and involved in ATP production, could have acquired (by chance) a new function. Therefore, F-ATP synthase, similarly to what happened for other important enzymes (Vianello et al., 2012), underwent an exaptation/cooptation to perform a very different role, such as mitochondrial PT. In other words, it is a wonderful example of the creativity of life, acting as a "bricoleur", as suggested by François Jacob (1977).

Materials and Methods

Plant material

Etiolated pea (*Pisum sativum* L. cv. Meraviglia d'Italia, Ingegnoli) stems were obtained by growing seedlings on sand for 7 days, in the dark, at 25°C and 60% relative humidity.

Isolation of mitochondria for membrane potential, Ca^{2+} fluxes, Ca^{2+} retention capacity (CRC), swelling and oxygen consumption experiments

CM from pea stem were isolated from 90 g fresh weight of stems, homogenized in a mortar at 4°C in 120 plus 100 ml of extraction buffer (0.3 M sucrose, 20 mM HEPES-Tris [pH 7.6], 1 mM EGTA, 1 mM DTE, 0.6% [w/v] PVPP, 0.1% [w/v] BSA). The homogenate was filtered through 6 layers of cellulose gauzes and centrifuged at 2500 *g* (SS34 Sorvall rotor) for 4 min at 4°C; the supernatant was then re-centrifuged at 28,000 *g* for 5 min. The pellet was resuspended and homogenized in 120 ml of the extraction buffer, without PVPP, and again centrifuged at 2500 *g* for 4 min. The supernatant was finally centrifuged at 28,000 *g* for 5 min. The resulting pellet was resuspended in approx. 1 ml final volume of 0.25 M sucrose, 10 mM MOPS-Tris (pH 7.4), 10 µM EGTA-Tris and 0.1% (w/v) defatted BSA (resuspension buffer). This fraction was kept on ice and immediately used in the following $\Delta\Psi$, Ca^{2+} fluxes, Ca^{2+} retention capacity (CRC), swelling and oxygen consumption experiments.

Isolation of sub-mitochondrial particles

SMP were isolated following the method described in Vianello et al. (1997) by sonicating CM (1 mg protein ml^{-1}) three times on ice-bath at 100 W for 30 s each pulse (Braun Labsonic 1510) in a buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.2) and 0.1% (w/v) defatted BSA. The suspension was centrifuged at 28,000 *g* (SS34 Sorvall rotor) for 5 min and the supernatant was centrifuged at 120,000 *g* (Ty 70 Ti Beckman rotor) for 50 min. The final pellet was resuspended in a buffer containing 0.25 M sucrose and 10 mM MOPS-Tris (pH 7.5).

Isolation of mitochondria for electrophoresis, ATP synthase in-gel activity assay, electrophysiology and immunoblotting experiments

CM were isolated as described above, with minor changes, in extraction buffer (0.3 M sucrose, 20 mM HEPES-Tris [pH 7.6], 5 mM EGTA, 1 mM DTE, 1 mM PMSF in 1% [v/v] EtOH, 0.6% [w/v] PVPP, 0.3% [w/v] BSA). CM were layered over a discontinuous Percoll density gradient (45%, 21% and 13.5% [v/v]) and centrifuged at 20,000 *g* (HB-4 Sorvall rotor) for 40 min at 4°C. PM were collected at the 21-45% interface, washed three times in 150 ml of wash buffer (0.25 M sucrose, 10 mM Tris-HCl [pH 7.2]) and centrifuged at 28,000 *g* for 5 min. The pellet from PM was finally resuspended in approx. 1 ml of wash buffer. Mitochondria were then frozen in liquid nitrogen and stored at -20°C for BN-PAGE, in-gel activity assay, SDS-PAGE and immunoblotting experiments.

Membrane potential measurement

Mitochondrial electrical membrane potential ($\Delta\Psi$) changes of CM were estimated by fluorescence quenching of Safranin O or Rhodamine 123 in a PerkinElmer LS50B spectrofluorimeter equipped with magnetic stirring, as described in Casolo et al. (2005). One

mg of CM was incubated in 2 ml of resuspension buffer without BSA, containing 5 μM Safranin O or 0.15 μM Rhodamine 123, 1 mM P_i-Tris and 10 μM of the calcium ionophore ETH129. The wavelengths were set at 495 and 586 nm (2.5 nm slit width) for excitation and emission, respectively.

Ca^{2+} fluxes and Ca^{2+} Retention Capacity (CRC) detection

Mitochondrial Ca^{2+} fluxes in CM were evaluated in a PerkinElmer LS50B spectrofluorimeter, equipped with magnetic stirring, incubating mitochondria in the resuspension buffer without BSA, in the same condition used for ΔΨ measures, but in the presence of 0.5 μM Calcium Green 5N (Molecular Probes), instead of Safranin O. The excitation and emission wavelengths were set at 506 and 532 nm (2.5 nm slit width), respectively.

Mitochondrial swelling measurement

Swelling in CM was monitored as absorbance changes at 540 nm, following the method of Pastore et al. (1999) and using an Agilent 8453 spectrophotometer. Mitochondria were resuspended in the assay buffer (0.25 M sucrose, 10 mM MOPS-Tris [pH 7.4] and 10 μM EGTA-Tris).

Assay of proton pumping and ATP hydrolysis

ATP-dependent proton transport was monitored as acridine orange (AO) fluorescence quenching in a PerkinElmer LS50B spectrofluorimeter. The SMP (50 μg ml^{-1}) were resuspended in 2 ml of 0.25 M sucrose, 10 mM MOPS-Tris (pH 7.4), 50 mM KCl, 5 μM AO. Bivalent cations were added were indicated as $MgCl_2$, $MnCl_2$ and $CaCl_2$. The reactions were started by the addition of 1 mM Tris-ATP. Hydrolysis of ATP was evaluated as P_i release following the method described by Cross and Briggs (1978), in the same buffer used for mitochondrial swelling measurements.

Measurement of mitochondrial respiration

Oxygen consumption in CM was evaluated by a Clark-type oxygen electrode (YSI, Yellow Springs, OH, USA) equipped with magnetic stirring, as described in Casolo et al. (2005). One mg of CM was incubated in 2 ml of 0.25 M sucrose, 10 mM MOPS-Tris (pH 7.4), 10 μM EGTA-Tris, 1 mM P_i-Tris and 10 μM ETH129.

Detection of cytochrome *c* release

CM were initially subjected to induction of PTP opening and then, at different times, the incubation mixture was collected from the cuvette and immediately used for the extraction of soluble proteins. Samples were ultra-centrifuged at 100,000 *g* for 40 min by a Beckman L7-55 centrifuge (Ty 70ti rotor) and the soluble proteins were concentrated by 5000 MWCO VIVASPIN 6 (Sartorius) at 10,000 *g* (SM24 Sorvall rotor) for 40 min. 30 μg of the concentrated proteins was later separated by 15% (w/v) SDS–PAGE and electroblotted onto a nitrocellulose membrane to detect the presence of the Cyt *c* released by mitochondria. Blots were incubated with anti-Cyt *c* rabbit polyclonal antibody (1:1000 dilution, from Agrisera). The reactive proteins were finally detected by nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate staining, after incubation with alkaline phosphatase-conjugated anti-rabbit IgG antibody (1:2500 dilution,

from Sigma).

Blue native (BN)-PAGE and in-gel activity assay

BN-PAGE was performed according to Wittig et al. (2006). PM were solubilized by digitonin with a protein/detergent ratio of 1/2 for a better visualization of F-ATP synthase dimers. Native-PAGE precast gel (3-12% Bis-Tris protein gel) and buffers were purchased from Invitrogen (Thermo Fisher Scientific). The electrophoretic run was performed at 150 V in the Dark Blue Cathode Buffer, replaced after 30 min by the Light Blue ones, and stopped after further 6 h. In order to visualize the bands corresponding to the native F-ATP synthase forms, BN-PAGE gel was transferred into the zymogram buffer (35 mM Tris [pH 8.3], 270 mM Glycine, 14 mM $MgSO_4$, 8 mM Tris-ATP, 0.2% [w/v] $Pb(NO_3)_2$) and incubated overnight at 25°C.

SDS-PAGE and immunoblotting

Bands corresponding to the dimeric form of F-ATP synthase, identified by the zymogram, were excised from the BN-PAGE gel and subjected to 12% (w/v) SDS-PAGE and electroblotted onto nitrocellulose membranes. The following primary antibodies were used: anti-β subunit (polyclonal, 1:10,000 dilution, from Agrisera), anti-OSCP (polyclonal, 1:2000 dilution, from Santa Cruz Biotechnology), anti-CyP-D (monoclonal, 1:2000 dilution, from Calbiochem), anti-plant VDAC1 (polyclonal, 1:10,000 dilution, from Agrisera), anti-ANT (polyclonal, 1:1000 dilution, from Sigma). The secondary antibodies (1:10,000 dilution, from Sigma) conjugated to either horseradish peroxidase or alkaline phosphatase were used for ECL or colorimetric development, respectively.

Electrophysiology experiments

Bands corresponding to dimers of F-ATP synthase were excised from BN gels and eluted with 25 mM tricine, 7.5 mM Bis-Tris, and 1% (w/v) n-heptyl β-D-thioglucopyranoside (pH 7.0) following the protocol by Rehling et al. (2003), and supplemented with 8 mM Tris-ATP and 10 mM $MgSO_4$. After overnight incubation at 4 °C, samples were centrifuged at 20,000 g for 10 min at 4 °C, and supernatants were used directly for reconstitution in electrophysiological studies. Bilayers of 150–200 picofarads of capacitance were prepared using purified soybean asolectin. The standard experimental medium was 500 mm KCl, 10 mm Hepes, pH 7.4. The experiments were performed as described in Szabò et al. (2011) and Giorgio et al. (2013).

Protein determination

Protein concentration was estimated by colorimetric reaction with Bradford reagent (Bradford, 1976). The calibration curve was obtained using BSA as a standard.

Reagents and statistics

All chemicals were of the highest commercially available purity and, unless otherwise specified, purchased from Sigma-Aldrich. Results are typical of at least three independent replicates for each experiment, and error bars refer to the SD.

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FIGURE 1 | Effect of Ca^{2+} and CsA on PTP opening in CM
Isolated CM (0.5 mg/ml) were incubated in 0.25 M sucrose, 10 mM MOPS-Tris (pH 7.4), 10 μM EGTA-Tris, 5 mM P_i-Tris, 10 μM ETH129. (**A**) ΔΨ was monitored as fluorescence decrease with 5 μM safranin O in the incubation medium (**B**) CRC was evaluated with 0.5 μM Calcium Green-5N as fluorescence increase. Where indicated, 5 mM succinate-Tris (*Succ*) and 80 μM Ca^{2+} pulses were added in sequence. In both panels A and B, *traces b* indicate CsA addition to the incubation medium at the concentration of 0.5 μM. In all traces, 5 μM alamethicin was added at the end for the complete collapse of ΔΨ.

FIGURE 2 | Swelling and release of cytochrome *c* in CM
(**A**) Experimental conditions were as indicated in Fig. 1. CM (0.5 mg/ml) were incubated in 0.25 M non-ionic osmoticum (sucrose or mannitol) (*trace a*) or 0.125 M ionic osmoticum (NaCl, KCl, Choline-Cl) (*trace b*). Swelling was monitored as absorbance decrease at 540 nm. (**B**) Immunoblot assay of released proteins from isolated mitochondria incubated in sucrose-based medium in the presence of different reagents, after SDS-PAGE. Mitochondrial protein (1 mg) was incubated in the assay buffer with 5 mM succinate, 1 mM P_i-Tris, 10 μM EGTA-Tris. From the left, lanes correspond to: addition of 5 μM alamethicin; 5 μM FCCP; 10 μM ETH129 and 300 μM Ca^{2+}; 10 μM ETH129; 300 μM Ca^{2+}.

FIGURE 3 | Effect of P_i on PTP opening induced by Ca^{2+}
Isolated CM (0.5 mg/ml) were incubated as in Fig. 1 and Ca^{2+} was added to induce PTP opening, measured as fluorescence change of 0.5 μM Calcium Green-5N, in the presence of increasing concentrations of P_i.

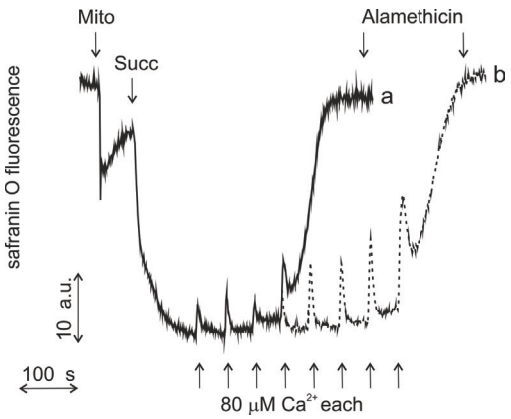
FIGURE 4 | Characterization of F-ATP synthase activity
(**A**) Evaluation of F-ATP synthase proton pumping activity as fluorescence quenching of AO induced by divalent metal (Mn^{2+}, Mg^{2+}, Ca^{2+}) and ATP in SMP (100 μg). (**B**) Evaluation of F-ATP synthase activity as ATP hydrolysis in SMP, in the presence of increasing concentrations of each divalent metal (Mn^{2+}, Mg^{2+}, Ca^{2+}) and ATP.

FIGURE 5 | Immunodetection of F-ATP synthase in PM and dimers
(**A**) β subunit, OSCP and CyP-D were detected in PM (20 μg). The bands detected at the predicted molecular weight are indicated in the boxes, while the additional bands suggest non-specific reactions or associations to other mitochondrial proteins. (**B**) PM (250 μg) were solubilized by digitonin and run on BN-PAGE. The active bands, corresponding to F-ATP synthase dimer (D), monomer (M) and F_1 were visualized by zymogram. (**C**) Immunoblot assay of β subunit, ANT and VDAC after SDS-PAGE of PM, F-ATP synthase dimers and monomers.

FIGURE 6 | Electrophysiology experiments with F-ATP synthase dimers
(**A**) Dimers eluted from BN-PAGE were added to the *cis* side of a purified soybean asolectin bilayer. Representative current traces were recorded upon incorporation of purified dimers, in the presence of 3 mM Ca^{2+} plus 100 μM PheAsO and 200 μM Bz-423, all added to the *trans* side. (**B**) Inhibitory effect in the presence of 2.1 mM Mg^{2+} added to the *trans* side on channel activity (n=1). In both panels A and B, *c* defines the closed state of the channel.

Fig. 1

A



B

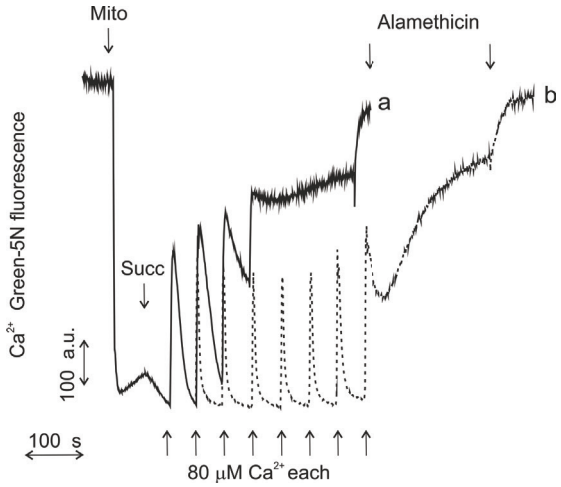
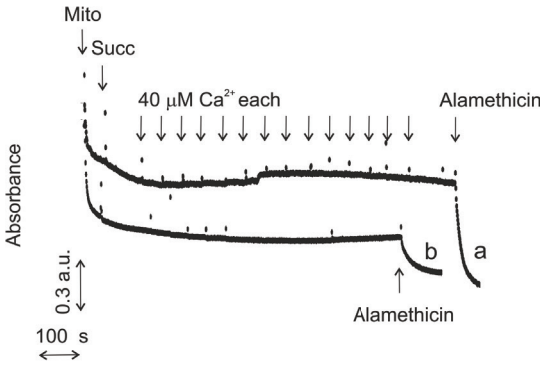


Fig. 2

A



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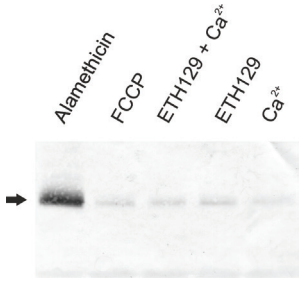


Fig. 3

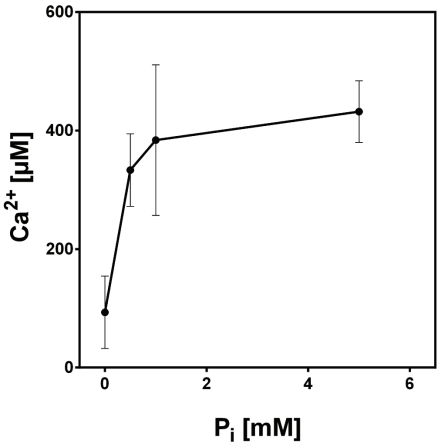
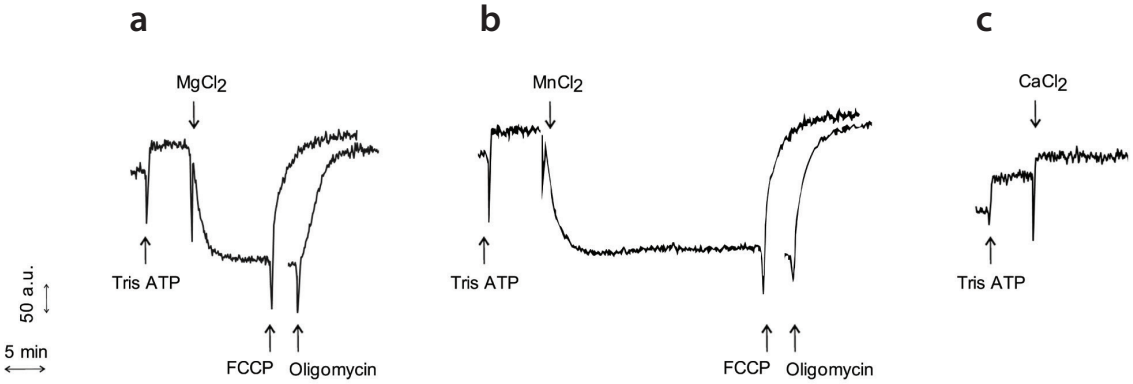


Fig. 4

A


a
b
c
MgCl2
MnCl2
CaCl2
Tris ATP
Tris ATP
Tris ATP
FCCP
Oligomycin
FCCP
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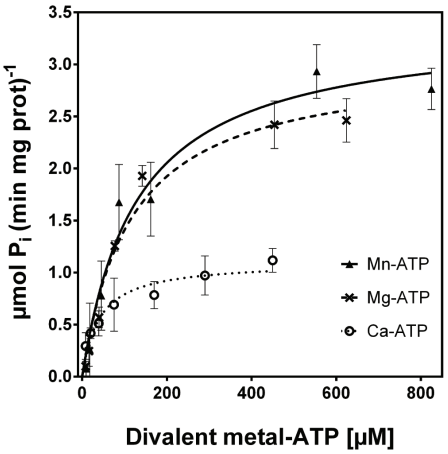
50 a.u.
5 min

B


µmol Pi (min mg prot)-1
Divalent metal-ATP [µM]
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Mg-ATP
Ca-ATP
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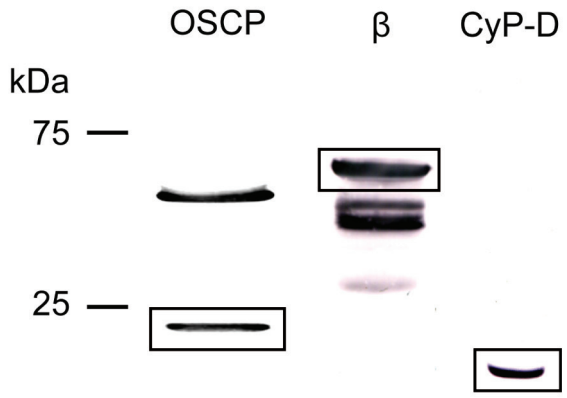
3.5
0
200
400
600
800

Fig. 5

A



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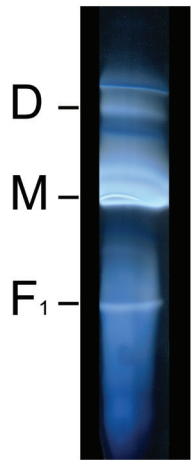


C

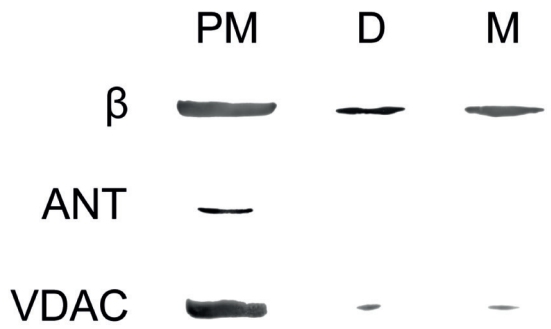


Fig. 6

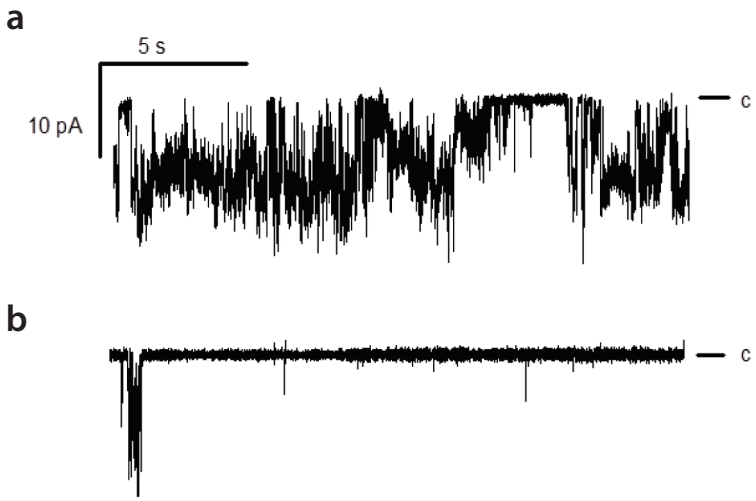


TABLE 1 | Effect of PTP modulators on CRC in CM

Isolated CM (0.5 mg/ml) were incubated in 0.25 M sucrose, 10 mM MOPS-Tris (pH 7.4), 1 mM P_i-Tris, 10 µM EGTA-Tris, 10 µM ETH129 and in the presence of Mg^{2+}/ADP or Mg^{2+}/ATP, 5 mM $MgCl_2$ and 1 µM oligomycin. CRC has been evaluated by fluorescence of Calcium Green. Data are means ± SD of at least 5 independent replicates.

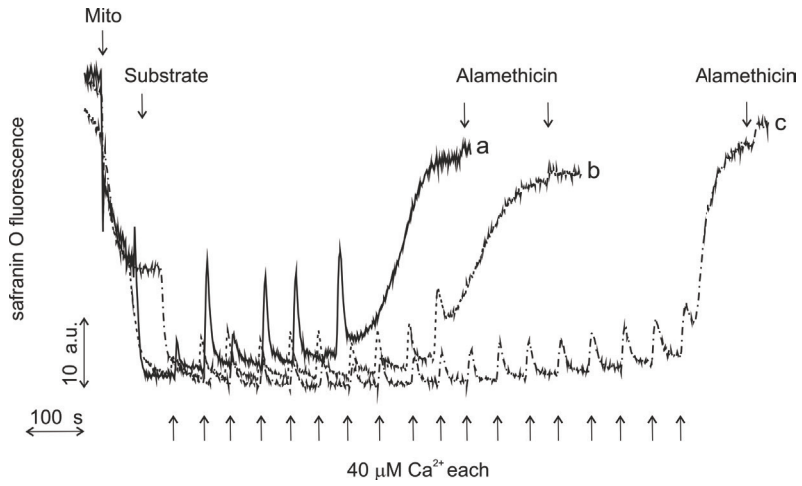
	Control	Mg^{2+}/ADP (2 mM)	Mg^{2+}/ATP (2 mM)	Diamide (2 mM)	PheAsO (50 µM)	Bz-423 (100 µM)
Ca^{2+} (µM)	382 ± 121	701 ± 258	681 ± 179	278 ± 80	188 ± 61	199 ± 66
%	100	190	184	76	51	54

Supplementary Material

FIGURE 1S | Effect of different respiratory substrates on PTP opening evaluated as ΔΨ collapse in CM

Isolated CM (0.5 mg/ml) were incubated in 0.25 M sucrose, 10 mM MOPS-Tris (pH 7.4), 1 mM P_i-Tris, 10 µM EGTA-Tris, 10 µM ETH129. The incubation medium was supplemented with 5 µM safranin O and ΔΨ was monitored as fluorescence decrease. Where indicated, respiratory substrates and pulses of 40 µM Ca^{2+} were added. Different substrates: 5 mM succinate-Tris (*trace a*), 5 mM succinate-Tris plus 1 mM pyruvate (*trace b*), or 10 mM malate plus glutamate (*trace c*). In all traces, 5 µM alamethicin was added for the complete collapse of ΔΨ.

Fig. 1S


Mito
Substrate
Alamethicin
Alamethicin
a
b
c
safranin O fluorescence
10 a.u.
100 s
40 μM Ca2+ each

2.C Monitoring ATP dynamics in isolated plant mitochondria using a fluorescent protein sensor

Preface

Modern sensing techniques have shown great potentialities in addressing bioenergetic questions. This contribution presents how the purified ATeam sensor was successfully used to follow the ATP produced and exported from isolated and functional *Arabidopsis thaliana* mitochondria. This sensor proved to be a flexible and reliable tool for exploring the ATP machinery in isolated organelle. Although the paper is mainly focused on the applicability in the context of *in vitro* assay, in its wider form it will be further completed by *in vivo* measurements with confocal imaging and by high throughput assays using the plate-reader and intact seedlings.

Outstanding questions

Can this novel methodological approach be applied to dissect *in vivo* F-ATP synthase activity? Can it also unravel what occurs to ATP level and dynamics in isolated mitochondria during the occurrence of PT?

Monitoring ATP dynamics in isolated plant mitochondria using a fluorescent protein sensor

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Key-words: ATP, plant mitochondria, fluorescent protein biosensor, F-ATP synthase, adenylate kinase, FRET, Arabidopsis thaliana

Abstract

ATeam is a genetically encoded sensor already used to explore the biology of intact and functional subcellular modules *in vivo*. In this work we report a novel application of the purified sensor for measuring ATP levels and dynamics in functional isolated plant mitochondria, in a highly controlled manner. The method allows to discriminate between ATP production by oxidative phosphorylation and adenylate kinase (AK) activity and the changes in ATP levels induced by specific inhibitors. Although this approach cannot clearly reflect the complexity of processes in an intact cell system, it can be usefully applied to dissect and understand dynamics in the bioenergetics of plant organelles. The method has great potential and applicability for use with other species and in different contexts.

Introduction

"Where there is life, there is ATP" (P. W. Atkins)

Adenosine 5'-triphosphate (ATP) is the universal energy currency for living cells. The establishment and breakage of the high-energy phosphate anhydride bonds of the molecule is the principle that allows energy transduction between the biochemical reactions and processes that sustain life. Metabolism, membrane transport, cellular movements, intracellular trafficking, signalling, are examples for the diverse biological functions in which ATP plays a critical role.

In plant cells, a bulk amount of the ATP required for cell maintenance is typically generated by mitochondrial oxidative phosphorylation (Gout et al., 2014). This applies not only to non-green tissues and green tissues in the dark, but also in the light. Although in green cells, chloroplasts generate large amount of ATP through the photosynthetic light reactions, direct export of ATP to the cytosol is thought to be inefficient (Gardeström and Igamberdiev, 2016). Energy flux out of the chloroplast is believed to occur mainly indirectly via metabolites synthesized in the chloroplast stroma under ATP consumption (Haferkamp et al., 2011). Yet the exact relative contributions to ATP provision in the illuminated plant cell are still under debate and both organelles may contribute to the ATP supply of the cytosol. How the organelles coordinate their functions to maintain energy supply for the cell under fluctuating conditions remains largely unresolved (Gardeström and Igamberdiev, 2016).

The core chemical players in this energy transduction system are ATP, ADP, P_i and Mg^{2+}.

Cell complexity is much larger, however, considering the balance between free and bound nucleotides, the equilibria of Mg^{2+} complexes, phosphorylating and de-phosphorylating systems, membrane transport machineries and their interplay within and between cell compartments. In the presence of oxygen, ATP/ADP ratios in the cytosol and in most other cell compartments are maintained by phosphorylation of ADP mainly in the mitochondrial matrix, catalysed by the F-ATP synthase. ATP is then efficiently exported across the inner mitochondrial membrane by highly active ATP/ADP translocases, such as the adenine nucleotide translocator ANT (Haferkamp and Schimitz-Esser, 2012; Gout et al., 2014). ATP generated on the matrix side must be exported, while ADP and phosphate (P_i) must be imported as substrates. The ATP/ADP exchangers facilitate this, actively exporting ATP and enriching ADP in the matrix, a processes driven by membrane potential (Haferkamp et al., 2011). P_i import is mediated by the highly abundant phosphate carrier (P_iC) and driven by the pH gradient (Haferkamp and Schimitz-Esser, 2012).

However, chemiosmotic phosphorylation is not the solely responsible for ATP synthesis and ATP/ADP ratios in mitochondria. It also depends on equilibration of the adenylate pools by adenylate kinase (AK), that sets a favourable environment for the large cellular network of ATP-dependent systems. AK catalyses the reversible disproportioning of 2ADP to AMP and ATP (2ADP <-> AMP + ATP), allowing stabilisation of the ATP/ADP ratio even when fluctuations in ATP production or consumption rate occur (Igamberdiev and Kleczkowski, 2001). AK has been found in a wide range of plant species (Carrari et al., 2005), in different subcellular locations including cytosol and bioenergetic organelles (Johansson et al., 2008). In plant mitochondria, this enzyme has been independently reported to reside in the intermembrane space (Day et al., 1979; Stitt et al., 1982; Busch and Ninnemann 1997; Roberts et al., 1997; Igamberdiev and Kleczkowski, 2001; Zancani et al., 2001), while it appears absent from the mitochondrial matrix, in contrast to mammalian mitochondria where matrix isoforms have also been reported (Igamberdiev and Kleczkowski, 2001; 2003; Dzeja and Terzic, 2009). Classical biochemical techniques have been used to estimate AK localization (e.g., fractioning of protoplast, lysis of mitochondria, enzymatic inhibition), indicating its presence on the outer surface of the inner membrane of potato (Day et al., 1979) and pea (Zancani et al., 2001) mitochondria. AK localisation in the mitochondrial intermembrane space makes AK able to react with the adenine nucleotides of the cytosol (Stitt et al., 1982; Igamberdiev and Kleczkowski, 2001), buffering the subcellular ATP/ADP ratio and supporting efficient ATP synthesis under variable physiological conditions (Igamberdiev and Kleczkowski, 2015; Gardeström and Igamberdiev, 2016).

The structural and physiological complexity of mitochondria has a colourful history of stimulating, yet challenging investigation. In this work we established a new method to dissect the role of plant mitochondria using a purified genetically encoded ATP-sensor, which allows monitoring ATP dynamics in real time. The use of respiratory substrate feeding in combination with pharmacological inhibitors enabled a detailed dissection of mitochondrial ATP homeostasis and demonstrated the usefulness of this new methodology.

Sensing ATP

Several methods for ATP quantification have been developed over the last decades (Strehler and Totter, 1952), but accurate and specific measurement techniques for ATP dynamics in intact

cells with subcellular resolution have been lacking (De Michele et al., 2014).

Classical and well-established methods use a chemiluminescence approach based on the luciferin-luciferase system (Lemasters and Hackenbrock, 1973); although the method is technically straightforward to use, it does not allow any measurement in living, intact cells and it only works for extracts in which cell integrity had been lost and subcellular adenylate pools mixed. As such it can provide measurements of an average ATP concentration across all compartments of a cell. Yet, detection of ATP changes in the cell lysate may then not necessarily reflect changes in cytosolic ATP (Zamaraeva et al., 2005) and measurements in isolated organelles remain problematic because of the risk of changes in ATP, even during very rapid subcellular fractionation (Gardeström and Wigge, 1988; Wigge et al., 1993). This limits the power of this approach in extracting *in situ* information.

Over the last decades, several ATP-specific probes were developed and combined with imaging, to enable qualitative and quantitative ATP detection in real-time and *in vivo* (Rajendran et al., 2016). The introduction of genetically encoded sensors has removed critical hurdles. Protein-based biosensors can be targeted to specific compartments by fusion with targeting proteins or peptides (De Michele et al., 2014).

Among the available biosensors for ATP, one class relies on chemiluminescence by firefly luciferase. The sensor has been targeted to the mitochondrial matrix and the cytosol (Maechler et al., 1998; Jouaville et al., 1999; Zamaraeva et al., 2005). A second class comprises fluorescent protein sensors that respond to ATP by changes in Förster resonance energy transfer (FRET). Two fluorescent proteins with different spectral properties are linked by an ATP-binding domain which modifies their relative position in response to substrate binding (De Michele et al., 2014). Among them, the ATeam (Adenosine 5'-Triphosphate indicator based on Epsilon subunit for Analytical Measurements; Imamura et al., 2009) sensor family relies on FRET. This family includes the original ATeam-mD/mA and the later improved version ATeam-nD/nA, which shows a higher YFP/CFP ratio (Kotera et al., 2010). Another variant is Go-ATeam, developed to follow simultaneously ATP and Ca^{2+} dynamics in the cytosol and the mitochondria (MitGo-ATeam; Nakano et al., 2011; Vevea et al., 2013).

The genetically encoded ATeam sensors offer the advantage that they can be introduced stably into cell lines or organisms and provide information with subcellular spatial resolution, highlighting the heterogeneous behaviour of individual cells (Vevea et al., 2013) and circumventing issues related to probe uptake (De Michele et al., 2014). Moreover, FRET-based indicators are ratiometric in their emission, meaning that they are independent of sensor concentration. They can respond to changes in physiological conditions in a rapid, quantitative and reversible manner, enabling fluorescent microscopic visualization of physiological events in space and time (Kotera et al., 2011; De Michele et al., 2014). Ideally, these sensors report local responses with minimal perturbation of the biological system investigated (Hamers et al. 2013); however whether this applies requires careful examination in each specific system.

ATeam was the first FRET-based fluorescent ATP indicator for visualizing ATP levels within single living cells. The sensor was engineered by inserting the ε subunit of *Bacillus subtilis* F-ATP synthase between two fluorescent proteins: a donor CFP (cyan fluorescent protein), and an acceptor, cpVenus (circularly permuted Venus, a variant of YFP, or yellow fluorescent protein). When binding ATP, the ε subunit undergoes a pronounced conformational change into a folded

form, bringing the two fluorophores into closer vicinity and resulting in an increase in FRET (Fig. 1) (Imamura et al., 2009; Kotera et al., 2010; Nakano et al., 2011; Hatsugai et al., 2012). Since ATP is not hydrolysed, but bound fully and reversibly instead, cell homeostasis remains largely unaffected (Imamura et al., 2009). Although a biological impact even of reversible binding can never be ruled out completely, the sensor is typically expressed to low μM levels, while ATP is present at ~ 1000 x higher levels, suggesting that any effect is likely to be small, especially since many endogenous proteins also bind ATP. ATeam has paved the way for diverse biological applications, mainly in the animal field; in plants, only one report so far has made use of the sensor to investigate hypersensitive cell death (Hatsugai et al., 2012).

In this study we developed a novel methodology that allows to measure ATP levels and dynamics in functional, isolated plant mitochondria. The purified ATeam sensor protein is added to the assay buffer where it can monitor the changes in ATP resulting from export or uptake by the organelle. Since the sensor cannot cross the outer mitochondrial membrane, it can evaluate ATP production by mitochondria in a simplified system where the buffer represents an 'artificial cytosol', well defined in composition and lacking most cytosolic components, including ATP-hydrolysing enzymes.

Results

Sensor stability

In preliminary experiments we observed that the dynamic range of ATeam 1.03-nD/nA decreased with the storage time of the purified sensor protein (data not shown). Hence we decided to test systematically the impact of different storage conditions on the highly pure sensor by measuring the change in relative dynamic range after one day or one week of storage at different temperatures (Fig. 2a). All the samples were loaded and analysed by SDS-PAGE (Fig. 2b). Storage at 4, 22 and 37°C showed protein degradation, where many protein fragments of different sizes appeared below the band corresponding to the intact sensor (67.7 kDa) (Fig. 2b), suggesting that the purified sensor was stable when stored frozen, but underwent gradual proteolysis in the fridge by an unknown mechanism.

In vitro characterization of purified ATeam 1.03 nD/nA sensor protein

We re-evaluated the key properties of the purified sensor as a basis for further application. ATP-dependence of the fluorescence spectra of ATeam 1.03-nD/nA could be confirmed (Fig. 3a). Consistent with previous results (Imamura et al., 2009), the emission peak of the donor CFP (475 nm) decreased with increasing ATP concentrations, while that of the acceptor cpVenus (527 nm) increased.

The sensor response to different adenine nucleotides (complexed with equimolar $MgCl_2$) was determined as emission ratio of the cpVenus (527 nm) over the CFP (475 nm), showing sensitivity for ATP changes in the high μM and low mM range (Fig. 3b). The maximal spectroscopic dynamic range was 3.97. The dissociation constant (K_d) for ATP was 0.74 mM at 25°C in the assay buffer (pH 7.5), in agreement with previous reports (Imamura et al., 2009). ADP and AMP had no effects on the sensor behaviour (Fig. 3b), confirming previous observations of high ATP specificity over other nucleotides, such as ADP, GTP, CTP, UTP (Imamura et al., 2009) and AMP. The pH dependence of ATeam 1.03-nD/nA was also tested, since pH variations

can occur in living cells and their effect on sensor readout needs to be taken into account. The sensor was fairly insensitive to pH above 7.5, but a sharp decrease occurred below 7.25 (Fig. 3c). Interestingly, the pH effect only occurred in the presence of ATP, suggesting a change of protonation in the ATP-binding domain rather than in the fluorescent proteins. These data confirm the pH stability of ATeam in the physiological range of the cytosol, although caution is required when minor FRET ratio changes are interpreted *in vivo*. In order to guarantee strict comparability, we used the same assay buffer (composition, pH buffer system, ionic strength) for both sensor characterization and assays with isolated mitochondria.

In vitro assays with isolated mitochondria and purified ATeam protein

In a next step, we used identical conditions as above, adding freshly purified, intact and functional mitochondria isolated from *Arabidopsis thaliana* seedlings to study on ATP dynamics (Fig. 4a). The FRET ratio of the sensor was unaffected by the addition of 10 mM succinate, used as electron transport substrate, and was increased by ADP addition (at different concentrations: 0, 0.5, 1 and 4 mM), indicating ATP production by the F-ATP synthase. When ADP was added before succinate (Fig. 4b), an increase in FRET ratio was observed. This increase was particularly pronounced at high ADP concentrations (4 mM), while it was very limited at lower ADP concentrations (0.5 mM). Subsequent addition of succinate resulted in an additional increase in FRET ratio, which was particularly pronounced with lower ADP levels. This indicates that AK activity is present in the mitochondria and is able to convert ADP, when present at high concentration, to ATP and AMP.

To explore the hypothesis that both AK and F-ATP synthase contribute to the ATP production observed by the sensing method, we used different approaches. First, to test for the possibility that isolated mitochondria contained any substrate for the electron transport chain, able to promote ATP synthesis in the presence of ADP, we performed oxygen electrode assays (Fig. 5a). The addition of 4 mM ADP, which gave rise to substantial ATP production (Fig. 4b), did not result in any increase of oxygen uptake rate above the baseline, while the addition of succinate initiated oxygen consumption (Fig. 5a). This showed that ATP production was not linked to electron transport in the absence of succinate. Next, we tested the hypothesis of AK-mediated ADP disproportioning to ATP and AMP by supplementing the assay with AMP at different concentrations. Four mM ADP added to non-energised mitochondria resulted in ATP production (Fig. 4a), but the reaction could be reversed by the addition of AMP at different concentrations (2, 4, 16 and 32 mM) (Fig. 5b). This clearly indicates that AK activity regulates the equilibrium between ADP, ATP and AMP. The curve in figure 5c indicates that 32 mM AMP abolished completely ATP production by AK. Consistent results were obtained when supplementing AMP at different concentrations before the addition of 4 mM ADP (data not shown). The use of P^1, P^5-di(adenosine-5')pentaphosphate (Ap5A; Roberts et al, 1997; Zancani et al., 2001), as a substrate analogue and competitive inhibitor of AK activity, was also tested, but the high concentration required to inhibit AK caused some interferences, probably with F-ATP synthase and/or ATP import/export (data not shown) and therefore not followed up further.

In order to evaluate ATP generation in isolated mitochondria by F-ATP synthase, we made use of our insights into the activity of AK. We exploited the differential usage of low ADP concentrations: 0.5 mM ADP alone caused a very limited increase in FRET ratio, while a strong

increase occurred in the presence of electron transport activity (Fig. 4). All assays to evaluate F-ATP-synthase activity were therefore performed with 0.5 mM ADP in the presence of 10 mM succinate. To investigate the dependence of the ATP production on the electron transport chain as a driver for ATP-synthase activity, we used different specific inhibitors (Fig. 5d), such as antimycin A (AA) for inhibition of complex III, potassium cyanide (KCN) for complex IV, oligomycin for F-ATP-synthase. Furthermore, carboxyatractyloside (cATR) was used as inhibitor of adenine nucleotide translocator (ANT) to block the exchange of ADP and ATP across the inner mitochondrial membrane. In succinate-energized mitochondria, the presence of all such inhibitors induced an almost complete reduction in ATP production (Fig. 5e).

We then investigated if the measured AK activity relies on ADP/ATP transport across the inner mitochondrial membrane, inferring information on the submitochondrial localisation of AK. For that purpose, we used cATR to block ANT, in combination with low and high ADP concentrations, i.e. to avoid or to trigger AK activity, respectively. To verify the inhibition of ANT, we subsequently added succinate to activate ATP production by ATP-synthase (Fig. 5f). When 4 mM ADP was added, ATP production was similar either in presence or absence of cATR. After subsequent energization with succinate, to activate F-ATP-synthase, a slight increase in ATP was only achieved in the absence of cATR. Those data confirmed that AK allowed ATP production and release even when inner membrane transport of nucleotides was blocked, while ATP production and release by F-ATP-synthase relied on inner membrane ADP/ATP transport. To substantiate this observation, the same assay was performed with 0.5 mM ADP to limit AK activity. Here succinate addition led to a very strong increase in ATP production that was fully blocked by cATR, confirming efficient blockage of ADP/ATP transport across the inner membrane. Together those observations show that all mitochondrial AK activity is located outside the inner mitochondrial membrane, in agreement with previous reports that have localized Arabidopsis AK1 and AK2 in the intermembrane space (Johansson et al., 2008), with no AK activity localized in the matrix.

Discussion

ATeam 1.03-nD/nA as a novel tool for visualizing ATP dynamics from mitochondria

Purified ATeam 1.03-nD/nA showed suitable properties as a sensor to follow dynamic changes in ATP levels in real time. This was exemplified by studying the production and export (as well as import and consumption) of ATP by isolated mitochondria, establishing a novel, dynamic ATP assay. While the fluorophores of FRET sensors may be affected differently by various factors with consequences on the sensor readout (Uslu and Grossmann, 2016), the assay system provides a highly controlled setup, with excellent properties not only for measuring ATP dynamics from intact organelles, but also as a bridge to link *in vitro* and *in vivo* investigations. As a FRET sensor, ATeam can only be functional as a single polypeptide chain and protein instability was identified as a potential pitfall, decreasing the maximal spectroscopic dynamic range that probably is due to the presence of the two non-connected chromophores (Fig. 2a, b). Since protease inhibitors were added during sensor purification, we speculate that the protein might possess an intrinsic instability. Yet, the problem of gradual fragmentation can be overcome by storage of the protein solution in a frozen state. In that context, it is interesting to note that the dynamic range that we observed for the pure, intact sensor (~4-fold) was larger than the one

reported before (~3.2-fold; Kotera et al., 2010). This may indicate that the same issues existed without being identified in previous work (Imamura et al., 2009; Kotera et al., 2010). While sensor integrity can be straightforwardly assessed and optimized *in vitro*, this is more difficult *in vivo*, and requires estimation, e.g. by calibration, to ensure meaningful measurements.

In vitro assays confirmed the spectral characteristics of ATeam 1.03-nD/nA, as described by Imamura et al. (2009) and Kotera et al. (2010). The sensor was specific for ATP and completely unresponsive to other adenine nucleotides such as ADP or AMP, which is an important prerequisite for the assay setup that we have established. ATeam 1.03-nD/nA is relatively pH stable and remains fully responsive to ATP at pH 7.5, which was set as buffer pH to mimic cytosolic conditions. The decrease of FRET ratio in more acidic conditions has been reported before (Imamura et al., 2009; Nakano et al., 2011). Although this has been ascribed to CFP quantum yield and YFP absorption, both of which are sensitive to acidic pH (Nakano et al., 2011), our data suggest that ATP-binding, rather than the fluorophores as such, is compromised at low pH. While acidification can represent an issue to consider when interpreting *in vivo* sensor data from subcellular compartments such as the cytosol or the chloroplast stroma, the experimental setup established here provides a stable pH environment set by a buffer system of high capacity.

Succinate-respiring mitochondria generate a proton motive force through the electron transport chain that allows oxidative phosphorylation performed by F-ATP synthase. In non-energized mitochondria, AK is able to generate ATP from ADP: at low ADP concentration (0.5 mM) we could detect only very little ATP, while at higher ADP concentrations (4 mM) ATP generation appeared to increase over-proportionally (Fig. 4b). This effect appears to be not fully accountable by the sigmoid relationship between FRET ratio and ATP concentration. Instead, ATP generation by AK appears to be over-proportionally favoured in the presence of higher ADP concentration, potentially based on a K_m below the mM range (3 µM for free ADP and 53 µM for MgADP for the forward reaction, 29 µM and 138 µM for the reverse reaction; Busch and Ninnemann, 1996). *In vivo* high ADP concentrations may indicate an ATP crisis, which can then be buffered by AK to maintain a high ATP/ADP ratio and bridge short-term increase in ATP demand or decrease in ATP production rate (Busch and Ninnemann, 1997).

In non-energised mitochondria, the addition of different concentrations of exogenous AMP shifted the AK equilibrium towards ADP by mass action. The reverse reaction of AK (ATP + AMP <-> 2ADP) could be equally visualized by the sensor (Fig. 5b). It has previously been shown that, in presence of both AMP and ATP, AK is active delivering ADP and supporting state III respiration rates (Busch and Ninnemann, 1997).

Different mitochondrial inhibitors were used to evaluate ATP production (Fig. 5e). Oligomycin was used to inhibit F-ATP synthase directly and to verify its contribution to ATP production. Antimycin A and potassium cyanide were used to inhibit electron transport at complexes III and IV and to prevent proton pumping as a prerequisite for F-ATP synthase activity in turn. Carboxyatractyloside (cATR), an ANT inhibitor, led to an equal reduction of FRET-ratio, confirming that all ATP generated by oxidative phosphorylation comes from the matrix. By contrast, cATR did not interfere with AK activity providing clear evidence that this enzyme is localized outside the inner mitochondrial membrane (Fig. 5f). This confirms previous findings from other plant species such as potato and pea, where AK activity has been associated

with the intermembrane space (Day et al., 1979; Stitt et al., 1982; Roberts et al., 1997; Busch and Ninnemann 1997; Igamberdiev 2001; Zancani et al., 2001), and contrasts the situation in mammalian mitochondria, where AK activity is localized in the intermembrane space and the matrix (Dzeja and Terzic, 2009). Arabidopsis contains seven genes predicted to encode adenylate kinase enzymes. Two isoforms have been predicted to be localized in mitochondria (AK1 and AK2; Johansson et al., 2008) but, to the best of our knowledge, neither direct biochemical nor cell biological localization had been proven yet in this species.

Another factor to be taken into account is the potential involvement of apyrase, an enzyme that has been suggested to be present also in the intermembrane space (Igamberdiev and Kleczkowski, 2015). Apyrase can hydrolyse ADP to produce P_i and AMP, which can be used by AK to regenerate ADP, using an additional ATP (Igamberdiev and Kleczkowski, 2015). The activity of apyrase would be expected to be present in our system, after the addition of 4 mM ADP and the establishment of a steady-state of ATP, in the absence of any electron transport substrate. Then apyrase should act as a net-consumer of phosphor-anhydride bonds, leading to a gradual decrease of ATP levels over time. We observed that, in the assays where ATP is produced by non-energized mitochondria, the FRET ratio decreased after reaching the plateau (data not shown), while, in the presence of succinate, no decreasing in ATP was observed. This drift could be attributed to apyrase activity, which appear to be very low in our system. The same argument applies for the inverse ATP-consuming mode of F-ATP-synthase, which appears to occur to a very limited extent. In pea mitochondria apyrase is bound to the cytosolic side of the outer mitochondrial membrane and washed off during mitochondrial purification (Zancani et al., 2001); a similar scenario may apply also to Arabidopsis mitochondria.

The use of purified mitochondria makes any significant contribution to ATP production by chloroplasts unlikely, removing an additional potential source that has to be considered *in vivo*. Chloroplasts possess specific AKs and ADP/ATP translocation systems (Busch and Ninnemann, 1997; Igamberdiev and Kleczkowski, 2003; Haferkamp et al., 2011; Gardeström and Igamberdiev, 2015) and it would be interesting in the future to apply the system that we established here for mitochondria also to chloroplasts.

ATeam shows the potential in monitoring ATP production and export from mitochondria. Here we have been able to discriminate between ATP production by oxidative phosphorylation and AK activity. The methodology we established and validated represents a powerful novel tool to evaluate ATP dynamics from organelles and it will be universally applicable for bioenergetic organelles of eukaryotes.

Conclusions

This work successfully validated several fundamental properties of the ATeam sensor and optimized a dynamic sensing technique that allows to monitor ATP in situ and in real time.

The genetically encoded sensor offers a novel tool for observing ATP production and export to the cytosol in isolated and functional *Arabidopsis thaliana* mitochondria in a highly controlled manner, since substrates and inhibitors can be added and directly controlled.

Albeit the active isolated organelles cannot fully reflect the processes in an intact cell system, this approach allows novel insights into the dynamic behaviour of bioenergetic organelles and shows the potential of genetically encoded sensors in exploring the biology of intact and

functional subcellular modules as opposed to isolated enzymes or enzyme complexes.

ATP export or uptake gives rise to measureable rates that would be masked by cytosolic consumption or production in vivo. In the cytosol, in fact, ATP supply represents a central component in the bioenergetics of plant cells, where large ATP turnover rates are required by sucrose and lipid synthesis, gene expression, polarisation of cellular membranes, transport, cytoskeletal maintenance and many other processes (Gardeström and Igamberdiev, 2016). In our assays, we have also followed the impact of AK as an important 'thermodynamic buffering enzyme' (Igamberdiev and Kleczkowski, 2003) on adenylate dynamics and tested the modulation of F-ATP synthase in presence of different inhibitors.

Although Arabidopsis mitochondria were used as a proof-of-concept, this methodology is expected to be equally applicable to isolated mitochondria of any species (no transgenic lines are required since the sensor is added externally), including mammals, and also to other isolated organelles such as chloroplasts. For plastids, this may be particularly useful to further explore ATP transport depending on photosynthetic activity. Other possible areas of investigations could be cytosolic ATP dynamics in response to changing conditions, such as pharmacological interference, dark and light transitions, stress exposure or activation of cell death pathways. It could be also used to illuminate the multifaceted role of F-ATP synthase, as central enzyme for ATP production, as crucial element involved in *cristae* architecture and, on the basis of recent findings, in permeability transition (Bernardi et al., 2015).

Materials and Methods

Chemicals

Chemicals were purchased from Sigma-Aldrich unless otherwise specified. ATP, ADP and AMP were complexed with equimolar amounts of magnesium chloride ($MgCl_2$) before each experiment. Where necessary, reagents were pH-adjusted prior to use.

Purification of ATeam 1.03-nD/nA

Escherichia coli (Theodor Escherich) strain Rosetta 2 (DE3) carrying the pETG10A-ATeam-1.03-nD/nA expression vector was grown in LB medium at 37°C to an OD_{600} of 0.2. The culture was transferred to 20°C until cells reached an OD_{600} of 0.6-0.8. Expression of 6×His-ATeam-1.03-nD/nA was induced by isopropyl β-D-1-thiogalactopyranoside at a final concentration of 0.2 mM over night at 20°C while shaking. Cells were collected by centrifugation at 4000 *g* for 10 min at 4°C and the pellet was resuspended in lysis buffer (100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10 mM imidazole). To preserve protein stability, one tablet of cOmplete protease inhibitor cocktail (Roche) was added and cells were chemically lysed after incubation for 30 min on ice in the presence of 1 mg/mL lysozyme and 0.1 mg/mL DNaseI (Roche). Cells were further lysed by sonication (3 × 2 min, 40% power output, 50% duty cycle) while kept in ice. Cell debris were pelleted through centrifugation at 40,000 *g* for 40 min at 4°C and the supernatant was loaded onto a Ni-NTA HisTrap column (GE Healthcare) pre-equilibrated with lysis buffer. The column was then connected to an ÄKTA Prime Plus chromatography system (GE Heathcare) and proteins were eluted with an imidazole gradient (10 – 200 mM in 100 mM Tris-HCl, pH 8.0, 200 mM NaCl). Fractions containing ATeam were pooled, concentrated by ultrafiltration

and applied to a HiLoad 16/600 Superdex 200 prep grade gel filtration column (GE Healthcare) pre-equilibrated with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. Eluted fractions with the intact sensor were pooled and concentrated by ultrafiltration. After the addition of glycerol at a final concentration of 20% (v/v), the sensor was aliquoted, frozen in liquid nitrogen and stored at -80°C.

SDS-PAGE

Purified ATeam 1.03-nD/nA was separated on 12% (w/v) SDS-polyacrylamide gels run at constant 90 V. Proteins were unspecifically stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 in 10% (v/v) acetic acid and 40% (v/v) ethanol. Gels were scanned after appropriate destaining of the background in 2% (v/v) acetic acid, 10% (v/v) ethanol.

Protein quantification

Protein concentration was quantified according to Bradford (1976). Five µl of sample were mixed with 195 µl Bradford reagent (Carl Roth) and absorbance at 595 nm was measured after 5 min of incubation in the dark. Dye absorbance was transformed into protein concentration using BSA as protein standard (0 – 0.8 mg/ml).

In vitro characterization of purified ATeam 1.03-nD/nA

Substrate specificity assays (ATP, ADP, AMP) were carried out on purified ATeam (1 µM final concentration) in 0.3 M sucrose, 5 mM KH_2PO_4, 50 mM TES-KOH, pH 7.5, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% [w/v] BSA) using a FP-8300 Spectrofluorometer (Jasco) at 25°C. CFP was excited at 435 ± 5 nm and the emission was recorded from 450 to 600 nm with a bandwidth of 5 nm.

Plant material and culture

Seedlings of *Arabidopsis thaliana* (L.) Heynh ecotype Columbia-0 were grown in hydroponic pots as described by Sweetlove et al. (2007). The seedlings were grown for 14 days and the photoperiod was set to 16 h light at 22°C (light intensity 50-75 µmol photons $m^{-2}s^{-1}$) and 8 h darkness at 18°C.

Mitochondrial isolation and oxygen consumption measurements

Mitochondria were isolated from two-week-old Arabidopsis seedlings as described by Sweetlove et al. (2007). Oxygen consumption was assayed as described by Sweetlove et al. (2002) using two Clark-type electrodes (Oxytherm, Hansatech).

Plate reader-based fluorimetry

ATeam 1.03-nD/nA was excited with monochromatic light at a wavelength of 435 ± 20 nm in a CLARIOstar plate reader (BMG Labtech). Emission was recorded at 483 ± 18 nm (CFP) and 539 ± 13 nm using bottom readings with a focal height of 4.9 mm in transparent 96-well plates (Sarstedt). Gain was adjusted to 70% using a well with sensor and either no (CFP channel) or a saturating (cpVenus channel) amount of ATP. The internal temperature was kept at 25°C and the plate was orbitally shaken at 400 rpm for 10 s after each cycle. Isolated mitochondria (20

μg) in basic incubation medium (0.3 M sucrose, 5 mM KH_2PO_4, 50 mM TES-KOH, pH 7.5, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% [w/v] BSA) were supplemented with 1 μM purified ATeam in a total volume of 200 μL per well. Fluorescent background of the basic incubation medium was recorded and subtracted from all data before further analysis. Data were normalized and plotted using GraphPad Prism version 6.01.

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Figure 1 | Hypothetical model of ATeam
The cyan fluorescent protein (CFP, PDB: 2WSN) and a variant of the yellow fluorescent protein (cpVenus, PDB: 3EKJ) are covalently linked by the ε subunit of *Bacillus subtilis* F-ATP synthase (PDB: 2E5Y, ATP bound; 4XD7, ATP unbound). In the absence of ATP, the ε subunit adopts an extended conformation and FRET efficiency between the donor (CFP) and acceptor (cpVenus) fluorophores is low. ATP binding induces a pronounced conformational rearrangement that results in an increase of FRET. Note that individual crystal structures have been manually linked.

Figure 2 | Stability of the purified sensor at different storage conditions
Sensor aliquots, maintained for one day or one week after purification at different temperatures, were tested for (**a**) change in their spectroscopic dynamic range (DR) and (**b**) proteolytic degradation products after SDS-PAGE separation. Freshly purified, intact sensor extract was used as reference (MW 67.7 kDa), indicated by the arrow.

Figure 3 | *In vitro* characterization of the purified ATeam sensor
(**a**) ATP-dependent fluorescence spectra of ATeam 1.03-nD/nA. The probe was excited at 435 ± 5 nm and the emission spectra collected between 450 and 600 nm in the assay buffer (0.3 M sucrose, 5 mM KH_2PO_4, 50 mM TES-KOH [pH 7.5], 10 mM NaCl, 2 mM $MgSO_4$, 0.1% [w/v] BSA) at 25°C, using different concentrations of ATP. Values are means from three technical replicates, error bars = SD (not visible in figure due to very low variation).
(**b**) ATP-dependent fluorescence emission ratio and selectivity of the purified ATeam 1.03-nD/nA in vitro. The points were obtained plotting the fluorescence ratio of cpVenus (527 nm) and CFP (475 nm) against nucleotide concentrations (log_{10} scale). The assay was performed in the assay buffer (0.3 M sucrose, 5 mM KH_2PO_4, 50 mM TES-KOH [pH 7.5], 10 mM NaCl, 2 mM MgSO4, 0.1% [w/v] BSA) at 25°C, using different concentration of ATP, ADP and AMP. Values are means from three technical replicates, error bars = SD (not visible in figure due to very low variation).
(**c**) pH-dependence of ATeam 1.03-nD/nA at different ATP concentrations (0, 0.5 and 4 mM). The range of pH between 6.5 and 8.5 was obtained using the assay buffer (0.3 M sucrose, 5 mM KH_2PO_4, 50 mM TES-KOH, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% [w/v] BSA) at 25°C. Values are means from three technical replicates, error bars = SD (not visible in figure due to very low variation).

Figure 4
The dynamic response of the FRET ratio of ATeam 1.03-nD/nA to ATP released by isolated Arabidopsis mitochondria (20 µg). The medium (0.3 M sucrose, 5 mM KH_2PO_4, 50 mM TES-KOH [pH 7.5], 10 mM NaCl, 2 mM $MgSO_4$, 0.1% [w/v] BSA) was supplemented with 1 µM of purified ATeam protein. Values are mean from three technical replicates; error bars = SD of technical replicates. A representative trace is shown which was obtained from experiments repeated three times, using independent biological material giving consistent results, error bars = SD of technical replicates.

(**a**) Mitochondria were supplemented with 10 mM succinate, followed by ADP (0, 0.5, 1 and 4 mM).
(**b**) Reverse order of substrate additions with respect to (a).

Figure 5

(**a**) Respiration in purified mitochondria. Representative polarographic traces illustrating the oxygen consumption of pure and intact mitochondria isolated from Arabidopsis seedlings. Mitochondria (40 μg/ml), 10 mM succinate and 0.25 mM ADP were added at the indicated time points. Numbers represent linear oxygen consumption rates in nmoles/min/mg protein (mean ± SD) from three technical replicates.
(**b**) Effect of AMP on ATP production by AK in non-energized mitochondria. After the addition of 4 mM ADP, AMP was added at different concentrations (2, 4, 16 and 32 mM), showing a decrease in ATP levels. Values are means from three technical replicates; error bars = SD of technical replicates.
(**c**) AK activity in the presence of increasing addition of AMP (2, 4, 16 and 32 mM). The AK activity is set to 100% at 4 mM ADP and 0 mM AMP Values are mean from three technical replicates; error bars = SD of technical replicates.
(**d**) Schematic representation of the mitochondrion. Complex III and IV of the electron transport chain are inhibited by AA and KCN respectively, while oligomycin acts as specific inhibitor of F-ATP synthase. cATR blocks the ATP/ADP exchange via ANT.
(**e**) ATP production in energized mitochondria. Inhibition of F-ATP synthase, complexes III and IV of the electron transport chain with 25 μM oligomycin, 10 μM AA and 4 mM KCN, respectively, and inhibition of ANT with 10 μM of cATR. The treated thesis consisted in the addition of the inhibitor in presence of 0.5 mM ADP and 10 mM succinate. Values are mean from three technical replicates; error bars = SD of technical replicates. Statistical differences were determinate using One-way ANOVA followed by Tukey's multiple comparisons test. Letters represent significant differences $p < 0.05$).
(**f**) Effect of the ANT inhibitor cATR on ATP production in energized mitochondria. Two different concentrations of ADP were used to highlight cATR effect on ATP production by AK and F-ATP synthase. Values are mean from three technical replicates; error bars = SD of technical replicates.

Fig. 1

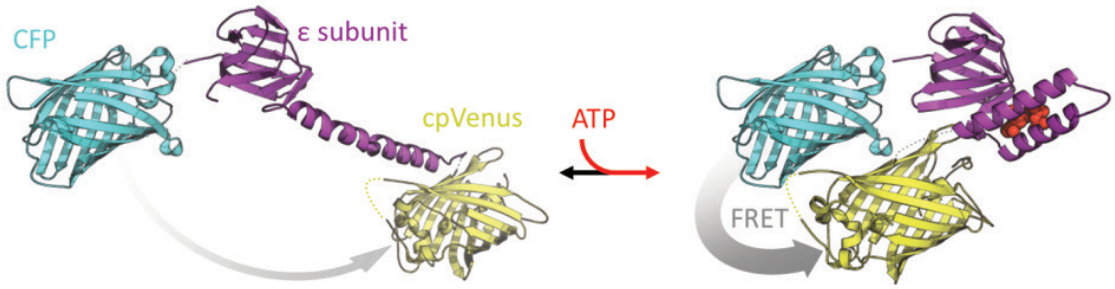

CFP
ε subunit
cpVenus
ATP
FRET

Fig. 2

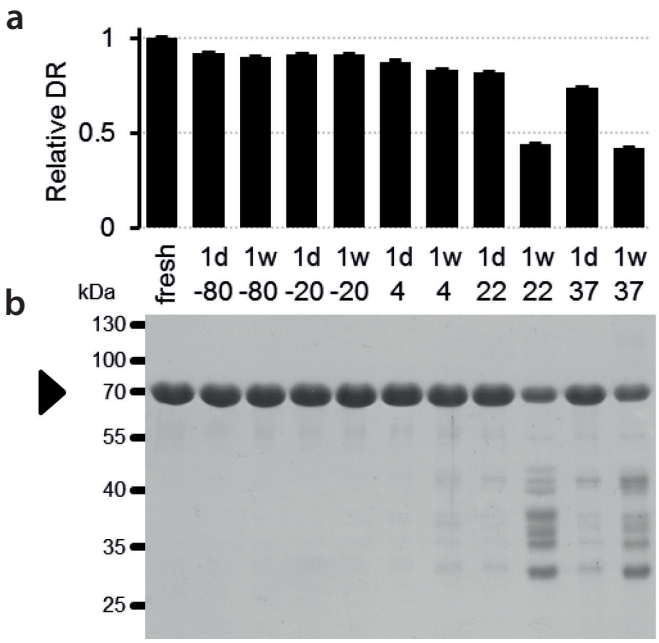
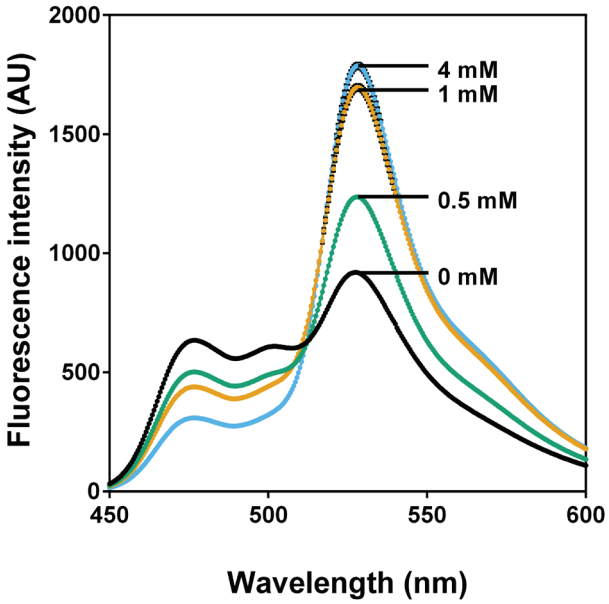
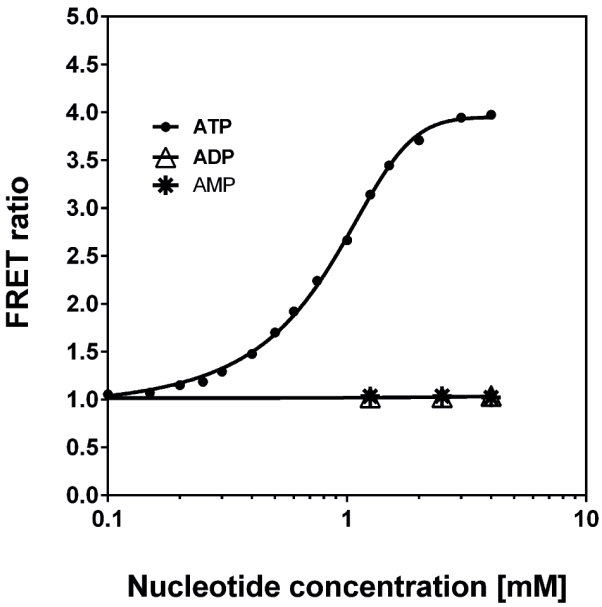

a
Relative DR
1
0.5
0
fresh
1d -80
1w -80
1d -20
1w -20
1d 4
1w 4
1d 22
1w 22
1d 37
1w 37
b
kDa
130
100
70
55
40
35
25

Fig. 2

a


2000
1500
1000
500
0
450
500
550
600
Fluorescence intensity (AU)
Wavelength (nm)
4 mM
1 mM
0.5 mM
0 mM

b


5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.1
1
10
FRET ratio
Nucleotide concentration [mM]
ATP
ADP
AMP

c

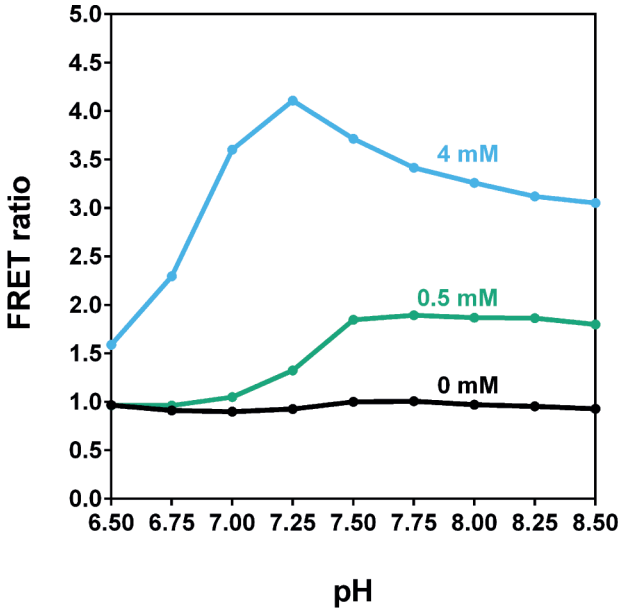
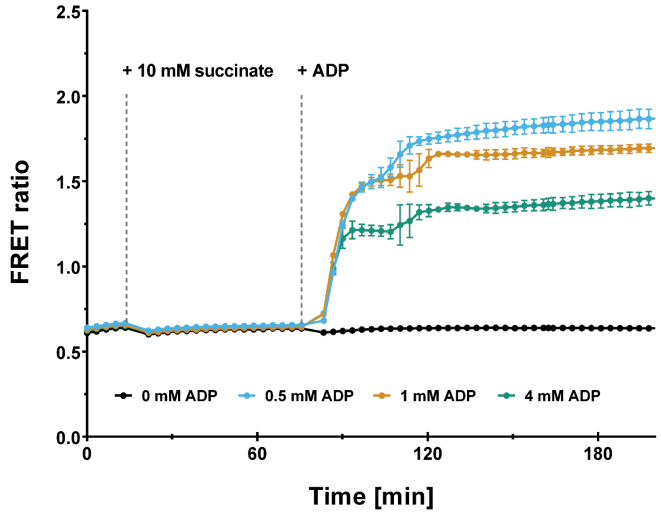

FRET ratio
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4 mM
0.5 mM
0 mM
6.50 6.75 7.00 7.25 7.50 7.75 8.00 8.25 8.50
pH

Fig. 4

a


+ 10 mM succinate
+ ADP
FRET ratio
2.5
2.0
1.5
1.0
0.5
0.0
0 mM ADP
0.5 mM ADP
1 mM ADP
4 mM ADP
0
60
120
180
Time [min]

b

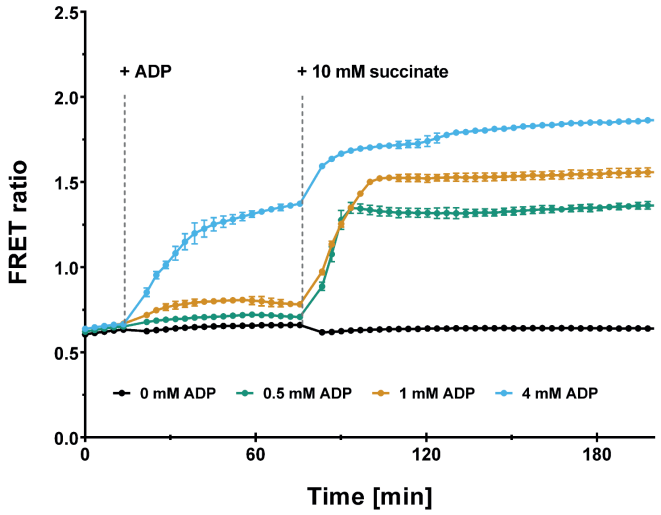
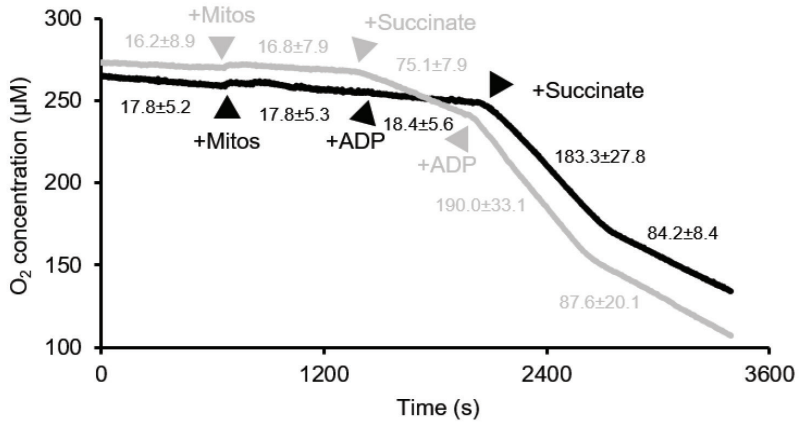
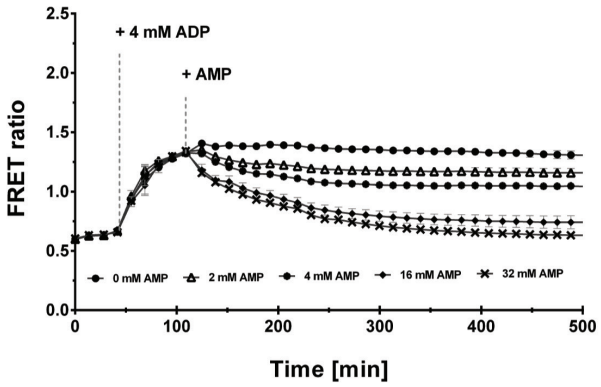

+ ADP
+ 10 mM succinate
FRET ratio
2.5
2.0
1.5
1.0
0.5
0.0
0 mM ADP
0.5 mM ADP
1 mM ADP
4 mM ADP
0
60
120
180
Time [min]

Fig. 5

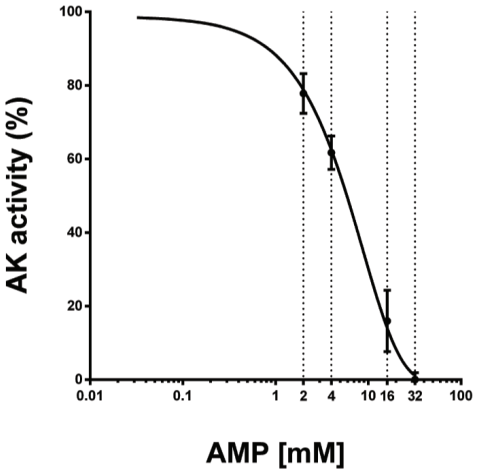
a


+Mitos
+Succinate
16.2±8.9
16.8±7.9
75.1±7.9
+Succinate
17.8±5.2
17.8±5.3
18.4±5.6
+Mitos
+ADP
+ADP
183.3±27.8
190.0±33.1
84.2±8.4
87.6±20.1
300
250
200
150
100
0
1200
2400
3600
O2 concentration (µM)
Time (s)

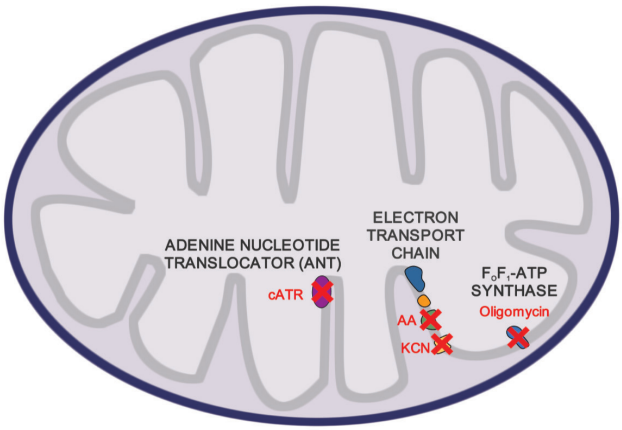
b


+ 4 mM ADP
+ AMP
FRET ratio
2.5
2.0
1.5
1.0
0.5
0.0
0 mM AMP
2 mM AMP
4 mM AMP
16 mM AMP
32 mM AMP
0
100
200
300
400
500
Time [min]

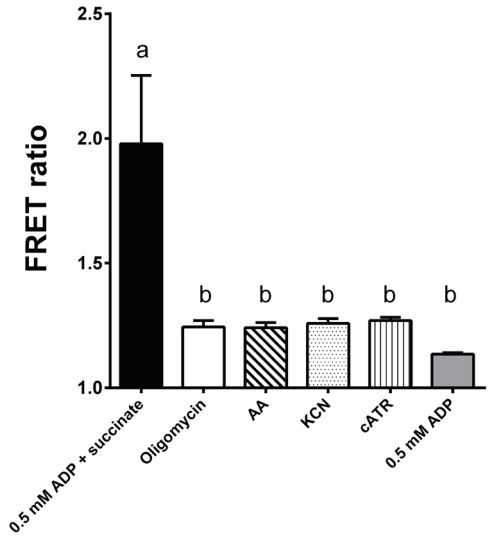
c


AK activity (%)
100
80
60
40
20
0
0.01
0.1
1
2
4
10
16
32
100
AMP [mM]

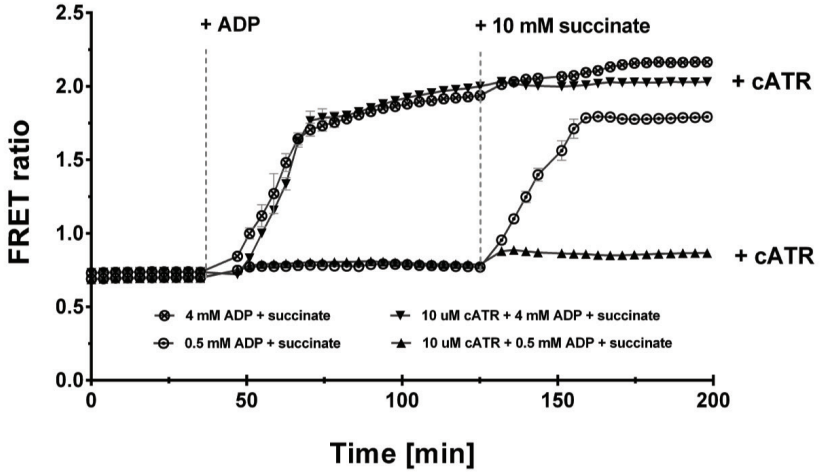
d


ADENINE NUCLEOTIDE TRANSLOCATOR (ANT)
cATR
ELECTRON TRANSPORT CHAIN
AA
KCN
F$_o$F$_1$-ATP SYNTHASE
Oligomycin

e


FRET ratio
2.5
2.0
1.5
1.0
a
b
b
b
b
b
0.5 mM ADP + succinate
Oligomycin
AA
KCN
cATR
0.5 mM ADP

f


+ ADP
+ 10 mM succinate
+ cATR
+ cATR
FRET ratio
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
Time [min]
4 mM ADP + succinate
0.5 mM ADP + succinate
10 uM cATR + 4 mM ADP + succinate
10 uM cATR + 0.5 mM ADP + succinate

3. Conclusion

PTP in plants is still considered one of the less known issue of cell biology (Vianello et al., 2012) and its molecular identity has been the subject of an extensive scientific debate for nearly three decades (Karch and Molkentin, 2014). So far, no definitive evidence has been obtained on plant PTP, or the molecular mechanisms involved in its occurrence.

However, the phenomenon of PT was observed in some studies (Vianello et al., 1995; Fortes et al., 2001; Arpagaus et al., 2002; Curtis and Wolpert, 2002; Virolainen et al., 2002) as a potentially harmful event, guided by mitochondria, for deciding the destiny of the entire cell.

The results obtained during these three years of research are in line with previous findings, although representing some mosaic tiles of a wider picture still out of focus. It is very difficult to formulate a definitive conclusion, giving that many bioenergetic mechanism and processes in plant physiology are not completely understood. In this sense, also Ca^{2+} uptake is not a well-defined system, despite its important role. In particular, Ca^{2+} represents one of the main character of PT, and the alteration of its homeostasis is a key factor for triggering PTP and different paradigms of cell death (Bernardi and von Stockum, 2012). We observed, in agreement with other researchers (Curtis and Wolpert, 2002; Virolainen et al., 2002), an apparent inability of Ca^{2+} uptake into energized pea stem mitochondria, where the Ca^{2+} ionophore ETH129 was needed to induce the import. Differences observed in isolated pea mitochondria indicated also that high Ca^{2+} threshold was needed for the PTP opening, P_i had an inhibitory effect and swelling combined with Cyt *c* release were absent.

It is tempting to speculate that plant PT could represent an alternative or even a simultaneous system (e.g., in combination with K^+ channels; Petrussa et al., 2004; Casolo et al., 2005) from which plant mitochondria take advantage to adjust their metabolic responses to different *stimuli*. Some of the inducers and modulators of PT were found to be shared among organisms and this could be an indication that PT is an evolutionarily conserved mechanism that probably appeared during the endosymbiotic process (Vianello et al., 2012).

In the light of this hypothesis, the model of F-ATP synthase dimers as the putative PTP is fascinating. For exploring this possibility, the structure of plant F-ATP synthase monomer and oligomers would need a better resolution, particularly with respect to the subunits embedded in the inner membrane domain. BN-PAGE is a valuable technique that already highlighted the presence of at least one subunit (g) that could be responsible for the dimerization in *Arabidopsis thaliana* F-ATP synthase (Lapaille et al., 2010; Klodmann et al., 2011), although information on the spatial position of the subunits and their interactions within the enzyme are still missing.

Another limitation encountered for this study was the low yield of pea stem mitochondria after isolation. This is not surprising, since mitochondria for many plant tissue are less numerous compared to mammalian high-energy demand ones (e.g., heart, liver, muscle, brain) (Whitehouse and Moore, 2013). In addition, we experienced technical challenges during the isolation of F-ATP synthase dimers, probably for their 'weaker' constitution (e.g., less proteins implicated in the dimerization, a weak protein-protein interaction). We observed that also the detergent

used (digitonin) could operate differently on the solubilisation of plant membranes and affect the dimer/monomer ratio. The dimeric form of F-ATP synthase appeared to be physiologically lower in abundance than the monomeric one (Eubel et al., 2003; 2004). There is therefore some doubt if this is the real picture or an artefact, since dimer rows on the mitochondrial *cristae* have a strong effect on respiratory activity, growth rates and mitochondrial morphology (Kühlbrandt and Davies, 2016). They can also guarantee the integrity of the F-ATP synthase structure (Buzhynskyy et al., 2007) and enhance ATP synthesis (Dabbeni-Sala et al., 2012). To add more complexity, another difference is that plant dimers appeared also to be orientated in a different displacement angle, with a more open conformation compared to dimers in *fungi* and animals (Davies et al., 2011; Stewart et al., 2013).

The validation of the hypothesis of F-ATP synthase dimers as the putative PTP will require more detailed studies using different approaches. Surely, the simplified *in vitro* approach we established in isolated mitochondria would need to be complemented also with an *in vivo* system.

F-ATP synthase appears therefore to be an even more appealing subject of investigation for its multifaceted roles: shaping mitochondrial *cristae* architecture, supporting life and becoming also a potential permeability system. It is not surprising that ATP represents a multifunctional player for many cellular processes, among which metabolism, transport and signalling. Novel approaches and methodologies could help understanding these function, like the fluorescent protein sensor ATeam that proved to be a powerful tool to monitor and follow ATP level and dynamics in isolated plant mitochondria. Such a probe could find applications for answering both general bioenergetic questions and specific aspects related to F-ATP synthase, the key enzyme in energy conversion.

The research on PTP remains still a fascinating challenge to unravel how mitochondria can be decision makers for the entire cell destiny and the hope is that a renewed interest on the topic will finally shed some light on this old mystery.

Further research

Biochemical characterisation of F-ATP synthase complex

Proteomic studies on plant F-ATP synthase will be essential for resolving enzyme composition and highlight complex abundance in different species and tissues. The resolution of the subunits that have eluded crystallographic analysis will require highly specialized expertise, but it could represent a great advance for understanding dimer composition and providing new insight into the structure and function of one of the most crucial enzymes.

Submitochondrial particles

The use of submitochondrial particles (SMP) have been used in many studies for investigating the properties of enzymatic activities in the inner mitochondrial membrane (Kay et al., 1985; Petit et al., 1991; Vianello et al., 1997). Thus, SMP would be useful for testing the effect of some conditions and active compounds on PTP, providing that the dimeric form of F-ATP synthase would be preserved during SMP preparation. This might offer an experimental condition where the F_1 portion and the Ca^{2+} binding site, normally facing the mitochondrial matrix side, are exposed and easily accessible to substrates and modulators (inside-out particles).

Arabidopsis mutants

The use of knockout mutants lacking the subunit(s) needed for the F-ATP synthase dimerization could give an insight into how dimers are functional for the mitochondrial morphology and for the ATP synthesis. Genetic manipulation of dimer-specific subunit(s) could have the effect of altering directly the mitochondrial architecture and indirectly the onset of the PTP. Mutants lacking of CyP-D could also be used to clarify the effect of this PTP regulator on PT modulation, in combination with P_i and CsA.

Monitoring ATP dynamics during PTP occurrence

The ATeam sensor was successfully used to follow ATP levels dynamically during the onset of plant PCD (Hatsugai et al., 2012). A challenge would be to take advantage of the versatility of the sensor and monitor what happens to ATP level during the occurrence of PT. In its broader application, cells expressing cytosolic ATeam will allow real-time imaging of ATP levels and dynamics through confocal imaging or plate-reader assay.

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Appendix

| 2014

M. Zancani,G. Lippe, E. Braidot, E. Petrussa, V. Casolo, C. Peresson, S. Patui, A. Bertolini, V. De Col, P. Bernardi, A. Vianello (2014) **Search for compounds able to modulate F_0F_1 ATP synthase in switching from life enzyme to cell death executor**, Cross-border Italy-Slovenia, Biomedical Research: are we ready for Horizon 2020?

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CROSS-BORDER ITALY-SLOVENIA
BIOMEDICAL RESEARCH:
ARE WE READY FOR HORIZON 2020?

SEARCH FOR COMPOUNDS ABLE TO MODULATE F_0F_1 ATP SYNTHASE IN SWITCHING FROM LIFE ENZYME TO CELL DEATH EXECUTOR

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Abstract — Mitochondria are the main site of energy power in eukaryotic cells. The enzyme F_0F_1 synthase is responsible for ATP production driven by the transmembrane proton gradient. The maintenance of a very low permeability of the inner mitochondrial membrane is crucial for this mechanism, since sudden opening of the permeability transition pore (PTP) leads to matrix swelling and outer membrane rupture, with release of pro-apoptotic factors. Recently, it has been suggested that dimers of ATP synthase in mammals could represent the main component of the mitochondrial PTP, a feature modulated by calcium and involving the matrix protein Cyclophilin D (CyPD). This study would help to develop new tools for the identification of plant secondary metabolites, in particular flavonoids, able to modulate PTP and therefore acting on the programmed cell death mediated by mitochondria. Therefore, this project would represent the first screening for plant molecules able to interfere with programmed cell death, as a preliminary study for the development of drugs active in PTP-related pathologies.

Index Terms — TRANS2CARE, Cyclophilin ATP synthase, permeability transition pore, programmed cell death, cyclophilin D

S. Patui, C. Peresson, V. De Col, E. Braidot, E. Petrussa, V. Casolo, A. Bertolini, G. Lippe, A. Vianello, M. Zancani (2014) **Characterization of F_oF_1 ATP synthase from pea stem mitochondria: does it form the mitochondrial permeability transition pore in plants?** Oral presentation at the Annual Meeting of the GIBB (Italian Group of Biomembranes and Bioenergetics), Matera – Italy.

GIBB
Gruppo Italiano di Biomembrane e Bioenergetica

CHARACTERIZATION OF F_OF_1 ATP SYNTHASE FROM PEA STEM MITOCONDRIA:

DOES IT FORM THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN PLANTS?

Dr. Sonia Patui

UNIVERSITÀ DEGLI STUDI DI UDINE
DIPARTIMENTO DI SCIENZE AGRARIE E AMBIENTALI

S. Patui, C. Peresson, V. De Col, E. Braidot, E. Petrussa, V. Casolo, A. Bertolini, G. Lippe, A. Vianello, M. Zancani (2014) **Does F_oF_1 ATP synthase form the mitochondrial permeability transition pore in plants?** XIII Congress of the Italian Federation of Life Sciences (FISV), Pisa – Italy.

Does F_oF_1 ATP synthase form the mitochondrial permeability transition pore in plants?

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UNIVERSITÀ DEGLI STUDI DI UDINE

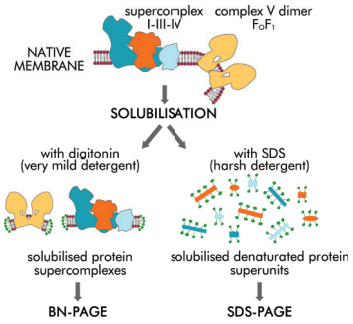
FISV 2014 XIII Congress Pisa, 24-27 September

Introduction

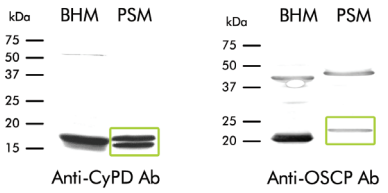
In aerobic conditions, the production of ATP in mitochondria is exerted by F_oF_1 ATP synthase. Nevertheless, mitochondria are also involved in the early stages of programmed cell death (PCD) through the release of pro-apoptotic factors from the intermembrane space. Such a release could be due to a sudden opening of the inner mitochondrial membrane permeability transition (PT) pore to molecules up to 1500 Da. This implies a matrix swelling, leading to partial outer membrane rupture and release of mainly cytochrome *c*. Recently, it has been proposed that ATP synthase in mammalian mitochondria, when present as a dimer, forms the PT pore in the presence of a high calcium concentration and thiol oxidants (Giorgio *et al.*, 2013; Bernardi, 2013). Consistently, the modulator of PT, the matrix protein Cyclophylin D (CyPD), has been also identified as an ATP synthase interactor (Giorgio *et al.*, 2009). In mammalian mitochondria, one of the targets of CyPD is the Oligomycin-Sensitivity Conferring Protein (OSCP), an essential component of ATP synthase for coupling proton transport to ATP synthesis (Antoniel *et al.*, 2014). The PT in plant mitochondria is still elusive and has been evidenced only in few peculiar cases (Vianello *et al.*, 2012). This research aims at verifying the involvement of ATP synthase also in plant PT and thus in PCD pathway.

Results

Mitochondria were purified from pea etiolated stems by a Percoll discontinuous gradient. Then, the organelles were solubilized with either SDS or digitonin, a mild detergent that could preserve the dimeric form of ATP synthase. After solubilisation, the proteins were separated by both SDS-PAGE and Blue Native (BN) - PAGE.

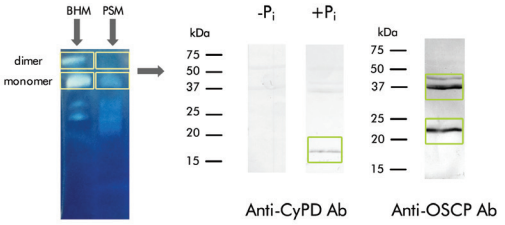


After SDS-PAGE and Western blotting, CyPD and OSCP were detected in Purified Stem Mitochondria (PSM) with anti-CyPD and anti-OSCP antibodies, respectively. In both cases the reaction was compared to that obtained with Bovine Heart Mitochondria (BHM). CypD was present in PSM and exhibited a molecular mass of approximately 18 kDa, a value very similar to the mammalian one. Immunodetection of OSCP in pea mitochondria revealed, instead, a protein with a slightly higher molecular mass compared to the bovine one.

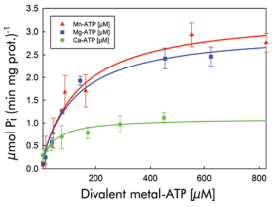


PSM were subjected to BN-PAGE and ATP synthase was identified by *in gel* ATPase activity. The white lead phosphate stains associated to the ATPase activity revealed few bands corresponding to oligomeric, dimeric, and monomeric forms, as well as isolated F_1 portion (BHM, control). The active bands, corresponding to monomer/dimer, were excised and the proteins were eluted from BN gel and then subjected to SDS-PAGE and Western blotting. Immunodetection showed that CyPD was bound to ATP synthase when mitochondria were resuspended with phosphate prior to solubilisation with digitonin and BN-PAGE.

The cross-reaction with anti-OSCP Ab confirmed the result obtained with PSM and showed one additional band, suggesting that OSCP could be still associated to other mitochondrial proteins.

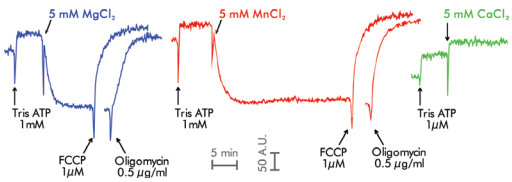


ATP synthase was also characterized in inside-out Sub-Mitochondrial Particles (SMP), evaluating its activity both as ATP hydrolysis and proton pumping. The scalar ATPase activity, evaluated by the Fiske-Subarrow method as inorganic phosphate (P_i) release, was dependent on divalent ions, such as Mg^{2+}, Mn^{2+} and Ca^{2+}. The concentration-dependence of hydrolysis rate with MnATP and MgATP showed similar affinity for the enzyme, whereas the value for CaATP was lower. In all these experiments oligomycin was able to inhibit ATP hydrolysis completely.



Substrate	Vmax μmol (min mg)$^{-1}$	K_M μM
Mn-ATP	3.4	128
Mg-ATP	3.0	115
Ca-ATP	1.1	40

ATP induced the formation of a proton gradient in SMP, evaluated as fluorescence quenching of acridine orange. In the presence of either Mg^{2+} or Mn^{2+}, the decrease in fluorescence was observed until a steady-state was reached. Subsequent addition of either oligomycin or the uncoupling agent FCCP induced the complete recovery of the fluorescence, indicating that ATP hydrolysis was coupled to the transmembrane proton flux. When Ca^{2+} was added to SMP, there was no change in fluorescence intensity and, therefore, no gradient was generated across the membrane.



Conclusions

Our results show that ATP synthase in plant mitochondria possesses structural and functional properties similar to the animal counterpart. In particular, the presence of CyPD in plant mitochondria and its interaction with ATP synthase dimers modulated by phosphate, suggests its putative role in PTP regulation, even if PT manifestation is only circumstantial in plants. Therefore, the ATP synthase could become a potential candidate to induce the release of cytochrome *c* and other pro-apoptotic proteins in the cytosol, a crucial step for the induction of PCD also in plants.

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| Other Contribution

V. De Col, V. Casolo, M. Tomasella, E. Braidot, T. Savi, A. Nardini (2014), **The importance of osmoregulation and ionic effects on xylem hydraulics in the invasive halophyte *Spartina patens* (Ait.) Muhl.**, XIII Congress of the Italian Federation of Life Sciences (FISV), Pisa – Italy.

The importance of osmoregulation and ionic effects on xylem hydraulics in the invasive halophyte *Spartina patens* (Ait.) Muhl.

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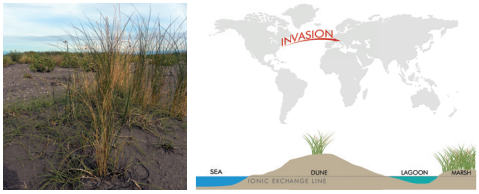
Introduction

Spartina patens is a grass native from the Atlantic salt marshes of North America, that spread into several European regions and, during the last decades, also in the Northern Adriatic coasts. *S. patens* possesses an extraordinary functional adaptability to sea-shore pioneer environments (characterized by low soil water potential, salinity, tidal inundation) and in particular it is able to colonize both dunes and back-barrier salt marshes. As a result of this biological invasion, populations of *S. patens* outcompete natural vegetation communities and endanger important hotspots of floristic and functional biodiversity in the Mediterranean area (Cowling *et al.*, 1996).



Spartina patens: the plant, the geographical barrier overcome, the spread in the invaded habitats.

How can *S. patens* be invasive in dune and salt marsh?

Two populations of *S. patens* growing under contrasting salinity levels, i.e. salt marsh and dune system, were compared in order to highlight the functional plasticity of the species as a factor contributing to its invasive potential. Therefore, leaf-level physiology was studied, analysing leaf water relations, osmoregulation and ionic effects on xylem hydraulics.

Evaluation of the biochemical and physiological traits

- Nitrogen concentration in soil and leaves;
- Leaf starch concentration;
- Total plant biomass;
- Leaf anatomy (diameter of all metaxylem vessel in primary veins, number of major veins per section, average distance between adjacent vascular bundles);
- Leaf water potential (Ψ_{leaf});
- Leaf hydraulics conductance (K_{leaf_area}) and vulnerability to drought stress (P_{50}) and response of leaf xylem hydraulic efficiency to increasing concentration of NaCl (Δk_{leaf_xylem}) (Brodribb and Holbrook 2003; Nardini *et al.*, 2014).

Results and discussion

Plants growing in the salt marsh experienced higher soil salinity level and had access to higher nitrogen availability. In fact, soil salinity was about 10-fold higher while soil [N] was about 3-fold higher in the marsh population than in the dune one (tab. 1).
The analysis of leaf-level water relations revealed a suite of mechanisms hypothetically adopted by the species to overcome salt and drought stress while maintaining relatively invariant leaf morphological traits (A_{leaf}, LMA) (tab.1) and overall biomass accumulation (tab. 1). Leaf [N] was significantly higher (about 50%) in marsh plants, while primary starch content showed an increasing trend along the same ecological pattern (tab. 1).

	Soil osmotic potential MPa (*)	Soil [N] mg g^{-1} (*)	A_{leaf} cm^2 (n.s.)	LMA mg cm^{-2} (n.s.)	Total biomass kgm^{-2} (n.s.)	Leaf [N] mg g^{-1} (*)	Leaf [starch] mg g^{-1} (n.s.)
Marsh	-1.09 ± 0.69	1.10 ± 0.44	9.3 ± 1.8	12.1 ± 1.7	0.67 ± 0.10	10.6 ± 1.9	24.6 ± 8.0
Dune	-0.12 ± 0.01	0.34 ± 0.10	10.7 ± 2.5	11.6 ± 1.5	0.55 ± 0.14	7.0 ± 1.8	19.0 ± 7.4

Tab. 1 Soil salinity, nitrogen [N] content, leaf nitrogen and starch content, leaf surface area (A_{leaf}), leaf mass per area (LMA) and total biomass accumulated by plants as recorded in the marsh and dune population of *S. patens*. Means are reported ± S.D. (*), P<0.05; (n.s.), not significant.

Apparently high soil salinity resulted in a more severe water stress experienced by marsh plants with respect to the dune population, as revealed by lower Ψ_{leaf}: the highest value was reached in July (fig. 1) for plants grown in the dune, while in September Ψ_{leaf} dropped to significantly more negative values in the marsh population.
Whole leaf hydraulic conductance (fig. 2) was clearly higher in plants from the marsh population than in the dune one, and in both plant groups K_{leaf_area} declined linearly upon leaf dehydration, with a steeper decline in marsh plants. Our data suggest that *S. patens* plants from marsh or dune habitats did not experience significant losses of hydraulic efficiency over the growing season.
Despite the higher whole leaf hydraulic conductance, the efficiency of water transport through the vein xylem system in marsh plants was lower than that from the dune population (fig. 3).

This was probably the consequence of narrower xylem conduits and suggests that reduced hydraulic diameter of xylem vessels apparently compensated at the whole-leaf level by higher efficiency of the extra-vascular water pathway. In both dune and marsh plants, the addition of NaCl in the solution perfused through leaves (fig. 4) induced a significant increase in k_{leaf_xylem}. In leaves from the marsh population, the k_{leaf_xylem} gradually increased (< 5% at 5 mM [NaCl]) in response to increasing salt concentration, showing a slightly higher capacity to enrich xylem sap with ions with respect to the dune population. Dune leaves showed a marked k_{leaf_xylem} increase (10% at 5 mM [NaCl]), revealing an inverse relationship between NaCl availability and sensitivity of the hydraulic system to changes in xylem sap salt concentration.

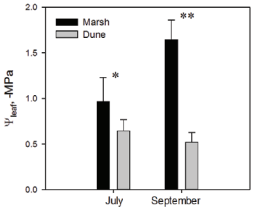
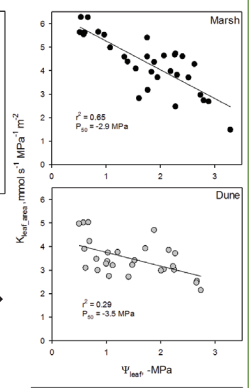


Fig. 4 Leaf water potential Ψ_{leaf} as measured in *Spartina patens* plants growing in a salt marsh area (black columns) or in the nearby dune system (grey columns). Means are reported ± SD. *, P<0.05.

Fig. 5 Leaf hydraulic conductance K_{leaf_area} and Ψ_{leaf} as measured in *Spartina patens* plants growing in a salt marsh area (black dots) or in the nearby dune system (grey dots). Each point represent a different leaf. The regression line is reported together with R^2. P_{50} indicates the water potential inducing 50% loss of K_{leaf_area}.



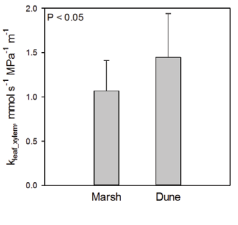


Fig. 6 Hydraulic conductivity of the leaf vein xylem as measured in *Spartina patens* plants growing in a salt marsh area or in the nearby dune system. Means are reported ± SD, and the P values is also shown.

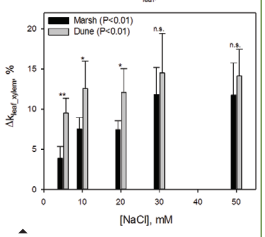


Fig. 7 Percentage increase of hydraulic conductivity of leaf vein xylem (Δk_{leaf_xylem}) in response to the enrichment of the perfused solution with increasing concentration of NaCl. Measurements were performed in leaves of *Spartina patens* sample from plants growing in a salt marsh area (black columns) or in the nearby dune system (grey columns). Means are reported ± SEM.

Conclusions

Our data revealed a suite of functional traits related to the ecological plasticity of the species, ranging from changes in leaf nitrogen concentration to mechanisms assuring leaf hydraulic efficiency and safety. Such modifications allowed S. patens to grow in saline habitats and to cope with restricted water supply. In particular, this study is the first experimental evidence for salt-mediated regulation of xylem hydraulic efficiency in a halophytic grass. These results highlight the functional plasticity of the species, that might represent a key trait underlying its invasive potential.

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UNIVERSITÀ DEGLI STUDI DI TRIESTE

| 2015

C. Peresson, E. Petrussa, V. Casolo, S. Patui, A. Bertolini, V. Giorgio, V. Checchetto, V. De Col, E. Braidot, G. Lippe, I. Szabò, A. Vianello, P. Bernardi, M. Zancani (2015) **Does ATP synthase form the PTP in pea stem mitochondria?** 9th International Conference for Plant Mitochondrial Biology, Wrocław – Polonia.

Does ATP synthase form the PTP in pea stem mitochondria?

Peresson C.[1], Petrussa E.[1], Casolo V.[1], Patui S.[1], Bertolini A.[1], Giorgio V.[2], Checchetto V.[3], De Col V.[1], Braidot E.[1], Lippe G.[4], Szabò I.[3], Vianello A.[1], Bernardi P.[2], Zancani M.[1*]

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INTRODUCTION

The mitochondrial Permeability Transition (PT) is a phenomenon mediated by the opening of mitochondrial inner membrane channel named Permeability Transition Pore (PTP). Recently, it has been demonstrated in mammals (Giorgio *et al.*, 2013), yeast (Carraro *et al.*, 2014) and *Drosophila* (von Stockum *et al.*, 2015) that the PTP is a channel formed by dimers of F_OF_1-ATP synthase. PTP opening in mammals is induced by matrix Ca^{2+} and thiol oxidation, while it is inhibited by Mg^{2+}, ADP, ATP and reductants. The interaction of Cyclophylin D (CyPD) with F_OF_1-ATP synthase, possibly through association with OSCP (Oligomycin Sensitivity-Conferring Protein), is favored by P_i. When CyPD is associated to the F_OF_1-ATP synthase, lower concentrations of Ca^{2+} are needed to induce PTP opening (Bernardi, 2013).
This work was undertaken to verify if plant mitochondrial F_OF_1-ATP synthase possesses features similar to those already seen in other species, as this would provide a significant contribution to understand the structure/function relationships of this enzyme and the evolution of its channel function.

RESULTS & DISCUSSION

PTP modulation

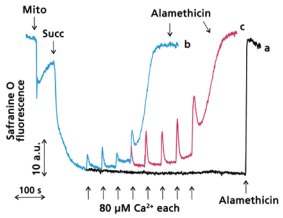
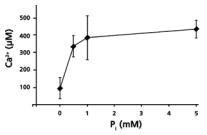


FIG. 1 | Electrical potential formation and PTP opening induced by Ca^{2+} in PSM



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	Ca^{2+} (μM)	%
Control	368 ± 127	100
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Calcium Retention Capacity (CRC)

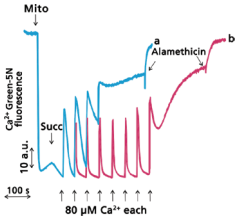
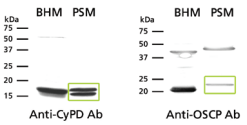


FIG. 3 | Calcium Retention Capacity in PSM

Calcium Retention Capacity (CRC) was evaluated using Calcium Green-5N **(Figure 3)**, in the presence of 5 mM P_i-Tris and 10 μM ETH129. These results show that PSM, energized by succinate and in the presence of ETH129, accumulated Ca^{2+} in the matrix, followed by the PTP opening and Ca^{2+} release. In the presence of 1 μM CsA **(trace b)**, a higher concentration of Ca^{2+} was needed to induce the PTP opening.

CyPD and OSCP detection



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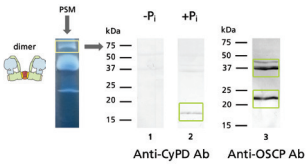
Dimers of ATP synthase



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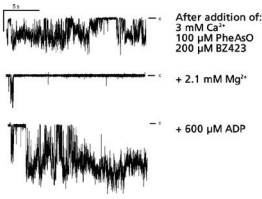

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CONCLUSIONS

Our results show that, even if apparently lacking an efficient Ca^{2+} uptake system, PSM possess a Ca^{2+}-induced PT similar to that described for mammals, *Drosophila* and yeast. In particular, PT in PSM shares some similarities with those from other species, but also shows some differences **(see Table II)**. Furthermore, these data provide preliminary evidence that gel-purified dimers of F_OF_1-ATP synthase from PSM, when incorporated into an artificial bilayer, are able to form a channel that showed features similar to those reported for the mammalian, yeast and *Drosophila* complexes.

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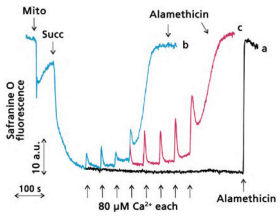

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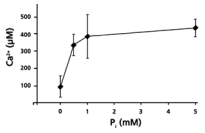

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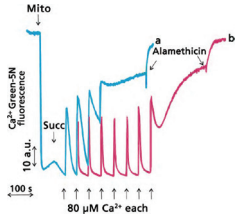

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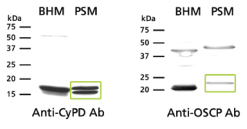

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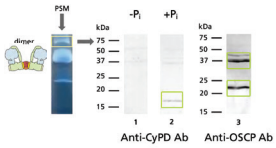

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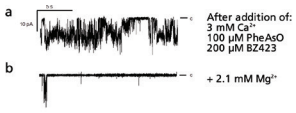

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Udine, 20th June 2015 Valentina De Col

| Other Contribution

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CSIRO PUBLISHING
Functional Plant Biology
http://dx.doi.org/10.1071/FP14172

Water relations of an invasive halophyte (*Spartina patens*): osmoregulation and ionic effects on xylem hydraulics

Valentino Casolo[A], *Martina Tomasella*[A,B,C], *Valentina De Col*[A], *Enrico Braidot*[A], *Tadeja Savi*[C] *and Andrea Nardini*[C,D]

[A]Department of Agricultural and Environmental Sciences, University of Udine, Via delle Scienze 91, 33100 Udine, Italy.
[B]Department of Ecology, Technische Universität München, Hans-Carl-von-Carlowitz-Platz 2, 85354 Freising, Germany.
[C]Department of Life Sciences, University of Trieste, Via L. Giorgieri 10, 34127 Trieste, Italy.
[D]Corresponding author. Email: nardini@units.it

Abstract. *Spartina patens* (Ait.) Muhl. is a grass native to the Atlantic coastal area of North America currently invading salt marsh ecosystems in several regions of Europe. We investigated leaf water relations and hydraulics, gas exchange, nitrogen and starch content in two populations of *S. patens* growing under contrasting salinity levels in a salt marsh and in a dune system in order to assess its functional plasticity as a factor contributing to its invasive potential. The analysis of leaf water relations revealed a suite of mechanisms adopted by *S. patens* to overcome salt and drought stress while maintaining relatively invariant leaf morphological traits and plant biomass. In particular, salt marsh plants experiencing severe water stress underwent greater osmoregulation and leaf hydraulic adjustment than dune plants. We also present the first experimental evidence for salt-mediated regulation of xylem hydraulic efficiency in a halophytic grass and suggest that it is an important functional trait allowing plants growing in saline habitats to cope with a restricted water supply. The functional plasticity of leaf water relations and xylem hydraulics emerges as a key trait underlying the competitive ability and invasive potential of *S. patens*.

Additional keywords: biomass, invasive plants, leaf hydraulics, nitrogen, osmotic potential, phenotypic plasticity.

Received 25 June 2014, accepted 29 October 2014, published online 26 November 2014

Introduction

The invasion of natural ecosystems by alien species is one of the most important components of ongoing global environmental changes (Vilà *et al*. 2011). Invasive alien plants are generally characterised by functional traits conferring high competitive ability in a wide variety of habitats (van Kleunen *et al*. 2010). Biological invasions are more commonly observed in high-resource environments, especially when affected by anthropogenic disturbance. Under these conditions, morphological and physiological traits favouring fast resource acquisition allow some alien plants to successfully out-compete native species (Cavaleri *et al*. 2014; Kimball *et al*. 2014). These traits include high specific leaf area and leaf area ratio, high foliar nitrogen and phosphorus contents, and high photosynthetic assimilation rates both on a leaf mass and a leaf area basis (Leishman *et al*. 2007; Osunkoya *et al*. 2010).

Some alien species are apparently able to invade also low-resource environments, including dry (Smith *et al*. 2000; Mangla *et al*. 2011) and saline areas (Mateos-Naranjo *et al*. 2010; Morais *et al*. 2012). These are often also important hotspots of biodiversity, like in the case of the Mediterranean area (Cowling *et al*. 1996), which is susceptible to biological invasions, especially in sandy coastal areas. Low-resource environments pose serious challenges to plant survival and productivity, and abiotic stress factors largely set the limits on plant establishment and reproductive success in these harsh habitats (He *et al*. 2012). Limited light, water or soil nutrient availability have selected for plant species with specialised mechanisms conferring both stress tolerance and the ability to acquire or retain limiting resources, including slow growth, high resource use efficiency, low specific leaf area and long leaf life-span (Reich *et al*. 1999).

Although invasive alien species occurring in low-resource environments have been reported to have higher nutrient use efficiency than native ones, the two groups are often quite similar in terms of water and light use efficiencies (Cavaleri and Sack 2010; Funk 2013). Low-resource environments are also often characterised by marked heterogeneity in the distribution of resources and stress factors (Bazihizina *et al*. 2012). Under these conditions, the ability to modify the functional phenotype in response to environmental factors, the so-called 'phenotypic plasticity' (Bradshaw 1965), might represent another important trait affecting the fitness of alien versus native plant species (Richards *et al*. 2008; Drenovsky *et al*. 2012). Indeed, a recent

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Do dimers of F-ATP synthase form the PTP in pea stem mitochondria?

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INTRODUCTION

The mitochondrial Permeability Transition (PT) is a phenomenon mediated by the opening of mitochondrial inner membrane channel named Permeability Transition Pore (PTP). Recently, it has been demonstrated in mammals (Giorgio *et al.*, 2013), yeast (Carraro *et al.*, 2014) and *Drosophila* (von Stockum *et al.*, 2015) that the PTP is a channel formed by dimers of F-ATP synthase. PTP opening in mammals is induced by matrix Ca^{2+} and thiol oxidation, while it is inhibited by Mg^{2+}, ADP, ATP and reductants. The interaction of Cyclophylin D (CyPD) with F-ATP synthase, possibly through association with OSCP (Oligomycin Sensitivity-Conferring Protein), is favored by P_i. When CyPD is associated to the F-ATP synthase, lower concentrations of Ca^{2+} are needed to induce PTP opening (Bernardi, 2013).

AIMS

This work was undertaken to verify if plant mitochondrial F-ATP synthase possesses features similar to those already seen in mammals, yeast and *Drosophila*. This would provide a significant contribution to understand the structure/function relationships of this enzyme and the evolution of its channel function.

RESULTS & DISCUSSION

PTP modulation

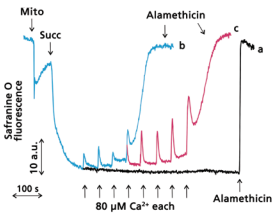

FIG. 1 | Electrical potential formation and PTP opening induced by Ca^{2+} in PSM

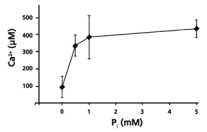

FIG. 2 | Inhibition of PTP opening by P_i in PSM

	Ca^{2+} (μM)	%
Control	368 ± 127	100
Mg ADP (2 mM)	701 ± 258	190
Mg ATP (2 mM)	681 ± 179	184
Diamide (2 mM)	278 ± 80	76
Phenylarsine oxide (50 μM)	188 ± 61	51

TAB. I | Effect of modulators on Ca^{2+}-induced PTP opening in PSM

Isolated Pea Stem Mitochondria (PSM) showed a succinate-dependent electrical potential formation **(Figure 1)**. The addition of several pulses of 80 μM Ca^{2+}, in the presence of 1 mM P_i, did not alter the potential, which was completely dissipated by 10 μM alamethicin **(trace a)**. In the presence of Ca^{2+} ionophore ETH129 (10 μM), after the addition of about 300 μM Ca^{2+}, PSM underwent a process of Ca^{2+} release **(trace b)** that was delayed by CsA **(trace c)**, suggesting that the process is mediated by PTP opening. Ca^{2+} release was inhibited by P_i in a concentration-dependent manner **(Figure 2)**. These results show that PSM were able to take up Ca^{2+} only in the presence of ETH129. Like the PTP of all species tested so far, Ca^{2+} release was inhibited by adenine nucleotides and stimulated by oxidative conditions **(Table I)**.

Calcium Retention Capacity (CRC)

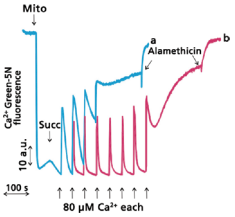

FIG. 3 | Calcium Retention Capacity in PSM

Calcium Retention Capacity (CRC) was evaluated using Calcium Green-5N **(Figure 3)**, in the presence of 5 mM P_i-Tris and 10 μM ETH129. These results show that PSM, energized by succinate and in the presence of ETH129, accumulated Ca^{2+} in the matrix, followed by the PTP opening and Ca^{2+} release. In the presence of 1 μM CsA **(trace b)**, a higher concentration of Ca^{2+} was needed to induce the PTP opening.

Detection of ß subunit, OSCP and CyPD

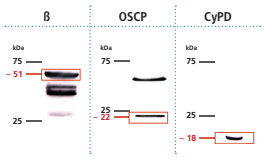

FIG. 4 | Detection of ß subunit, OSCP and CyPD in purified PSM

ß subunit, OSCP and CyPD were detected in purified PSM **(Figure 4)** with an apparent molecular mass of 51, 22 and 18 kDa, respectively. The additional bands suggest unspecific reactions or associations to other mitochondrial proteins. ß and OSCP were considered as markers of F_1F_o-ATP synthase, while CyPD as a crucial molecule for PTP modulation.

F-ATP synthase dimers and monomers

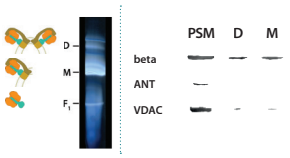

FIG. 5 | Identification of ATP synthase by in-gel assay and Western blotting in purified PSM, dimers and monomers

In-gel activity of PSM F-ATP synthase was detected after Blue native PAGE (BN-PAGE) **(Figure 5, left panel)**. The bands corresponding to the dimers (D) and monomers (M) were eluted from the gel and antibodies against ß were used to confirm the presence of the F-ATP synthase. ANT was completely absent in dimers and monomers, while there was only a little contamination by VDAC **(Figure 5, right panel)**.

Calcium-induced currents

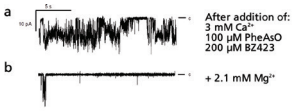

FIG. 6 | Lipid planar bilayer preliminary experiments

Bilayers were prepared using purified soybean asolectin. Dimers eluted from the gel were added to the cis side. Representative current traces were recorded, following the addition of 3 mM Ca^{2+} plus 100 μM Phenylarsine oxide (PheAsO) and 200 μM benzodiazepine 423 (BZ423) (all added to the trans side) **(Figure 6a)**. The addition of 2.1 mM Mg^{2+} to the trans side caused an inhibitory effect on channel activity **(Figure 6b)**.

CONCLUSIONS

Our results show that, even if apparently lacking of an efficient Ca^{2+} uptake system, PSM possess a Ca^{2+}-induced PT similar to that described for mammals, *Drosophila* and yeast. In particular, PTP in PSM shares some similarities with those from other species, but also shows some differences **(Table II)**. Furthermore, these data provide preliminary evidence that gel-purified dimers of F-ATP synthase from PSM, when incorporated into an artificial bilayer, are able to form a channel that showed features similar to those reported for the mammalian, yeast and *Drosophila* complexes.

	Bovine	Drosophila	Yeast	Pea
Ca^{2+} induction	YES	YES	with ETH129	with ETH129
Inhibition by CsA	YES	NO	NO	YES
Effect of P_i	ACTIVATION	INHIBITION	INHIBITION	INHIBITION
Swelling	YES	NO	YES	NO?

TAB. II | Comparison among some features of mitochondrial PTP from different species

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