ION TRANSPORT IN CHLOROPLAST AND MITOCHONDRIA PHYSIOLOGY IN GREEN ORGANISMS

EDITED BY: Cornelia Spetea, Ildikò Szabò and Hans-Henning Kunz
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ION TRANSPORT IN CHLOROPLAST AND MITOCHONDRIA PHYSIOLOGY IN GREEN ORGANISMS

Topic Editors:
Cornelia Spetea, University of Gothenburg, Sweden
Ildikò Szabò, University of Padova and Institute of Neurosciences, Italy
Hans-Henning Kunz, Washington State University, USA

Photograph of a leaf with chloroplast and mitochondria ion transport illustrated in white color
Illustration by Emil Wiklund and photograph by Cornelia Spetea Wiklund

Chloroplasts and mitochondria both have a prokaryotic origin, carry essential genes on their own highly reduced genome and generate energy in the form of ATP for the plant cell. The ion composition and concentration in these bioenergetic organelles impact photosynthesis, respiration and stress responses in plants.

Early electrophysiological and biochemical studies provided strong evidence for the presence of ion channels and ion transporters in chloroplast and mitochondrial membranes. However, it wasn't until the last decade that the development of model organisms such as *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* along with improved genetic tools to study cell physiolgy have led to the discovery of several genes encoding for ion transport proteins in chloroplasts and mitochondria. For the first time, these discoveries have enabled detailed studies on the essential physiological function of the organellar ion flux.

This Research Topic welcomed updated overviews and comprehensive investigations on already identified and novel ion transport components involved in physiology of chloroplasts and mitochondria in green organisms.

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EDITORIAL
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Editorial: Ion Transport in Chloroplast and Mitochondria Physiology in Green Organisms

Cornelia Spetea[1†], Ildikò Szabò[2,3†] and Hans-Henning Kunz[4†]*

[1] Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden, [2] Department of Biology, University of Padova, Padova, Italy, [3] Institute of Neurosciences, CNR, Padova, Italy, [4] Plant Physiology, School of Biological Sciences, Washington State University, Pullman, WA, USA

Keywords: ion transport, chloroplast, mitochondria, plant physiology, green organisms

Editorial on the Research Topic

Ion Transport in Chloroplast and Mitochondria Physiology in Green Organisms

This Research Topic represents a collection of articles either focusing on specific ion transport mechanisms or providing updated overviews of the research and transport mechanisms awaiting identification in chloroplasts and mitochondria. Some articles also cover detailed mechanisms of action and regulatory processes for already identified ion transport components. Other contributions unravel new possible players and interactions underlying the physiology of chloroplasts and mitochondria in plants, and green organisms in general.

Patch clamping of membranes is the most direct method used to characterize ion channel activities. Pottosin and Dobrovinskaya review the challenges in using this method for characterization of activities in chloroplast thylakoid membranes. Predominantly, these complications arise from the high protein/lipid ratio, the low phospholipid content, and presence of bulky ATP synthase subunits. All three aspects result in extremely fragile membrane patches. In addition, the authors discuss patch clamping of chloroplast envelope membranes together with the evidence for ion channel activities and identity of the genes involved.

Luesse et al. explore if loss of the mechanosensitive ion channels MSL2 and MSL3 in the chloroplast inner envelope interferes with common pathways for leaf development. MSL2 and MSL3 are required to maintain normal organelle size, shape, and ion homeostasis. Instead of constructing higher-order mutants, they apply RNA sequencing on *Arabidopsis msl2msl3* mutants and several other mutants similarly compromised in leaf morphology. Although the mechanism behind defective leaf morphology could not be solved, the authors generated a massive publicly available transcriptomics dataset, representing a valuable community tool for future studies on organelle dysfunction, ion homeostasis, and leaf variegation. The authors conclude that either RNA sequencing is not suitable to reveal the leaf developmental genetic network or that mechanisms determining leaf shape are even more complex than anticipated thus far.

The thylakoid-located putative Cl^- channel CLCe was hypothesized to play role in photosynthetic regulation based on initial analyses of *Arabidopsis* loss-of-function *clce* mutants (Marmagne et al., 2007). Herdean et al. reveal that the *clce* mutants are disturbed in chloroplast Cl^- homeostasis, leading to re-arrangements of the electron transport chain components in the thylakoid membrane. Moreover, while another thylakoid Cl^- channel was found important for fine-tuning photosynthesis in fluctuating light environments (Duan et al., 2016; Herdean et al., 2016), CLCe appears to function in the same process following dark adaptation. These findings speak for well-defined roles the two proteins may play in regulation of photosynthesis via Cl^- flux across the thylakoid membrane.

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Steven Carl Huber, Agricultural Research Service (ARS)-USDA, USA

***Correspondence:**
Cornelia Spetea
cornelia.spetea.wiklund@bioenv.gu.se

† These authors have contributed equally to this work.

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Höhner et al. review all currently known H^+-dependent transport mechanisms in plastids. These carriers transport a variety of co-substrates from essential ions to other solutes, and impact several physiological processes, such as cation and pH homeostasis, osmoregulation, coupling of secondary active transport, and photosynthesis. Unfortunately, most activities are mediated by thus far elusive proteins, preventing exploitation of genetic tools to definitively prove their involvement in the above processes. More strikingly, also the existence of a potential H^+ pump in the chloroplast envelope is still unclear, and thus should have high research priority in the future.

An extensive review on the role of ions in regulating light harvesting for photosynthesis is provided by Kăna and Govindjee. The authors focus on the molecular interactions taking place at the negatively charged surface of the thylakoid membrane. This interaction results in so-called screening effects of the electric field on the membrane surface. Furthermore, they discuss the impact of ion-membrane interactions on thylakoid membrane stacking, state transition, and non-photochemical quenching. The review is dedicated to Jim Barber, who first recognized the importance of membrane surface charges for photosynthesis (Barber, 1980).

Transition metals such as iron, copper, and manganese are essential for chloroplast processes including photosynthesis. The review by López-Millán et al. deals with proteins involved in iron transport, storage, and assembly in the chloroplast as important players for homeostasis and photosynthetic performance. While the thylakoid iron transporter is still unknown, several systems function in acquisition of iron into the chloroplast across the inner envelope. In addition, the authors discuss the mechanisms for crosstalk between chloroplasts and mitochondria, both major control points of iron homeostasis.

The review by Aguirre and Pilon addresses the current knowledge about ATP-driven copper-transporters in chloroplasts, including envelope PAA1, and thylakoid PAA2, that work in concert to supply stroma and lumen with Cu ion. Insights into the regulatory mechanism of PAA2 are provided, such as the importance of miRNAs during low copper availability to prioritize delivery to plastocyanin. Since loss-of-function *paa1* and *paa2* mutants are suppressed by high copper levels in the growth media and the double *paa1paa2* is lethal, the authors suggest the possibility of mistargeting of PAA1 and PAA2 within the chloroplast, and the need for an alternative copper delivery route using low-affinity transport systems yet to be identified.

Three reviews on mitochondrial ion transport in plants are also part of this Research Topic. Carraretto et al. provide an update on the current knowledge about Ca^{2+} transport. Such knowledge is important because transient changes in Ca^{2+} concentration act as signals for transcriptional and metabolic responses, setting for an optimal performance of mitochondria. The molecular players involved in Ca^{2+} transport into mitochondria have been either identified or hypothesized.

Trono et al. review the knowledge about the mitochondrial ATP-dependent K^+ channel discovered about 15 years ago in wheat (Pastore et al., 1999). This channel is compared to other known K^+ channels, and the mechanism of regulating proton motive force and reactive oxygen species production in hyperosmotic stress is discussed.

Zancani et al. provide an updated overview of the permeability transition (PT) linked to programmed cell death in mitochondria, impacting plant development, and stress responses. Based on recent works suggesting that the mitochondrial ATP synthase functions as PT in mammals, the authors speculate that this could be the case in plants as well. Future studies are required to validate the role of mitochondrial ATP synthase as a PT channel in plant programmed cell death.

In summary, by analyzing loss-of-function and gain-of-function mutant phenotypes over the last years, exciting insights into the physiological significance of specific ions and the importance of their transport proteins in chloroplasts and mitochondria have been gained. However, more research is still required to identify the many elusive ion transport systems of these two fundamental organelles. Besides their initial discovery, studies of the transport mechanisms, structure/function, post-translational regulatory modifications, and connections to other transport proteins have great potential to improve our knowledge about ion transport in the physiology of chloroplasts and mitochondria, and may open new avenues for biotechnological applications, for instance to improve photosynthetic efficiency and stress tolerance.

AUTHOR CONTRIBUTIONS

All listed authors made substantial contribution to the Research Topic. CS wrote the original version of the editorial. H-HK wrote and edited sections and contributed to the discussion. IS commented on the content. All authors read and edited the final version.

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REVIEW
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Ion Channels in Native Chloroplast Membranes: Challenges and Potential for Direct Patch-Clamp Studies

*Igor Pottosin * and Oxana Dobrovinskaya*

Centro Universitario de Investigaciones Biomédicas, Universidad de Colima, Colima, Mexico

Photosynthesis without any doubt depends on the activity of the chloroplast ion channels. The thylakoid ion channels participate in the fine partitioning of the light-generated proton-motive force (p.m.f.). By regulating, therefore, luminal pH, they affect the linear electron flow and non-photochemical quenching. Stromal ion homeostasis and signaling, on the other hand, depend on the activity of both thylakoid and envelope ion channels. Experimentally, intact chloroplasts and swollen thylakoids were proven to be suitable for direct measurements of the ion channels activity via conventional patch-clamp technique; yet, such studies became infrequent, although their potential is far from being exhausted. In this paper we wish to summarize existing challenges for direct patch-clamping of native chloroplast membranes as well as present available results on the activity of thylakoid Cl^- (ClC?) and divalent cation-permeable channels, along with their tentative roles in the p.m.f. partitioning, volume regulation, and stromal Ca^{2+} and Mg^{2+} dynamics. Patch-clamping of the intact envelope revealed both large-conductance porin-like channels, likely located in the outer envelope membrane and smaller conductance channels, more compatible with the inner envelope location. Possible equivalent model for the sandwich-like arrangement of the two envelope membranes within the patch electrode will be discussed, along with peculiar properties of the fast-activated cation channel in the context of the stromal pH control.

Keywords: chloroplast envelope, cation channel, ClC channel, magnesium, patch-clamp, porin, proton-motive force, thylakoid

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Ildikò Szabò,
University of Padova, Italy

Reviewed by:
Ingo Dreyer,
Universidad de Talca, Chile
Katrin Philippar,
Ludwig-Maximilians-University Munich, Germany

****Correspondence:***
Igor Pottosin
pottosin@ucol.mx

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INTRODUCTION

Chloroplasts originated from endosymbiosis of an ancestral cyanobacterium and a primitive eukaryotic cell. The two envelope membranes, outer (OE), and inner (IE) ones are homologous to external and plasma membranes of Gram-negative bacteria, which is confirmed by the presence of galactolipids and β-barrel proteins (porins) in the OE and external membrane of free-living Gram-negative bacteria (Inoue, 2007; Gould et al., 2008; Breuers et al., 2011). The two envelope membranes are aligned close to each other, separated by only 1–2 membrane thickness (5–10 nm as compared to a typical chloroplast size of 3–4 μm); IE and OE come to even closer proximity at contact sites (Inoue, 2007). Membrane/compartment arrangements in chloroplasts are different from those in mitochondria. Whereas, the outer mitochondrial membrane may be compared with the OE,

the inner mitochondrial membrane combines both energy-coupling and metabolite exchange functions. As the two mitochondrial membranes are mostly separated, the activity of outer and inner membrane channels could be directly studied by patch-clamp technique, using intact mitochondria or swollen mitoplasts, respectively (Szabo and Zoratti, 2014). Double-membrane bound chloroplasts represent technically a more challenging task, as will be discussed in this paper. The stroma of chloroplasts, however, may be compared with the mitochondrial matrix: it is a slightly alkaline (compared to the cytosol) compartment with a high biosynthetic potential. Nine out of twenty essential amine acids are synthesized exclusively in the stroma, as well as are fatty acids, carbohydrates and triose phosphates, NADPH, purines, and a variety of secondary metabolites (Breuers et al., 2011; Rolland et al., 2012). The inner envelope contains a variety of solute transporters, mediating export of photoassimilates and import of substrates, as well as ion exchange (Weber and Linka, 2011). Both functions can be complemented by the activity of inner envelope ion channels. In addition, as it will be discussed in this review, cation channels, a putative H^+-ATPase, and monovalent cation/H^+ exchangers of the IE could assist maintenance of metabolically optimal alkaline pH in the stroma and control chloroplast volume. In the OE, transport activity of porin-like channels appears to dominate in both ion and metabolite traffic (Duy et al., 2007) and their differential substrate selectivity and regulation will be discussed.

The thylakoid membrane is an internal membrane of the chloroplast, representing a complex network of grana stacks connected by stromal lamellae (for thylakoid structure, see recent review by Pribil et al., 2014). The thylakoid membrane, being a site for light-driven photosynthetic reactions, harbors photosynthetic pigments and protein complexes of the electron transfer chain as well as F-type H^+-ATPase, which performs photophosphorylation. In variance to mitochondria, chloroplasts store a proton-motive force (p.m.f.), which fuels the ATP-synthesis, mainly as ΔpH rather than the transmembrane electric potential difference ($\Delta\Psi$). Thus, thylakoid lumen represents a unique acidic compartment. Interconversion between ΔpH and $\Delta\Psi$ across the thylakoid membrane is under environmental control and the steady state p.m.f. partitioning critically depends on the activity of thylakoid ion channels (Kramer et al., 2004). Partial dissipation of $\Delta\Psi$, generated by light-driven H^+-pumping into thylakoid lumen is achieved via passive fluxes of anions (Cl^-), K^+, and Mg^{2+} (Hind et al., 1974). Accordingly, activities of anion and nonselective cation channels were revealed by direct patch-clamping of native thylakoid membranes, whereas activity of thylakoid K^+-selective channels was assayed in a reconstituted system (**Figure 1**). Specific functional properties of these channels will be discussed in the following.

This review is centered in chloroplast ion channels, which could be directly measured by patch-clamp technique. For a broader overview of the chloroplast ion transport system in the physiological context an interested reader could consult a recent review by Pottosin and Shabala (2015).

ION TRANSPORT ACROSS THE THYLAKOID MEMBRANE

Ion Fluxes Assist the Conversion of Electrical Potential Difference to ΔpH

The difference of electrochemical potential for H^+ ($\Delta\mu H^+$), generated under light, is an obligatory intermediate for the photo-phosphorylation, ATP synthesis by H^+-transporting F-type ATPase. Often, instead of ($\Delta\mu H^+$), the related parameter, proton-motive force (p.m.f.) is used, p.m.f. = $\Delta\Psi$-ZΔpH (Mitchell, 1966), where Z is approximately 59 mV at room temperature, $\Delta\Psi$ and ΔpH represent the differences in electrical potential and pH across the thylakoid membrane, respectively. Thermodynamically, electrical and chemical components of the p.m.f. are equivalent; however, measurements of turnover rates with isolated F-ATPases demonstrated that the mitochondrial and bacterial F-ATPases critically require the presence of a substantial $\Delta\Psi$, whereas chloroplast F-ATPase depends on the $\Delta\Psi$ less critically (Fischer and Gräber, 1999; Cruz et al., 2001). It was considered for a long time that under a steady state light the p.m.f. in chloroplasts consists almost completely of ΔpH, whereas the steady state $\Delta\Psi$ is negligibly small ($\sim$−10 mV stroma negative) (e.g., Bulychev et al., 1972; Remiš et al., 1986). *In vivo* studies, however, demonstrated that $\Delta\Psi$ may yield up to 50% of p.m.f. or −60 mV (Cruz et al., 2001; Kramer et al., 2003, 2004; Klughammer et al., 2013). Thus, ΔpH, which yields at least a half of the p.m.f., is about 1–1.5 pH units under light. Assuming a constant stromal pH of 8 under light (see the section on stromal pH control), this yields pH 6.5–7 in the lumen. Under extreme conditions (strong light, low CO_2), luminal pH may drop to 6–5.5 (Tikhonov et al., 1981; Schönknecht et al., 1995; Takizawa et al., 2007). On one side, an acidic pH in the lumen is necessary for the stimulation of non-photochemical quenching, which prevents the photodamage of the reaction center of the Photosytem II (PSII) and reactive oxygen species (ROS) generation at excessive light. On the other side, a luminal pH below 6.5 strongly reduced the linear electron transport flow from the cytochrome b6f complex to the reaction center of Photosystem I (PSI), and a pH below 6 may even cause a loss of function of the water-splitting complex (exclusive electron donor for the PSII) and of the plastocyanin, electron carrier between b6f and Photosystem I, due to the replacement of the functionally important Ca^{2+} and Cu in these proteins by protons (Kramer et al., 2003). Obviously, partitioning of p.m.f. into $\Delta\Psi$ and ΔpH should be finely tuned. Existence of a (stroma) negative steady-state $\Delta\Psi$ under light implies a driving force for anion efflux from and cation influx to the stroma. If the thylakoid membrane conductance for physiologically abundant cations or anions were large, $\Delta\Psi$ will collapse, and excessive lumen acidification will result. As it did not happen, one has to assume that the functional expression of respective ion channels, their selective permeability, the availability of transported ions, and the control of channels' open probability by physiologically relevant factors (membrane voltage, pH, Ca^{2+}, etc.) should be set exactly to balance the light-driven H^+ pump-generated current at *given* $\Delta\Psi$ *and pH*

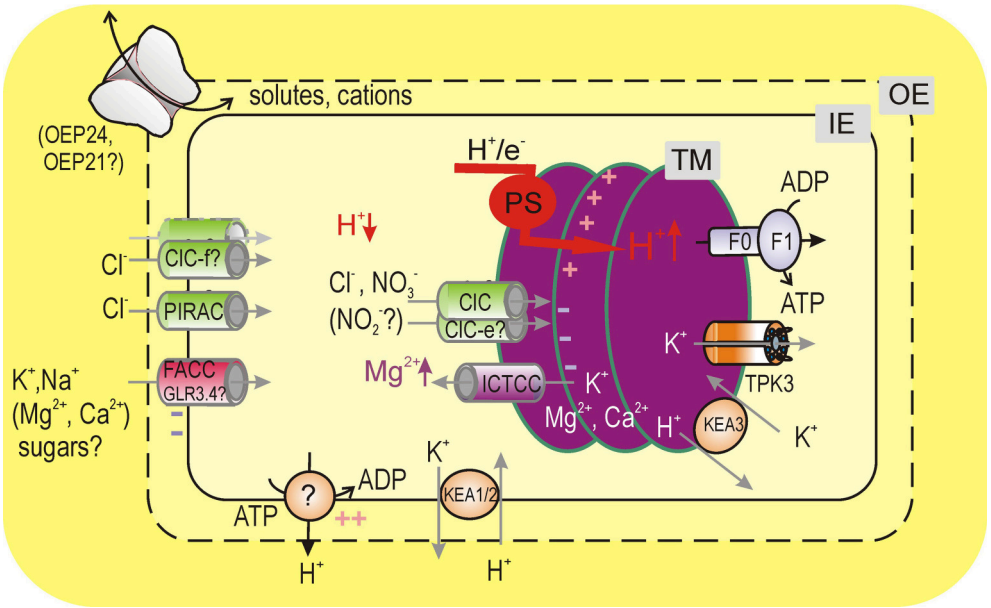

FIGURE 1 | Chloroplast ion transport under the light. Light-driven export of H^+ into the thylakoid lumen by photosynthetic electron transfer chain (PS) causes a hyperpolarization of the thylakoid $\Delta\Psi$. At steady state, this voltage difference is partly dissipated by channel-mediated fluxes of anions, K^+, and Mg^{2+}. Light-driven H^+ and parallel Cl^- fluxes to the thylakoid lumen cause the depletion of these ions in stroma, which is compensated by their uptake across the envelope. For maintenance of alkaline stromal pH, H^+ could be actively extruded to cytosol by the IE H^+ pump, which requires a counter influx of monovalent cations across the envelope for electrical balance. K^+/H^+ exchange across the envelope is essential for control of the chloroplast volume and stromal pH. Abbreviations: TM, IE, and OE are thylakoid, inner envelope, and outer envelope membranes, F0F1 is thylakoid ATP-synthetase (F-type H^+-ATPase), TPK3 (tandem-pore K^+ 3 channel, functionally characterized in recombinant system). *In situ* functionally (by patch-clamp) detected channels were: ClC (anion-selective channel from a ClC family), ICTCC (intermediate-conductance thylakoid cation channel), FACC (fast activating chloroplast cation channel), PIRAC (protein import related anion channel), and outer envelope porins (most possibly, active OEP24 or OEP21). Other: GLR3.4 (glutamate receptor type 3.4 channel) and KEA1/2 (cation/proton antiporters from family 2, CPA2). Another member of the CPA2 family, the thylakoid-localized KEA3, accelerates dissipation of the transthylakoid ΔpH upon the light offset.

in stroma and lumen. Current, generated by the light-driven H^+ pump (photosynthetic electron transfer chain), was directly evaluated by patch-clamp technique and could reach up to tens of pA for a single chloroplast under strong light (Bulychev et al., 1992; Muñiz et al., 1995). Above considerations emphasize the importance of *quantitative* measurements of the activity of thylakoid ion channels *in situ*, under conditions close to the physiological ones.

Patching of Thylakoids is Technically Challenging but Feasible

First patch-clamp recording on the photosynthetic membrane was achieved by Schönknecht et al. (1988), who employed hypotonic shock to inflate the thylakoid compartment. Chloroplasts, poised into the hypotonic medium (isotonic saline, diluted 4–5 times with pure water) rapidly broke down, releasing large (10–40 μm in diameter) transparent vesicles (blebs), which are swollen thylakoids. Such a large size of blebs is a consequence of the interconnection of all thylakoids within the network, which encloses therefore a common lumen (Schönknecht et al., 1990; Shimoni et al., 2005). Thylakoid blebs conserved light-induced membrane polarization, photochemical activity, and are able to photophosphorylate (Campo and Tedeschi, 1985; Allen and Holmes, 1986; Hinnah and Wagner, 1998). Forming of a high-quality gigaOhm seal between glass microelectrode and bleb represented, however, a major problem, which may be at least partially caused by a very low free lipid and high protein content of the thylakoid membrane. Together with another energy-coupling membrane, the inner mitochondrial one, thylakoids display the highest protein to lipid ratio of 3.4:1 (w/w); lipids cover only 20% of the membrane surface and 60% of total lipid is immobilized within the first protein solvation shell (Kirchhoff et al., 2002; Kirchhoff, 2008). In comparison, the outer and inner chloroplast envelope membranes present a protein to lipid w/w ratio of 0.35:1 and 1.1:1, respectively (Inoue, 2007). Abundance of integral proteins, protruding far from the bilayer (e.g., F-type H^+-ATPase), which have to denaturate against the glass, to allow direct contact between glass and lipid, would at least make the overall sealing process longer (Suchyna et al., 2009). Yet, although the inner mitochondrial membrane displays the same high protein to lipid ratio, respective studies greatly outnumber those available so far for thylakoids (Schindl and Weghuber, 2012; Szabo and Zoratti, 2014). The difference in lipid composition of the two membranes may be an additional problem. The mitochondrial inner membrane, similar to the plasma membrane, endoplasmic reticulum, Golgi, and endosomal membranes, is rich in phospholipids, including a high percentage (about 20%) of negatively (2-) charged cardiolipin (diphosphatydyl glycerol), specific for mitochondria (van Meer et al., 2008). Contrary to this, the thylakoid membrane is mainly

made of galactolipids (84%), with 7% of negatively charged sulfolipids and phosphatydylglycerol as a sole phospholipid (Block et al., 2007). The adhesion energy between glass and lipid bilayer varies by up to one order of magnitude as a function on the lipid composition (Ursell et al., 2011). This has been proven for different combinations of phospholipids, while respective data for galactolipids is missing. However, as gigaOhm seal formation is mainly stabilized by van der Waals forces (Suchyna et al., 2009), the presence of fixed dipoles in phospholipids as compared to glycolipids, would facilitate a tight seal formation in the first case. Although the first successful patch-clamp study was performed on a species with abnormally large (up to 40 μm in diameter) chloroplasts, *Peperomia metalica* (Schönknecht et al., 1988), bleb size does not appear to be a problem for tight sealing with a patch-pipette tip. Moreover, in our hands, blebs originated from *Peperomia* chloroplasts were proven to be more difficult to patch as compared to more typical chloroplasts of spinach. A critical moment was the time spent between a bleb formation in the experimental chamber and the attempt to obtain a tight seal, which should not exceed 10–15 min. (Pottosin and Schönknecht, 1995a, 1996; Hinnah and Wagner, 1998). Failure in fulfilling this condition resulted in the absence of stable tight seals (Enz et al., 1993). The presence of high divalent cation concentrations (e.g., 5 mM $MgCl_2$) at both membrane sides was also mandatory. It should also be noted that albeit achievement of high (up to 10 GOhm) resistance seals between the patch pipette and a thylakoid bleb could be done routinely, obtained membrane patches were extremely fragile and rarely withstood voltages higher than 40 mV by absolute value. So far, any attempt to get access to the bleb interior (whole thylakoid configuration) by application of a short pulse of high voltage or of strong suction resulted in the loss of the sample in 100% of cases. Yet, a very promising perspective to gain low resistance access to the thylakoid lumen without loss of a tight seal may be patch perforation by incorporation of channel-forming antibiotics (e.g., gramicidin, Schönknecht et al., 1990) into the patch pipette tip.

An alternative for a direct patch-clamping of the intact thylakoid membrane could be a dilution of the thylakoid lipid, either by its fusion into azolectin liposomes (Enz et al., 1993) or by incorporation of thylakoid membranes or purified channel protein into artificial lipid bilayers (Tester and Blatt, 1989; Li et al., 1996; Carraretto et al., 2013). Yet, it should be noted that incorporation of any external material into the lipid bilayer could produce an artificial channel. This may not necessarily be a protein responsible for such activity, as defects caused by lipid peroxidation, detergents, and/or bacterial contamination could be equally problematic (Labarca and Latorre, 1992; Pelzer et al., 1993). Consequently, one needs to have additional criteria. Usage of specific channel agonists or antagonists should be a solution, but as far as we know such a test was not performed for thylakoid channels, and even reproducibility of channel characteristics may be considered a problem in this case. This is not surprising, as an artificial environment can alter the channels' function or even induce a channel-like behavior in proteins, which do not form channels under physiological conditions, as it is true, for instance, for the chloroplast triose phosphate/phosphate translocator or phosphate carrier of inner mitochondrial membrane (Schwarz et al., 1994; Herick et al., 1997).

Anion-Selective Channel: Evidence for a Functional ClC in the Thylakoid Membrane

A 100 pS (in 100 mM KCl) voltage-dependent channel, with almost perfect selectivity for anions (Cl^-, NO_3^-) over K^+ was first time reported for intact thylakoid membrane from chloroplasts, isolated from leaves of *P. metalica* (Schönknecht et al., 1988). Later on, quite similar in their conductance (**Figure 1**), selectivity, and voltage-dependent kinetics channels were reported also for thylakoids of a Charophyte alga *Nitellopsis obtuse* and spinach (Pottosin and Schönknecht, 1995a,b). Notably, no such channel could be detected upon reconstitution of spinach thylakoid membranes into giant azolectin liposomes (Enz et al., 1993), which emphasizes the importance of studies on native membranes. Thylakoid anion channel by its conductance is reminiscent of the so-called "mitochondrial Centium picoSiemens" (mCS) anion channel of the inner membrane. Yet, mCS channel has a low Cl^-/K^+ selectivity, is inhibited by Mg^{2+}, and has a distinct voltage dependence (Sorgato et al., 1987; Borecký et al., 1997; Tomaskova and Ondrias, 2010; Szabo and Zoratti, 2014) as compared to the thylakoid channel. The voltage dependence of the thylakoid anion channel was bell-shaped, with a maximal open probability at zero voltage and a steep decrease of it at both positive and negative potentials, so that at ±50 mV the steady open probability was close to zero (Pottosin and Schönknecht, 1995a, 1996). Increase of Cl^- concentration on the luminal side tended to increase the channel's open probability, which may serve as a positive feedback mechanism upon light-induced anion accumulation in thylakoids (Pottosin and Schönknecht, 1995a). It has been shown that the thylakoid anion channel voltage-dependence is controlled by the two gating processes: a rapid (ms) one, which tends to favor open probability at stroma-positive potential, and a slow (sub-seconds to seconds), which, in contrast, favors the channel opening at stroma-negative potentials. Consequently, changing the voltage polarity evoked transient channel openings, relaxing slower when the potential was switched from positive to negative values (Schönknecht et al., 1988; Pottosin and Schönknecht, 1995a). Change of the polarity of transthylakoid $\Delta\Psi$ naturally occurs upon light-dark transitions, with the light onset or offset making stromal side more negative (**Figure 1**) or positive, respectively (Kramer et al., 2003). In this context, transient "on demand" activation of the anion channel may be especially important upon the light onset, when the channel-mediated current will accelerate the achievement of a steady state $\Delta\Psi$ at the light. Often, thylakoid patches displayed the activity of multiple anion channels. Whereas, fast close-open transitions of these channels were independent each of other, a common slow gate was detected, which could close a pair of channels simultaneously, with a probability far above being merely a coincidence (Pottosin and Schönknecht, 1995a). Thus, thylakoid anion channels displayed a coupled ("double-barreled") behavior, which is a hallmark characteristic for anion channels from the ClC family.

The ClC family is dominated by $2Cl^-/1H^+$ antiporters, and only a small part of it displays channel-like behavior. Such "broken" transporters preserve the activation by external protonation and have some remnant permeability to H^+, which drives their slow gating cycle out of equilibrium (Miller, 2006; Zifarelli and Pusch, 2010a,b). ClC channels and transporters, however, present only minor structural differences. A ClC protein possesses two homologous extended Cl^- pathways, which may be locked open in channels, resulting in long-living transmembrane pores. The presence of those two homomeric Cl^- pores, whose opening may be controlled by a common gate, serves as a natural explanation for a "double-barreled" behavior. In the ClC transporter structure, two glutamate residues, external, and internal ones, are essential for H^+ sensing and transport, respectively. Consequently, channel members of ClC family lack the "H^+ transport" glutamate, but preserve the external one, controlling the fast gate (Miller, 2006; Zifarelli and Pusch, 2010a). In *Arabidopsis*, of seven ClC members only two, AtClC-e and AtClC-f, are potentially channel-forming, according to this criterion (Zifarelli and Pusch, 2010a). A ClC-f product was localized in the outer chloroplast envelope and in Golgi membranes, whereas ClC-e is targeted at the thylakoid membrane (Teardo et al., 2005; Marmagne et al., 2007). One problem, however, is related to these tentative ion channels: they are lacking a highly conserved sequence, underlying Cl^- (GSGIP) or NO_3^- (GPGIP) selectivity: in ClC-e and –f these are replaced by ESAGK and EILDQ, respectively. Thus, current state-of-art approaches may not predict to which selectivity this arrangement may account (Zifarelli and Pusch, 2010a). The knockout of either of these genes disturbed nitrate homeostasis, although it remains unclear, whether it was a consequence of the cessation of NO_3^- and/or or NO_2^- transport by these channels, because multiple parameters were affected, including decrease of nitrate uptake by roots and expression of high-affinity nitrate transporters, increased nitrite and decreased nitrate levels (Monachello et al., 2009).

Unanswered questions include before all an unambiguous identification of the thylakoid anion channel. If it is ClC-e, as proposed by us and by others (Checchetto et al., 2013; Pfeil et al., 2014), then a direct proof should be the presence or absence of anion channels activity in thylakoids of WT and knock-out plants, respectively. This reinforces the need of a direct patch-clamping of *Arabidopsis* thylakoids, the problem is technically not solved yet, but is definitely a rewarding one. Lack of ClC-e function plants did not show a clear phenotype (Marmagne et al., 2007), but p.m.f. partitioning was not addressed so far, which calls for a further study. We also believe that testing the pH regulation of the thylakoid anion channel activity is physiologically quite relevant, due to the large changes of luminal and stromal pH upon dark/light transitions and due to the existence of a strong regulation of ClC-type channels by protons. Finally, although up to now no specific blocker of ClC channels is known, non-specific blockers like 9-anthracene carboxylate, flufenamic or niflumic acid, and clofibric acid derivates are available (Zifarelli and Pusch, 2012) and should be tested directly against thylakoid anion channels, to reveal their potential for further usage in diverse functional assays (e.g., p.m.f. steady state partitioning).

DIVALENT CATION-PERMEABLE CHANNELS

Mg^{2+} influx from thylakoids into stroma is recognized as a part counterbalancing process for light-driven H^+ transport into the lumen for a long time, although its contribution decreases upon the increase of external (cytosolic) K^+ (Hind et al., 1974). However, light onset caused a very substantial increase of stromal Mg^{2+}, from 0.5–1 to 2–3 mM (Portis and Heldt, 1976; Ishijima et al., 2003). Given relatively low envelope permeability for Mg^{2+} (Gimmler et al., 1974), this change is due to the mobilization of Mg^{2+} from thylakoids. It should be noted that as specific volumes of thylakoids to stroma are related as 1:15 (Heldt et al., 1973), a 2 mM increase of stromal Mg^{2+} implies a decrease of total thylakoid Mg^{2+} concentration by 30 mM. This suggests the involvement of a high capacity Mg^{2+}-transporting system in the thylakoid membrane, most likely an Mg^{2+}-permeable channel (**Figure 1**).

Patch-clamp study of Pottosin and Schönknecht (1996) revealed the presence of a non-selective cation channel in 75% of patches, derived from intact spinach thylakoids. This (here: ICTCC) intermediate conductance (55 pS in symmetric 100 mM KCl) thylakoid channel was virtually impermeable for Cl^-, and has a lower conductance, but comparable relative permeability for Ca^{2+} and Mg^{2+} as compared to K^+. ICTCC appears to be relatively robust, as similar channel activity could be registered also upon reconstitution of spinach thylakoids in giant liposomes or artificial bilayers (Enz et al., 1993; Li et al., 1996). A similar channel was also reported in a direct patch-clamp study on swollen thylakoids from pea leaves (Hinnah and Wagner, 1998). ICTCC was weakly voltage-dependent, with an increase of open probability from 0.1 at −50 to ∼0.5 at +50 mV (stroma *minus* lumen). Thus, under light conditions (stromal side made more negative, voltage gradually declining to a steady state), time-averaged current through a single ICTCC would be approximately constant (∼ −0.3 pA), as a decrease of electrical driving force would be compensated by the increase of open probability (Pottosin and Schönknecht, 1996). As a permeability of ICTCC for K^+ and Mg^{2+} is comparable, ICTCC-mediated K^+ and Mg^{2+} fluxes ratio will be determined by the ratio between luminal K^+ and Mg^{2+} activities. Assuming that it is 10:1, which is probably an upper estimate for the Mg^{2+} flux fraction, a single ICTCC will increase total stromal Mg^{2+} by 1 mM in just 20 s. Free stromal Mg^{2+} increased by 1 mM in the light in 5–10 min (Ishijima et al., 2003). However, only 5% of the chloroplast Mg^{2+} is free, whereas 95% is bound to the thylakoid surface, phosphorylated compounds and dicarboxylates (Portis, 1981). Therefore, the ICTCC—mediated Mg^{2+} flux may account for observed light-induced increase in stromal free Mg^{2+}. A curious feature of the light-induced Mg^{2+} influx to the stroma is its insensitivity to La^{3+} and a high sensitivity to ruthenium red (Ishijima et al., 2003), which should be addressed by a direct test with ICTCC.

A light-induced increase of free stromal Mg^{2+}, although not being large by absolute value, is essential for activation of the two key enzymes of CO_2 fixation cycle, fructose -1,6-bisphosphatase (FBPase) and sedoheptulose-1,7- bisphosphatase,

SBPase (Portis et al., 1977; Hertig and Wolosiuk, 1980; Wolosiuk et al., 1982). Mg^{2+}-deficient plants are characterized by extreme light sensitivity and chlorotic lesions. This was associated with the impairment of the CO_2 fixation cycle, limiting the overall photosynthetic rate, over-reduction of the electron transport chain, and consequent photodamage due to ROS accumulation (Cakmak and Kirkby, 2008). So far, ICTCC represents the only known Mg^{2+}-permeable channel in thylakoids. Important regulatory role of light-induced Mg^{2+} increase on stromal metabolism calls for further studies on the ICTCC molecular identity, distribution, pharmacology, and functional contribution (e.g., existence of alternative ways of Mg^{2+} shuttling between cytosol and stroma).

A separate, potentially more important perspective for ICTCC, is related to its Ca^{2+}-permeability. In the last years, ample evidence for the accompaniment of responses to different stresses of the cytosolic Ca^{2+} signal by stromal Ca^{2+} increase has been obtained (Nomura and Shiina, 2014). Moreover, in several occasions stromal Ca^{2+} occurred simultaneously to cytosolic Ca^{2+} increase or even preceded it (Nomura et al., 2012). Stromal Ca^{2+} may be increased by a voltage-driven uniport across the envelope from the cytosol (Roh et al., 1998). As at the rest cytosolic and stromal Ca^{2+} levels are approximately the same, ∼150 nM (Johnson et al., 1995), Ca^{2+} uptake into stroma requires a negative potential difference across the envelope, which is the case, especially under light (see below). However, this route may have its limitations. For instance, although light-induced Ca^{2+} uptake to the stroma was readily recorded (Muto et al., 1982), this did not lead to a measurable stromal free Ca^{2+} increase, due to increased stromal Ca^{2+} buffering capacity at more alkaline pH on the light and/or efficient Ca^{2+} uptake by energized thylakoids, which occurs via Ca^{2+}/H^+ antiport mechanism (Ettinger et al., 1999). So, alternatively, passive Ca^{2+} release from thylakoid lumen to stroma may be considered as a cause of a stromal Ca^{2+} signal. A possible role of ICTCC in it requires further elucidation.

OTHER CHANNELS

Light-induced K^+ influx into the stroma, together with anion efflux via anion-selective channels of the thylakoid membrane, is indisputably important for electric balance of the light-driven H^+ uptake by thylakoids, especially at physiologically relevant K^+ concentrations (Hind et al., 1974). Activity of thylakoid anion or cation channels, due to the opposite direction of respective ion fluxes, will cause thylakoid swelling or shrinkage under light. Thus, volume control may be one of the reasons for the implementation of approximately equivalent fluxes of cations and anions across the thylakoid membrane (**Figure 1**). The activity of ICTCC may be relevant for a mediation of the light-induced K^+ flux, but perhaps insufficient, as the whole thylakoid cation current has to be in the range of several pA. Recently, a tandem-pore K^+-selective channel (TPK3) was localized in *Arabidopsis* stromal thylakoid lamellae (Carraretto et al., 2013). Silencing of TPK3 resulted in *Arabidopsis* plants with reduced growth and altered thylakoid morphology. More specifically, TPK3-silenced plants displayed a reduced non-photochemical quenching, due to a decrease of a steady state ΔpH between stroma and lumen (hence, more alkaline luminal pH). Thus, TPK3 has a strong impact on the steady state p.m.f. partitioning at the light. Preliminary characterization revealed that recombinant TPK3 forms a voltage-independent Ba^{2+}-sensitive and TEA^+ insensitive channel, with a conductance of ∼30 pS. It conducts K^+, but seems to be not perfectly selective for K^+ over Cl^- and its relative selectivity for physiologically relevant cations is unknown. TPK3 likely requires Ca^{2+} for its activity, but whether it is regulated by Ca^{2+} within its physiological range of free concentrations remains to be elucidated. It is instructing that two other explored members of TPK family in *Arabidopsis*, TPK1, and TPK4, are activated by cytosolic H^+ (Allen et al., 1998; Becker et al., 2004). Preliminary data by Carraretto et al. (2013) demonstrated a strong stimulation of the reconstituted TPK3 by the acidification from pH 7.4 to 6.75, but the sidedness of the effect is unclear. Bearing the large changes of pH in stroma and lumen upon light-dark transitions in mind, the pH-dependence of TPK3 should be addressed. Last, but not least: it is highly desirable to demonstrate the TPK3 activity *in situ*, i.e., in thylakoid blebs of *Arabidopsis*.

For the sake of completeness, an electroneutral (hence, not detectable by the patch-clamp) K^+/H^+ antiporter KEA3, localized in the thylakoid membrane, should be considered (**Figure 1**). It works oppositely to TPK3, reducing the ΔpH gradient across the thylakoid membrane and takes K^+ up into the lumen (Kunz et al., 2014). Its activity is very essential upon naturally occurring intermittent light conditions. Specifically, upon the transition from a high to a low light condition, KEA3 accelerates the ΔpH dissipation and luminal pH increase, which led to the recovery of the photosystem II quantum efficiency (Armbuster et al., 2014). The presence of two K^+ transporting systems, TPK3, and KEA3, mediating K^+ transport in opposite directions and oppositely affecting the lumen acidification is analogous to the presence of a K^+-permeable channel and KEA1/2 in the inner envelope (see below); a balance between their opposite activities is essential for the chloroplast volume regulation and pH homeostasis (Kunz et al., 2014).

ENVELOPE ION AND SOLUTE CHANNELS

Outer Envelope Contains Large-Conductance Porins

Outer envelope (OE) is an important interface between cytosol and chloroplast. The classical view on OE is that it is a low selective molecular sieve, which is freely permeable to small molecules and proteins with molecular weights of up to 10 kDa (Flügge and Benz, 1984). This view could be reconsidered, however, in light of finding of relatively high cation-selective ($P_{K+}/P_{Cl-} = 14$) porin-like channels OEP23 and OEP37 (Goetze et al., 2006, 2015), and OEP16, a large conductance channel with a striking substrate selectivity for amines and amine acids (Pohlmeyer et al., 1997; Steinkamp et al., 2000; Duy et al., 2007; Pudelski et al., 2010). Moreover, OEP16 excludes sugars, which, basing on their size should pass the channel pore if it was a simple

sieve (Pohlmeyer et al., 1997). It was demonstrated for at least one OEP16 isoform, OEP16.2, preferentially expressed in seeds, that its lack of function caused a metabolic imbalance (primarily, changes in free amino acid contents), ABA-hypersensitivity, and early germination (Pudelski et al., 2012). It is also notable, that a relatively weakly selective OEP21 could change its rectification and ionic selectivity from weakly anionic to cationic one upon binding of ATP and phosphorylated carbohydrates exclusively from the intermembrane space (Bölter et al., 1999). This fact argues for a possibility that OE may exert barrier functions for a metabolite exchange between chloroplast and the rest of the cell (Flügge, 2000; Soll et al., 2000; Breuers et al., 2011). As it follows then, the intermembrane space between OE and inner envelope (IE) may represent a compartment on its own, with properties different from those of cytosol. Finally, OEP24 represents the only porin with a low ion selectivity and a broad range of transporting substrates, including amino acids, sugars, and phosphorylated sugars, dicarboxylates, ATP, and inorganic cations (Bölter et al., 1999). It appears that OEP24 relative expression is correlated with a requirement of a higher metabolic flux across the envelope (Bräutigam et al., 2008; Breuers et al., 2011), at which condition its function could not be compensated by higher selective porins. Interestingly, upon heterologous expression in yeast, OEP24 is targeted to the outer envelope membrane of mitochondria, where it can compensate the lack of function of the VDAC (Röhl et al., 1999), principle mitochondrial porin, which controls ions and metabolite traffic across the outer mitochondrial membrane (Colombini, 2012a). Most of the OE porins display bell-shaped voltage dependence, being open at voltages close to zero and closing at large potentials of either sign (Pohlmeyer et al., 1997, 1998; Bölter et al., 1999; Goetze et al., 2006). It is believed that this property does not have a physiological importance, as there is no potential drop across the OE except for a (supposedly small) Donnan potential difference due to accumulation of large weakly permeable anionic species in the intermembrane space. There are some studies for mitochondria, however, which estimate the Donnan potential difference could be as large as 20–40 mV (cytosol positive; Porcelli et al., 2005). At +25 mV VDAC is switched to a subconductance state, and VDAC-mediated ATP transport stops (Colombini, 2012b). A similar situation may exist with OEP21, which is blocked by ATP and at the same time seems to transport it in a "tight fit manner" (Hemmler et al., 2006). Notably, OEP21 has the steepest voltage dependence among chloroplast porins, which is similar to that of VDAC, and at large potentials it is switched to a subconductance state of about 20% of maximal conductance (Bölter et al., 1999). A maximum of time-averaged conductance for OEP21 is observed at +25 mV, whereas at zero voltage it is close to minimal one. Therefore, for this porin, in contrast to other OEPs, it would be essential, whether the voltage difference across the OE is zero or +25 mV (the value, close to that for the Donnan potential difference in mitochondria).

Proteomic analysis of the OE demonstrated that OE is enriched in five non-redundant transport proteins: an ABC transporter with unknown function and aforementioned OEP 16, 21, 24, and 37, plus a protein translocon channel, formed by TOC75 (Gutierrez-Carbonell et al., 2014). Although this list may be non-exhaustive, it can serve as a first-aid guide for the identification of porins, measured in intact chloroplast membranes. Large conductance weakly selective ($P_K+/P_{Cl-} \sim 2.3$) porins, with a bell-shaped voltage dependence could be detected in intact chloroplasts of higher plants (Muñiz et al., 1995). All these properties are reminiscent of those for pea OEP24 (Pohlmeyer et al., 1998). Measurements on intact chloroplasts of a Charophyte alga *Nitellopsis obtusa* revealed two types of large-conductance porin-like channels and an anion selective channel (Pottosin, 1992). Anion selective-channel by its conductance and voltage dependence was very similar to its counterpart in the thylakoid membrane (Pottosin and Schönknecht, 1995a), albeit being less selective for Cl^- over K^+, $P_{Cl-}/P_K^+ \sim 12$. A weakly selective anion channel with a similar conductance and identical voltage dependence was registered upon reconstitution of spinach envelope membranes into lipid bilayers (Viérick et al., 2003). These findings corroborate the report on the localization of ClC-f in the OE (Teardo et al., 2005), although an expected "double-barreled" gating of this tentative channel remains to be demonstrated functionally. The most abundant weakly selective cation porin in a *Nitellopsis* chloroplast was reminiscent of VDAC by its voltage dependence, substates occurrence, and effect exerted by the synthetic König's polyanion, known gating modulator of VDAC (Colombini et al., 1987; Pottosin, 1993). When compared to pea porins, its voltage-dependent behavior (position of maximum at ∼+20 mV, steepness, closure to a subconductance state of about 20% of maximal at large potentials of either sign) better fits that of OEP21. OEP21 displayed cation selectivity only in the presence of submillimolar to low millimolar ATP in the intermembrane space (Bölter et al., 1999). Such a condition, however, might also naturally occur in experiments on intact chloroplasts on *Nitellopsis*. Summarizing, patch-clamp measurements on relatively large (about 10 μm) and robust chloroplasts of *N. obtusa* revealed ion channels, which, on the basis of their activity and large conductance, are more likely to be located in the OE. However, working on smaller chloroplasts from higher plants, we were forced to make different assumptions on the patch configuration upon stable recording.

Is it Possible to Measure Ion Channel Activity from the Inner Envelope of an Intact Chloroplast?

Patch-clamp measurements of porin-like channels in attached configuration on intact pea chloroplasts could be done only within a short period, due to a spontaneous patch isolation and chloroplast lysis (Pottosin et al., 2005). Before the collapse, currents through porin-like channels were distorted and decreased in amplitude as if a membrane vesicle was formed on the tip of the pipette (Hamill et al., 1981). We believed that, due to a very close alignment of the OE and IE membranes (5 nm space) and existence of tight contact sites between the two envelope membranes, it was hardly possible that this vesicle was formed by the OE alone, stripped from the IE. Most likely, the sealing of the intermembrane space took place upon patch

isolation, so that a sandwich-like arrangement of OE and IE resulted (**Figure 2**). Insulation of the transmembrane space is required for a switch of a recording on the OE to that on the IE. As electrical resistance of the OE, due to the presence of high conductance pores, is much lower than that of the IE, command voltage will fall mainly across the IE and the IE will limit the overall ion flux. The fraction of voltage drop across the OE would be close to zero, so porins would be mainly locked in the open state, fixing this condition over the whole experiment in inside-out patch configuration. The same conclusion was drawn by van den Wijngaard and Vredenberg (1997; van den Wijngaard et al., 2000)—another group, which succeeded in stable patch-clamp recordings on the intact chloroplast envelope of pea.

Fast-Activated Cation Channel in the Pea Envelope Membrane

Spontaneous isolation of the envelope patch resulted in a quite different pattern of channels' activity. The dominant smaller conductance channels were rapidly activated ($\tau \sim 1$–2 ms) by large membrane polarizations of either sign and were highly selective for K^+ over Cl^-, $P_{Cl-}/P_K^+ < 0.03$, but weakly differentiated between K^+, Na^+, Ca^{2+}, or Mg^{2+} (Pottosin et al., 2005). It was termed FACC, for the fast activating chloroplast cation channel (**Figure 1**). By its permeability to divalent cations and conductance value, FACC may be compared with the glutamate receptor iGLR3 channel, which was found in the IE of *Arabidopsis* chloroplasts and assayed upon fusion of IE vesicles with an artificial planar lipid bilayer (Teardo et al., 2010). It should be interesting, then, to test effects of iGLR3 channel agonists and antagonists against FACC. Physiologically, FACC will mainly mediate the exchange of K^+ (and Na^+) across the envelope, because free concentrations of Ca^{2+} and Mg^{2+} at both envelope sides are lower than those for K^+ by a factor of 10^6 and 10^2, respectively. FACC displayed a U-shaped voltage dependence of the open probability, which was reminiscent of this for so-called fast vacuolar channel, FV (Tikhonova et al., 1997). However, current via FV channel was almost completely abolished by sub- mM cytosolic Ca^{2+} or Mg^{2+} (Tikhonova et al., 1997; Brüggemann et al., 1999) and the FACC-mediated currents could be measured in the presence of high concentrations of these cations. Yet, a reduction of cytosolic Ca^{2+} from 2 mM to physiological level of 200 nM caused an increase of the FACC open probability almost 10-fold in the physiological voltage range (Pottosin et al., 2005). Trans-envelope voltage difference is negative at stromal side and decreased by absolute value upon an increase of cytosolic KCl concentration, from −110 mV at 5 mM to −20 mV at −100 mM KCl under light; in dark conditions voltage difference is depolarized by ∼20 mV (Demmig and Gimmler, 1983). FACC also sensed stromal pH changes within the range, correspondent to light- dark transition: its open probability increased several-fold upon the pH change from 8 to 7.3 (Pottosin et al., 2005). Such a behavior may be expected from a channel, which tended to oppose stromal acidification.

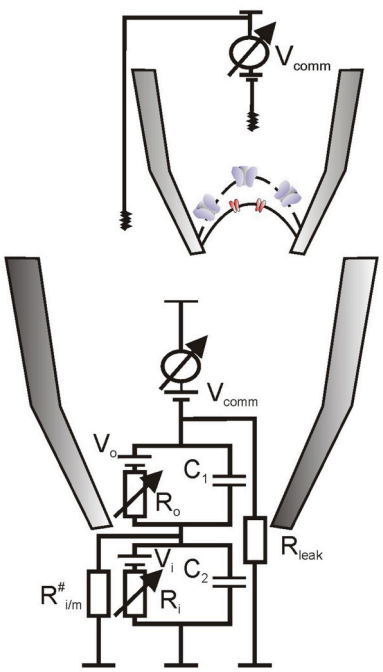

FIGURE 2 | Hypothetical equivalent electrical circuit for recording on a sandwich-like envelope patch. It is assumed that the intermembrane space was tightly sealed ($R^{\#}_{i/m} >> R_o$) and that inner envelope electrical resistance was much higher than of the outer envelope, $R_i >> R_o$ (due to the presence of multiple porins in the latter), so that command voltage (V_{comm}) dropped almost entirely across the inner envelope membrane patch ($V_{comm} \sim V_i$). R_o represents the access resistance, which was ∼1 GOhm for measurements on pea chloroplasts, equivalent to a presence of few (2–3) open porins in the outer patch membrane. Average patch capacitance was about 0.35 pF; on the basis of specific capacitance for biological membranes (∼1 $\mu F/cm^2$) this transforms to 35 μm^2 or 70% of the pea chloroplast surface, additional argument for the presence of a double membrane vesicle in the patch (Pottosin et al., 2005).

An alkaline stromal pH (∼8) is optimal for the activity of FBPase and SBPase, whereas at pH below 7.3 the turnover rate of the CO_2 fixation cycle became very low (Werdan et al., 1975; Chardot and Meunier, 1991). Light-driven uptake of H^+ and a parallel flux of Cl^- across the thylakoid membrane were extended to a decrease of activity of these ions in the cytosol, a stable one for Cl^- and a transient one for H^+ (Thaler et al., 1991). The reversibility of the light-induced cytosol alkalinization may argue for the existence of an active mechanism of the H^+ extrusion from stroma across the envelope. Functional evidence suggested that H^+ evacuation from stroma is mediated by yet non-identified IE primary H^+ pump (Maury et al., 1981; Berkowitz and Peters, 1993). Function of an electrogenic H^+ pump will polarize the trans-envelope potential (**Figure 1**), which calls for a balancing counterion flux. It is long known that stromal acidification may be reversibly induced by depletion of the external medium of monovalent cations, K^+ and Na^+, which could act, therefore, as counterions for active H^+ extrusion (Demmig and Gimmler, 1983; Wu et al., 1991; Wu and Berkowitz, 1992; Heiber et al., 1995). The respective K^+ influx to and H^+ efflux from stroma are in the range of 100 and 20 nmol/min per mg of chlorophyll at 10–20 and 100 mM external K^+, respectively (Demmig and Gimmler, 1983; Wu et al., 1991); a decreased K^+ influx at higher external

K^+ may be dealt with by a decrease of the K^+-driving force (trans-envelope voltage difference). These values transform to electrical current of 0.2 and 0.04 pA per single chloroplast; the latter value (for 100 mM cytosolic KCl) may be more physiologically relevant. At physiological pH, Ca^{2+} and voltage a single FACC mediated ∼1 pA current and had an open probability of 1–2% (Pottosin et al., 2005), which implies that a single FACC conducted the time-averaged K^+ current of approximately 10–20 fA. Thus, the activity of few FACC channels as typically observed in isolated chloroplast patches (Pottosin et al., 2005) might be sufficient to balance the IE pump-mediated H^+ extrusion. As will be discussed below, the chloroplast volume appears to be finely balanced by channel-mediated K^+ influx and K^+/H^+ antiporters- mediated K^+ efflux across the envelope (**Figure 1**). On the quantitative basis, therefore, we believe that high-conductance and poorly regulated K^+ permeable channels, reported by others to be present in the IE (Mi et al., 1994), are likely artifacts of reconstitution procedures. Indeed, a single channel of this type will conduct a time-averaged current of few pA, which is by two orders of magnitude above the demand. It will collapse the transenvelope voltage difference and will always overweight the K^+ flux, generated by IE K^+/H^+ antiporters, making their contribution to volume control insignificant and the overall function (stromal acidification) counterproductive.

An important note should be made on the thermodynamics of $K^+(Na^+)/H^+$ exchange across the envelope. H^+ extrusion from stroma to cytosol is against the H^+ gradient and may be only active. This active transport will fuel a downhill channel-mediated $K^+(Na^+)$ uptake into the stroma. The direction of transports, thus, is exactly opposite to those mediated by conventional $K^+(Na^+)/H^+$ antiporters. In the chloroplast IE two K^+/H^+ antiporters, *KEA1* and *KEA2*, and Na^+/H^+ antiporter *NHD1* were identified (Kunz et al., 2014). These cation/H^+ antiporters transport H^+ downhill *into* the stroma (hence, acidifying it), and *export* K^+ (or Na^+) to the cytosol (thus, assisting the chloroplast regulated volume decrease). Consequently, triple *kea1kea2nhd1* lack-of-function mutants displayed a severe phenotype, with swollen or broken chloroplasts (Kunz et al., 2014). These two different types of the IE $K^+(Na^+)/H^+$ antiport systems, one based on $K^+(Na^+)/H^+$ antiporters and another consisting of a non-selective cation channel and of an H^+ pump, should be both essential for stromal pH control and chloroplast volume regulation, exerting the effects opposite one to another. It was shown that osmotic adjustment in chloroplasts, crucial for the photosynthesis efficiency, requires transport of organic osmolytes in addition to inorganic ions and that the permeability of the IE for sugars is increased upon acute water stress (Robinson, 1985; McCain, 1995). This argues for the activity of mechanosensitive channels, which pores are wide enough to transport sugars. As far as we know, mechanical stretch-stimulation was never tested upon *in situ* patch-clamp measurements on intact chloroplasts, which may be addressed for future studies. However, the presence of MscS-like MSL 2 and 3 channels in the IE and their impact on the chloroplast volume was demonstrated, suggesting that they form functional mechanosensitive channels in *Arabidopsis* (Haswell and Meyerowitz, 2006). Ionic selectivity of MSL 2 and 3 is unknown, whereas a related MSC1 channel in *Chlamydomonas* chloroplasts is selective for anions over K^+ (Nakayama et al., 2007). On the other hand, FACC was permeable to N-methyl D-glucamine (Pottosin et al., 2005), and, therefore, possesses a pore wide enough to transport monosaccharides.

Anion Channel (PIRAC) in the Pea Envelope Membrane

In an interesting variation with our data, direct patch-clamping of pea chloroplasts by other group revealed that the envelope conductance in their case was dominated by a voltage-independent 50 pS anion channel (van den Wijngaard and Vredenberg, 1997, 1999; van den Wijngaard et al., 1999). Its open probability was negatively regulated by protein precursors (preferredoxin), so it was named PIRAC (for protein import related anion channel) and its interaction with the IE protein translocon (Tic) was considered, although it probably did not form a part of the Tic pore (van den Wijngaard and Vredenberg, 1999; van den Wijngaard et al., 2000). An anion channel has to be present in the IE (and possibly, also in the OE) due to the fact of a large cytosolic Cl^- decrease, caused by the light-driven anion uptake by chloroplasts (Thaler et al., 1991). Because PIRAC activity was studied in a relatively narrow voltage range, ±40 mV, where FACC open probability did not exceed 2%, FACC could be easily overlooked. However, how could we, in turn, overlook the activity of PIRAC, which open probability was close to 1? Comparison of our experimental conditions with those employed by van den Wijngaard and co-workers, revealed a single potentially important difference: for some reasons these authors used Mg^{2+}-free solutions, whereas in our case minimal free Mg^{2+} concentration at either membrane side was 1 mM (Pottosin et al., 2005). It is well known that the activity of mCS anion channel of the inner mitochondrial membrane is completely abolished by inclusion of 1 mM Mg^{2+} in solutions (Borecký et al., 1997). High intracellular Mg^{2+} also inhibits volume-regulated anion channels (Nilius and Droogmans, 2003). This opens a very interesting perspective, as Mg^{2+} may act as a natural regulator of PIRAC.

OUTLOOK

In vitro studies of individual OE porins, either purified native or recombinant proteins, is undoubtedly a very important source of information on their detailed properties. However, it is believed, albeit not proved experimentally, that all or at least some OEPs are negatively controlled *in vivo* and *in situ*, so that they are closed most of time and open only on demand- the situation, obviously, rather different from their high open probability *in vitro* (Bölter and Soll, 2001; Duy et al., 2007). Direct patch-clamping of the intact chloroplast envelope may be the way to prove or disprove this hypothesis. Up to the moment, only two groups, our lab and that from Prof. Vredenberg were succeeded in direct intact chloroplast patch-clamping, and most of available information for a higher plant chloroplast was obtained on a single species—the pea. The pea played an important role in OEP studies, as OEPs were first identified in this species and only afterwards in *Arabidopsis*. Nevertheless, there is really a

need to extend the list of explored species. *Arabidopsis* and rice chloroplasts are on the top of this list. We are unaware of such attempts in the past and may not judge, whether the absence of respective information is due to an extremely low success rate or just unwillingness to try. However, as pea chloroplasts were not uniquely patch-clamp friendly, we believe that it is worth to pursue the patch-clamp trials. We are also thinking that a concurrent implementation of a non-invasive MIFE technique to study ion fluxes on chloroplasts arrays, similar to the previous application of MIFE to studies on microorganisms (Shabala et al., 2006), may be of importance to elucidate the trans-envelope ion transport *in situ*. Our observations on electrical behavior of pea chloroplasts upon patching raised the possibility to register currents from the two envelope membranes in series. This model, of course, requires further proofs. However, a tightly regulated membrane conductance, revealed in our experiments as compared to a vast variety of poorly regulated conductance states in reconstituted studies (Mi et al., 1994; Heiber et al., 1995), appears to be more consistent with barrier properties of the IE. Thylakoid membrane patching, and, in particular, patching native thylakoid membrane of *Arabidopsis*, represents an interesting and potentially rewarding task for the future. Again, available data are too scarce for generalizations. Nevertheless, the dominant anion channel seems to be conserved as it was detected in only two distantly related dicotyledonous species and in alga chloroplasts. Its identity with ClC (ClC-e?), regulation by protons, and pharmacological profile remain to be elucidated. Divalent cation-permeable channel so far was found only in spinach thylakoid membrane (Pottosin and Schönknecht, 1996). Do similar channels exist in thylakoid membranes of other higher plant species? If so, what is their molecular identity and actual roles in stromal Ca^{2+} signaling and control of CO_2 fixation by Mg^{2+}? Finally, an interesting approach of thylakoid transport studies may be the achievement of a whole thylakoid configuration, using a perforated patch technique. It will allow not only to record currents mediated by all present ion channels, but in conjunction with it also measure light-induced electron and H^+ transport under strictly controlled voltage and pH conditions. This may represent a more direct way to study the effects of pH and voltage on the photosynthetic transfer and to reveal particular contributions of different ion channels into a dynamic control of the thylakoid $\Delta\Psi$.

AUTHOR CONTRIBUTIONS

IP was in charge of general planning, writing, editing, and figures design. OD performed database search, writing, editing, and proof-reading.

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RNA Sequencing Analysis of the *msl2msl3, crl,* and *ggps1* Mutants Indicates that Diverse Sources of Plastid Dysfunction Do Not Alter Leaf Morphology Through a Common Signaling Pathway

Darron R. Luesse[1]*, *Margaret E. Wilson*[2] and *Elizabeth S. Haswell*[2]

[1] Department of Biological Sciences, Southern Illinois University Edwardsville, Edwardsville, IL, USA, [2] Department of Biology, Washington University in Saint Louis, Saint Louis, MO, USA

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***Correspondence:**
Darron R. Luesse
dluesse@siue.edu

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Determining whether individual genes function in the same or in different pathways is an important aspect of genetic analysis. As an alternative to the construction of higher-order mutants, we used contemporary expression profiling methods to perform pathway analysis on several *Arabidopsis thaliana* mutants, including the *mscS-like* (*msl*)*2msl3* double mutant. MSL2 and MSL3 are implicated in plastid ion homeostasis, and *msl2msl3* double mutants exhibit leaves with a lobed periphery, a rumpled surface, and disturbed mesophyll cell organization. Similar developmental phenotypes are also observed in other mutants with defects in a range of other chloroplast or mitochondrial functions, including biogenesis, gene expression, and metabolism. We wished to test the hypothesis that the common leaf morphology phenotypes of these mutants are the result of a characteristic nuclear expression pattern that is generated in response to organelle dysfunction. RNA-Sequencing was performed on aerial tissue of *msl2msl3 geranylgeranyl diphosphate synthase 1* (*ggps1*), and *crumpled leaf* (*crl*) mutants. While large groups of co-expressed genes were identified in pairwise comparisons between genotypes, we were only able to identify a small set of genes that showed similar expression profiles in all three genotypes. Subsequent comparison to the previously published gene expression profiles of two other mutants, *yellow variegated 2* (*var2*) and *scabra3* (*sca3*), failed to reveal a common pattern of gene expression associated with superficially similar leaf morphology defects. Nor did we observe overlap between genes differentially expressed in *msl2msl3, crl,* and *ggps1* and a previously identified retrograde core response module. These data suggest that a common retrograde signaling pathway initiated by organelle dysfunction either does not exist in these mutants or cannot be identified through transcriptomic methods. Instead, the leaf developmental defects observed in these mutants may be achieved through a number of independent pathways.

Keywords: ion homeostasis, MscS-Like, RNA-seq, plastid, retrograde signaling, variegation, leaf morphology

INTRODUCTION

Ion homeostasis across organelle membranes is critical for plant survival. Proper solute concentrations in the stroma are essential for organelle size and shape, and the ions themselves are required for a wide variety of metabolic processes. The establishment and maintenance of ion homeostasis in the plant plastid is therefore a critical yet complex biological process. The genetic disruption of $Fe^{2+/3+}$ (Duy et al., 2007; Jeong et al., 2008), Na^+ (Müller et al., 2014), or K^+ (Kunz et al., 2014) homeostasis in the plastid can lead to impaired chloroplast function, reduced photosynthetic capacity, and stunted growth.

Two proteins likely to be involved in ion homeostasis in plastids are MscS-Like (MSL)2 and MSL3, two Arabidopsis homologs of MscS, a well-studied mechanosensitive ion channel from *Escherichia coli* (Hamilton et al., 2015a). Several lines of evidence support the proposal that MSL2 and MSL3 function as mechanically gated ion channels. At least two members of the Arabidopsis MSL family have been shown to function as mechanically-gated ion channels by single-channel patch clamp electrophysiology (Maksaev and Haswell, 2012; Hamilton et al., 2015b), and MSL3 is capable of rescuing an *E. coli* strain that lacks key mechanosensitive channels (Haswell and Meyerowitz, 2006).

MSL2 and MSL3 localize to the plastid envelope, and are semi-redundantly required for normal plastid size and shape, plastid division, and plastid osmoregulation (Haswell and Meyerowitz, 2006; Wilson et al., 2011; Veley et al., 2012). The large, round phenotype of epidermal leaf plastids in the *msl2msl3* mutant can be suppressed by a variety of genetic, physiological, and media manipulations that result in increased cytoplasmic osmolarity, indicating that the primary defect in this mutant is an inability to release osmolytes, most likely ions, from inside the plastid during hypoosmotic stress (Veley et al., 2012).

msl2msl3 mutants also exhibit a variety of leaf architecture phenotypes. Most prominently, they exhibit white or light green patches on the leaf surface, corresponding to regions of leaf tissue with large air spaces between mesophyll cells (Haswell and Meyerowitz, 2006). This indicates a tissue- as well as a cell-level defect, and in this respect, *msl2msl3* mutants resemble variegation mutants. The classic variegation mutants—some of the first mutants ever to be isolated in Arabidopsis—are derived from defects in chloroplast biogenesis and include *chloroplast mutator (chm)*, *yellow variegated (var)1, yellow variegated (var)2,* and *immutans (im)* (Foudree et al., 2012). The *var2* mutant phenotype is caused by a mutation in a subunit of the thylakoid-localized FtsH complex, which is likely involved in chloroplast biogenesis and development (Chen et al., 2000; Sakamoto et al., 2003). Complete loss of the FtsH complex leads to arrest of chloroplast development, while slight disruptions result in variegated plants (Liu et al., 2010). *var2* mutants show variegation phenotypes of varying intensity. While most have at least small white patches around the leaf periphery, more severely affected leaves are smaller than wild-type and have larger, irregularly-shaped white segments throughout the leaf, as well as scalloping at the leaf margin (Liu et al., 2010).

In addition to patches of white on the leaves, *msl2msl3* mutants are also dwarfed and have rumpled leaves and uneven leaf margins (Jensen and Haswell, 2012; Wilson et al., 2014). A straightforward explanation for these data is that MSL2 and MSL3 form MS ion channels in the plastid envelope, and their absence leads to defects in plastid osmoregulation and ion homeostasis, which in turn leads to pleiotropic effects on leaf development. We note that a defect in plastid ion homeostasis in the *msl2msl3* mutant has not yet been directly shown, and it is currently unclear how disruptions in plastid ion homeostasis might impinge upon pathways that regulate leaf development.

It has previously been proposed that leaf development is sensitive to organelle function (Streatfield et al., 1999; Tan et al., 2008; Moschopoulos et al., 2012), possibly through the action of an organelle-to-nucleus, or retrograde, signaling pathway (recent reviews on this topic include Barajas-López Jde et al., 2013; Jarvis and López-Juez, 2013). In favor of this argument, a large number of mutants with lesions in nuclear genes that encode plastid- or mitochondrial-targeted proteins exhibit similar leaf phenotypes (summarized in Moschopoulos et al., 2012). Patchy leaf color and leaf morphology defects are observed in plants harboring mutations in chloroplast division (Asano et al., 2004), chlorophyll synthesis (Ruppel et al., 2013), double strand break repair (Maréchal et al., 2009; Lepage et al., 2013), ribosomal RNA synthesis and processing (Bellaoui and Gruissem, 2004; Hricová et al., 2006), organellar tRNA synthesis (Uwer et al., 1998; Moschopoulos et al., 2012), and mitochondrial protease activity (Gibala et al., 2009). While these leaf morphology defects show no obvious link to the primary function of their causal mutation, all of these lesions are likely to produce physiological conditions that lead to organelle dysfunction. One possible explanation for these results is an organelle-to-nucleus signaling pathway initiated by a variety of plastid dysfunctions, which then produces a characteristic nuclear gene expression pattern that leads to defective leaf morphology.

Like the *msl2msl3* mutant, the *crumpled leaf* (*crl*) mutant also exhibits overall small stature and white sectors on its leaves, which have a ruffled surface and irregular margins (Asano et al., 2004). *CRL* encodes a novel protein of the chloroplast outer envelope and *crl* mutants have a defect in plastid replication so severe that some leaf cells in the mature plant lack any plastids (Chen et al., 2009). While chlorophyll a and b content is decreased in the *crl* mutant—likely a result of fewer plastids—photochemistry and thylakoid organization are indistinguishable from wild type plants, suggesting normal chloroplast biogenesis (Asano et al., 2004). Spontaneous microlesion formation and enhanced resistance to bacterial infection have been documented in *crl* mutants (Šimková et al., 2012). Cell cycle regulation and root meristem cell differentiation are also disrupted (Hudik et al., 2014).

A subset of the leaf morphology phenotypes observed in the *msl2msl3*, *var2*, and *crl* mutants are displayed by the *ggps1-1* mutant, which harbors a temperature-sensitive allele of *GERANYLGERANYL DIPHOSPHATE SYNTHASE (GGPS)1. GGPS1* is an essential gene that encodes a key branch point

Abbreviations: LFC, Log_2 Fold Change; FDR, False Discovery Rate; MSL, MscS-Like; CRL, Crumpled Leaf; GGPS, Geranyl Geranyl Diphosphate Synthase; RNA-seq, RNA sequencing.

enzyme required for plant isoprenoid biosynthesis (Bouvier et al., 2005). The GGPS1 protein is localized to the plastid (Okada et al., 2000). T-DNA insertions that disrupt the DNA sequence encoding the chloroplast targeting peptide and the C-terminus of the GGPS1 protein lead to seedling albino and embryo lethal phenotypes, respectively, while the EMS-induced single amino acid change in the *ggps1-1* allele was shown to produce temperature-sensitive variegation with reproducible patterning (Ruppel et al., 2013). Mutant plants grown at 21–23°C have an albino region in the center of the leaf and green tissue at the periphery, and have leaves that are typically smaller than wild type with a slightly rumpled surface and scalloped edges. Growth at higher temperatures leads to an increase in both the size of albino region and the severity of scalloping at the leaf margin. Transmission electron microscopy of white sectors revealed plastids with poorly developed thylakoid membranes.

A number of other Arabidopsis mutants show variable leaf color, rumpled leaves, and serrated or scalloped leaf edges; all harbor lesions in genes that encode plastid- or mitochondria-targeted proteins. These proteins include CAB UNDEREXPRESSION (CUE)1, a phosphoenolpyruvate (PEP)/phosphate translocator of the plastid inner envelope membrane (Li et al., 1995; Streatfield et al., 1999; Staehr et al., 2014); WHIRLY1/WHIRLY2, which is involved in double strand break repair and plastid genome stability (Maréchal et al., 2009; Lepage et al., 2013); DEFECTIVE CHLOROPLASTS AND LEAVES (DCL), which is involved in plastid ribosomal RNA processing in Arabidopsis (Bellaoui and Gruissem, 2004); SCABRA3, a plastid-localized RNA polymerase (Hricová et al., 2006); EMBRYO DEFECTIVE DEVELOPMENT (EDD1), an organellar glycyl-tRNA synthetase (Uwer et al., 1998; Moschopoulos et al., 2012); and the mitochondrial protease AtFtsH4 (Gibala et al., 2009). Similar mutants have been isolated in *Antirrhinum*, tobacco, and tomato (Chatterjee et al., 1996; Keddie et al., 1996; Wang et al., 2000; Wycliffe et al., 2005).

Taken together, these data suggest that the proper function of endosymbiotic organelles is required for normal leaf patterning. Because lesions in such a large number of genes implicated in apparently unrelated plastid or mitochondrial processes result in similar defects in leaf morphology, it is unlikely that these phenotypes are a direct result of the disruption of a specific metabolic or biosynthetic pathway within an organelle. Thus, while the primary defects are only similar in that they are defects in plastid function—they disrupt different pathways and likely generate variegated leaves through different mechanisms—all the mutants take on a similar appearance in the development of the leaf surface and edges. One possible explanation is that a characteristic nuclear gene expression pattern may be generated in response to a variety of organellar dysfunctions, and that this expression pattern leads to defects in leaf development and morphology. A second possible explanation is that the defects in leaf development and morphology seen in these mutants may be produced by different pathways that only coincidently produce similar developmental phenotypes that are not related at the molecular level.

One way to test for the presence of a gene pathway leading from plastid division to leaf development would be to make higher-order mutants, combining lesions in in the same background to look for genetic interactions (Huang and Sternberg, 1995; Koornneef et al., 2006). Instead, we characterized the gene expression profiles of *msl2msl3*, *crl*, and *ggps1* mutant leaf tissue by RNA sequencing, and compared the results to previously published expression profiles of two other mutants, *var2* and *sca3* and to 39 genes previously identified as a possible retrograde core response module (Glaßer et al., 2014). We reasoned that a high degree of overlap between the transcript profiles of these mutants would lend support to the proposal that lesions affecting different aspects of organelle physiology result in similar whole-cell and plant phenotypes because they alter gene expression networks in the same way. On the other hand, little overlap between the gene expression profiles of the mutants would suggest that diverse gene expression networks can still produce highly similar leaf phenotypes. It was expected that either of these outcomes would help illuminate the genetic pathway or pathways that link defects in plastid function in general (and in plastid ion homeostasis in particular) to disruptions in leaf development.

MATERIALS AND METHODS

Plant Growth

Seeds were sown on moist potting mix and stratified at 4°C for 48 h. Plants were grown in a growth chamber under 16 h photoperiods with a light intensity of 100–120 μmol m^{-2} s^{-1} at 21°C. The *ggps1-1* and *msl2-3* (GK-195D11) alleles are in the Columbia (Col-*0*) background. The *msl3-1* allele is in the Wassilewskija (Ws) background (Haswell and Meyerowitz, 2006). The *crl-3* mutant line (GK_714E08) was identified in the GABI-Kat collection of T-DNA insertion lines (Rosso et al., 2003) and is in the Col-*0* background.

Microscopy

Confocal laser scanning microscopy was performed on the youngest leaves of 3-week-old soil-grown plants using a FLUOVIEW FV1000 (Olympus), and images were captured with FVIO-ASW software. Chlorophyll autofluorescence was excited at 635 nm and emissions collected with a 655–755 nm band-pass filter.

Semi-Quantitative Reverse Transcription PCR

RNA was isolated from pools of 2-week-old Col-*0* and *crl-3* seedlings using the RNeasy Plant Mini kit (Qiagen) with an on-column DNase digestion. One milligram of RNA was used for subsequent cDNA synthesis with an oligo dT primer. The following primers were used to amplify *CRL* transcript levels: (a) 5′-ATGGGTACCGAGTCGGGTT-3′, (b) 5′-GGAGCAAGCGTCAAGAGATCCTG-3′, and (c) 5′-CCAGGTGAAGTTGAGCCTTCGTAC-3′. The *ACTIN* gene was used as a loading control and was amplified with the primers ACT.F2 5′-TACGCCAGTGGTCGTACAAC-3′ and Actin.8 5′-AACGACCTTAATCTTCATGCTGC-3′.

Tissue Collection, RNA Isolation, and RNA Sequencing

Aerial tissue from 3-week-old *msl2msl3* and *crl* mutants was harvested in triplicate, along with co-grown wild-type Col-*0* controls. This time point was chosen to collect the largest rosettes possible prior to bolting. Newer leaves near the center of the rosette were collected from 5-week-old *ggps1-1* mutants and controls in triplicate. These plants were co-cultivated with the *msl2msl3* and *crl* mutants, but were harvested later to allow leaves to reach sufficient size for dissection. After harvesting, leaves were dissected with a razor blade to isolate white tissue for sequencing.

RNA was isolated from three biological replicates per mutant using the RNeasy Plant Mini kit (Qiagen) with an on-column DNase digestion. Each sample had a 260:280 ratio of at least 2.0 and a RIN value above 8.0. Library preparation and RNA-seq was performed at the Genome Technology Access Center at Washington University. Samples were enriched for mRNA using a Dyna mRNA Direct kit (Life Technologies). One biological replicate from each mutant and its corresponding wild-type control were barcoded and run together using paired-end reads on an Illumina Hi-seq 2500. This was performed in triplicate, resulting in 15 individual sequenced samples.

Analysis of RNA-Sequencing Reads

Individual reads from each sample were aligned to the TAIR10 Arabidopsis reference genome provided by http://support.illumina.com/sequencing/sequencing_software/igenome.html using STAR (Dobin et al., 2013). A list of differentially expressed genes was determined for each sample in edgeR by comparison to the appropriate wild-type control (Robinson et al., 2010). Significant expression changes in *msl2msl3* and *crl* backgrounds were determined by comparison to the 3-week-old wild type controls grown and harvested with these mutants. For *ggps1*, significant expression changes were determined by comparison with 5-week-old wild-type leaf tissue that was grown and harvested with the mutant. Different wild-type standards were used to eliminate genes from consideration that showed altered expression based on age or tissue type. Lists were filtered to omit genes with a false discovery rate (FDR) below 0.05. A $\log_2$ transformation was performed on all fold-change data.

Analysis of RNA-Sequencing Results

The hypergeometric distribution test was used to assess the likelihood that any overlap between the differentially expressed genes in two mutants is nonrandom. The hypergeometric *P*-value is calculated using the equation $p = ({}_kC_x)\ ({}_{(n-k)}C_{(n-x)})/{}_NC_n$, where N is the number of genes in the genome, k is the number of genes identified in the first sample, n is the number of genes identified in the second sample, x is the number of overlapping genes and ${}_kC_x$ is the number of possible gene combinations.

The online tool g:Profiler was used to search for significantly over-represented GO terms within the gene lists (Reimand et al., 2007). *Arabidopsis thaliana* was set as the reference organism. The default g:SCS algorithm was used to determine the significance threshold and correct for the problem of multiple testing. For these analyses, a transformed *p*-value of 0.05 was established as the cutoff for significantly represented GO terms.

Comparison with Previously Reported Microarray Results

Microarray results for the *sca3* mutant were previously published (Hricová et al., 2006). While raw data were not available, supplementary information included a list of all genes from the original analysis with an absolute fold change greater than ±1.5-fold and a *p*-value less than 0.05. Microarray data from *var2* mutant seedlings (Miura et al., 2010) were obtained from NCBI's Gene Expression Omnibus (GEO Accession GSE18646) and analyzed using the GEO2R tool. Gene expression from white sectors of *var2* leaves was compared to leaves from the control wild-type Col-*0*, also available from GEO as part of the original Miura et al data set. A list of potential candidate genes that may be involved in retrograde signaling in response to alterations of chloroplast performance was obtained from Glaßer et al. (2014).

RESULTS

RNA-Sequencing Data Collection

Three mutants with diverse plastid defects—in osmoregulation and likely ion homeostasis (*msl2msl3,* **Figure 1B**), in plastid division and partitioning (*crl* **Figure 1C**), and in chlorophyll biosynthesis (*ggps1,* **Figure 1D**)—all display patchy, rumpled leaves with notched margins (arrows in **Figures 1B–D**). While the primary defect and the likely mechanisms by which variegation is created are different in the mutants selected for analysis, the secondary leaf morphology defects are at least superficially similar. To determine if these secondary leaf morphology defects were more than superficially similar, and were instead generated by a common signaling pathway triggered by plastid dysfunction, RNA-seq analysis was performed on leaf tissue from *msl2-3 msl3-1* double mutants (referred to as *msl2msl3* hereafter), *crl-3* single mutants (referred to as *crl* hereafter), and albino leaf tissue from *ggps1-1* (referred to as *ggps1* hereafter). The *msl2-3* allele is a T-DNA insertion in the first exon of *MSL2* (At5g10490, (Wilson et al., 2011) and the *msl3-1* allele is a T-DNA insertion in the last exon of *MSL3* (At1g58200, Haswell and Meyerowitz, 2006). A previously undescribed *CRL* mutant allele(At5g51020), named here *crl-3*, is a T-DNA insertion in the first exon of the gene. *CRL* transcript levels downstream of the T-DNA insertion site were similar to those observed in wild-type plants; however, no transcript spanning the T-DNA insertion site was detected (**Figures 1E,F**). These data suggest that *crl-3* mutant plants are unable to produce a functional full-length protein, either as a result of the incorporation of T-DNA sequences into the coding portion of the mRNA and/or truncation of wild type N-terminal sequences. The *crl-3* mutant is small, with crumpled leaves and enlarged chloroplasts (**Figures 1C,H**), as shown for other *crl* mutant alleles (Asano et al., 2004; Šimková et al., 2012). The *ggps1-1* allele contains a missense point mutation in a conserved aspartic acid of GGPS1 (encoded by At4g36810), resulting in a temperature-sensitive phenotype (Ruppel et al., 2013).

RNA was collected from three biological replicates of each mutant and co-cultivated wild-type control plants. Between 43

and 63 million read pairs were mapped to the Arabidopsis reference genome for each genotype and its appropriate control using STAR (Dobin et al., 2013). For each genotype, this represented at least 94% of the total reads uniquely mapping, greater than 21,000 genes identified, and at least 13,000 genes with five counts per million per replicate. The ribosomal fraction was less than 0.25%. Gene-level transcript abundance and differential expression was determined by edgeR (Robinson et al., 2010).

Significant expression changes in *msl2msl3* and *crl* backgrounds were determined by comparison to the 3-week-old wild type controls grown and harvested with these mutants. In the *msl2msl3* mutants 4777 genes with an FDR less than 0.05 were identified. The *crl* mutant showed differential expression of 3499 genes. For *ggps1*, significant expression changes were determined by comparison with 5-week-old wild-type leaf tissue that was grown and harvested with the mutant. Different wild-type standards were used for *ggps1* to enhance downstream comparisons with *msl2msl3* and *crl*, eliminating genes from consideration that showed altered regulation based on age or tissue type. In the *ggps1-1* white tissue, 8121 genes with an FDR less than 0.05 were identified. The larger number of differentially regulated genes discovered in *ggps1* when compared to the other mutants may be a result of the greater severity of the albino phenotype (**Figure 1D**).

In agreement with a role for MSL2 and MSL3 in maintaining plastid ion homeostasis, 148 out of the 2247 (6.6%) differentially regulated genes exhibiting a $\log_2$ fold change (LFC) of 1.17 (2.25-fold) or more were associated with the GO terms "transition metal ion transport" and "ion transport" ($p < 0.01$; marked in green in Supplementary Data Sheet 1). For further analysis, an LFC of ± 1.65 was implemented as a cutoff. We chose this LFC cut-off because it corresponds to a fold change of approximately 3.1, a level of differential expression that is likely to be physiologically relevant. This resulted in 931 up-regulated and 520 down-regulated genes in the *msl2msl3* double mutant, 670 up-regulated and 211 down-regulated genes in *crl*, and 1117 up-regulated and 1266 down-regulated genes in *ggps1-1* white tissue, all compared to their respective wild type (**Figures 2A,B**; these gene lists are provided in Supplementary Data Sheet 1).

Our Selected Mutants Show Distinct Transcriptomic Profiles

Differentially expressed genes from each mutant were compared and a hypergeometric test performed for genes identified in each

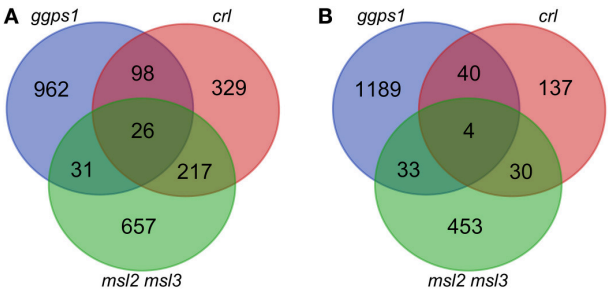

FIGURE 2 | Overlap of differentially expressed genes in *msl2msl3*, *crl*, and *ggps1* mutants. Lists of up-regulated genes **(A)** or down-regulated genes **(B)** were compared for the three mutants. Venn diagrams include all genes with an FDR below 0.05 and an absolute LFC greater than 1.65.

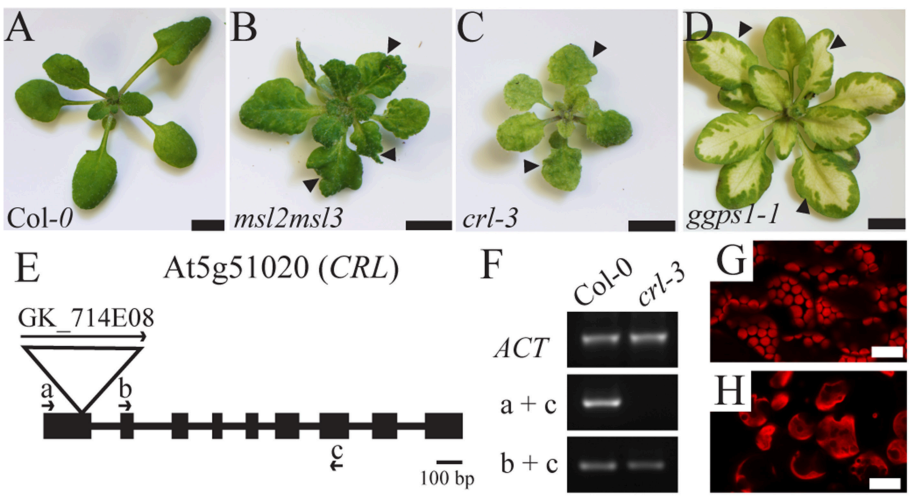

FIGURE 1 | Aerial phenotypes of *msl2msl3*, *crl*, and *ggps1* mutants and characterization of *crl-3* allele. Images of 3-week-old Col-*0* **(A)**, *msl2msl3* **(B)**, *crl* **(C)**, and 5-week-old *ggps1* **(D)** mutants. Arrows in **(B–D)** indicate notched leaf margins. Scale bar = 5 mm. Schematic of the *CRL* gene. The T-DNA insertion in *crl-3* is indicated by a triangle. According to GABI-KAT, the insertion site is 134 bp downstream of the *CRL* start codon, near the end of exon 1 **(E)**. Boxes and lines indicate exons and introns, respectively. Arrows indicate the location of primers used in **(F)**. RT-PCR on cDNA derived from Col-*0* and *crl-3* seedlings **(F)**. No *CRL* transcript was detected using primer pairs spanning the T-DNA insertion site in the *crl-3* mutant background. Confocal micrographs of mesophyll chloroplasts of Col-*0* **(G)** and *crl-3* **(H)** rosette leaves. Scale bar = 20 μm.

sample in a pair-wise fashion to reveal potential relationships between transcriptomic profiles of individual mutants (**Table 1**). The hypergeometric distribution test is a statistical measure of the probability that the number of genes differentially expressed in two different mutants could arise by chance, given the number of possible genes. Most pairwise comparisons had a hypergeometric distribution *p*-value of less than 0.01, suggesting that the similarities in the gene expression patterns of *msl2msl3* and *crl* (or *crl* and *ggps1)* are unlikely to have occurred randomly. The one exception was comparison between *msl2msl3* and *ggps1*. These mutants showed very little overlap in either up-regulated or down-regulated genes and both hypergeometric distribution *p*-values were greater than 0.01. This suggests that *msl2msl3* and *ggps1* respond to plastid dysfunction with distinct genetic responses, while the other pairs of mutants may share pathways or pathway components.

We next attempted to identify a common set of differentially expressed genes present in all samples. Twenty-six genes were identified as up-regulated in leaf tissue from *msl2msl3* double mutants, *crl-3* mutants, and dissected white tissue from *ggps1-1* seedlings (**Table 2** and **Figure 2A**). Surprisingly, none of the protein products from these genes are predicted to localize to plastids (Ashburner et al., 2000). Six of these proteins are predicted to reside within mitochondria and five to the plasma membrane. Two of the plasma membrane proteins, At3g28600 and At5g45570, are predicted to function as hydrolases, and two others are potential receptors, an S-locus receptor kinase that may bind mannose and a Leucine Rich Repeat receptor-like protein. At3g16450 is another potential sugar sensor and a predicted nuclear protein. Five proteins from this group are also predicted to reside within the cell wall, and may play a role in cell expansion. Finally, seven of the 26 proteins in this set are predicted to localize to the nucleus and alter transcription.

Venn analysis also revealed four genes that were down-regulated in all three mutants (**Table 3** and **Figure 2B**). The product of one of these, PUMILLO9, is localized to the cytoplasm and involved in mRNA stability (Francischini and Quaggio, 2009). Two others potentially have enzymatic activity; At3g45940 encodes a glycosyl hydrolyase that is found in the apoplast and At1g12010 encodes a cytoplasmic oxidase (Ashburner et al., 2000).

The Overlapping Gene List for All Three Mutants Does Not, Though Some Pairwise Lists Do, Contain Statistically Over-Represented Go Terms

While the 30 genes differentially expressed in *msl2msl3, crl*, and *ggps1* mutants are candidates for a shared response pathway, their differential expression does not guarantee that they are involved in the manifestation of the rumpled leaf phenotype. To determine if the two sets of overlapping differentially expressed genes share specific biological pathways or functions, the Gene Ontology (GO) terms associated with each were analyzed with g:Profiler (Reimand et al., 2007). This tool analyzes the GO terms associated with each gene in a list and determines if any specific biological processes, cellular locations, or molecular functions are represented in the list beyond what is expected by chance. Analysis of both the up-regulated and the down-regulated genes did not reveal significant enrichment for any specific GO terms. This suggests that these genes are unlikely to work in a coordinated manner. However, it is also possible that the genes are working together in an undescribed pathway that is not reflected in the GO terms.

We also performed GO-term analysis of differentially regulated genes found in just two genotypes (Supplementary Data Sheet 2). Lists of differentially regulated genes found in both *ggps1* and *crl* showed significant ($p < 0.05$) representation of several GO terms indicating secondary metabolic processes, immune and defense response, peptide-methionine (R)-S-oxide reductase activity, and responses to external and biotic stimuli, other organisms, stress and iron starvation. Analysis of genes differentially regulated in both *msl2msl3* and *crl* showed significant ($p \leq 0.05$) over-representation of genes involved in S-glycoside catabolic process, nitrile biosynthetic process, and biotin transport and metabolism. There were no significantly over-represented GO terms in the overlapping genes from *msl2msl3* and *ggps1*.

Including Gene Expression Data from *var2* and *sca3* Does Not Reveal a Core Set of Differentially Expressed Genes

If the set of genes that are differentially expressed in the *msl2msl3, crl,* and *ggps1* mutants represent a core set of genes responsible

TABLE 1 | Pairwise hypergeometric probability test shows a statistically significant overlap in differentially expressed genes between *msl2msl3* and *crl* and between *crl* and *ggps1*.

Change compared to the wild type	Mutant 1 (Number of genes)	Mutant 2 (Number of genes)	Number of genes altered in both Mutant 1 and Mutant 2	Hypergeometric probability
Up-regulated	*msl2msl3* (931)	*crl* (670)	243	$4.48\ e^{-55}$
Up-regulated	*msl2msl3* (931)	*ggps1* (1117)	57	0.95
Up-regulated	*crl* (670)	*ggps1* (1117)	124	$6.88\ e^{-44}$
Down-regulated	*msl2msl3* (520)	*crl* (211)	34	$1.27\ e^{-20}$
Down-regulated	*msl2msl3* (520)	*ggps1*(1266)	37	0.021
Down-regulated	*crl* (211)	*ggps1* (1266)	44	$2.99\ e^{-16}$

Probability of the observed overlap based on size of the differentially expressed gene list for each (in parentheses) was calculated. Gray shading indicates significance with a cutoff of 0.01.

TABLE 2 | Twenty-six genes were up-regulated in *msl2msl3, crl,* and *ggps1.*

Gene	Description	*msl2msl3*		*crl*		*ggps1*	
		LFC	FDR	LFC	crl FDR	LFC	FDR
AT1G19610*	Pathogen-related defensin-like protein	2.49	5.1 e^{-08}	3.71	3.3 e^{-16}	3.23	9.1 e^{-21}
AT2G02990	*RNS1,* Ribonuclease 1	2.37	8.6 e^{-07}	4.20	1.2 e^{-20}	1.86	1.7 e^{-03}
AT5G14180	*MPL1, Myzus persicae*-induced lipase 1 involved in lipid metabolism	2.11	1.8 e^{-04}	3.42	3.5 e^{-11}	5.89	6.1 e^{-30}
AT5G40990*	*GLIP1,* GDSL lipase 1	4.04	1.4 e^{-03}	5.28	8.1 e^{-06}	5.48	4.2 e^{-03}
AT3G20370	TRAF-like family protein	2.12	7.8 e^{-07}	1.83	3.4 e^{-05}	4.41	3.2 e^{-03}
AT1G21310*	*ATEXT3,* Extensin 3	2.22	4.6 e^{-21}	3.13	2.2 e^{-38}	2.06	2.1 e^{-05}
AT3G01600*	*ANAC044,* NAC domain containing DNA binding protein 44	3.91	7.7 e^{-32}	2.94	3.1 e^{-17}	2.62	1.7 e^{-02}
AT5G61890	Encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family	3.54	1.2 e^{-07}	2.43	3.2 e^{-03}	3.35	2.6 e^{-06}
AT5G18270*	*ANAC087,* NAC domain containing DNA binding protein 87	3.07	1.1 e^{-34}	2.91	2.3 e^{-31}	1.70	2.0 e^{-05}
AT5G46590	*ANAC096,* NAC domain containing DNA binding protein 96	2.77	5.8 e^{-08}	1.90	1.0 e^{-03}	1.72	4.9 e^{-02}
AT2G38250*	Homeodomain-like superfamily DNA binding protein	2.54	1.4 e^{-14}	2.38	1.4 e^{-12}	2.45	6.5 e^{-08}
AT5G13330	*Rap2.6L*, Encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family	2.51	4.7 e^{-15}	1.95	8.9 e^{-09}	4.04	1.7 e^{-18}
AT3G16450	Mannose-binding lectin superfamily protein	2.22	1.6 e^{-07}	2.74	7.1 e^{-11}	2.61	3.7 e^{-02}
AT3G06490	*MYB108,* Putative transcription factor myb domain protein 108	1.70	3.9 e^{-02}	1.76	4.2 e^{-02}	1.89	3.4 e^{-06}
AT4G29200	Beta-galactosidase related protein	9.54	7.9 e^{-34}	9.10	9.6 e^{-28}	5.30	5.6 e^{-03}
AT4G04030	*ATOFP9,* Ovate family protein 9	6.05	1.3 e^{-04}	5.16	6.0 e^{-03}	5.21	6.2 e^{-03}
AT5G15360	unknown protein	5.93	9.1 e^{-12}	4.83	1.3 e^{-06}	7.34	8.6 e^{-01}
AT5G03090	BEST *Arabidopsis thaliana* protein match is: mto 1 responding down 1	4.90	1.5 e^{-06}	3.64	6.6 e^{-03}	8.03	3.8 e^{-15}
AT3G01345	Expressed protein. InterPro DOMAIN/s: Glycoside hydrolase, family 35 (InterPro:IPR001944)	3.45	2.2 e^{-07}	1.99	1.9 e^{-02}	4.07	1.5 e^{-07}
AT1G65500#	Unknown protein	1.74	9.0 e^{-11}	2.14	1.2 e^{-15}	1.78	5.5 e^{-05}
AT5G45570	Ulp1 protease family protein	4.66	2.5 e^{-02}	4.56	4.4 e^{-02}	4.20	3.9 e^{-04}
AT2G32660*	*ATRLP22,* Receptor like protein 22 with Leucine Rich Repeat	3.52	1.8 e^{-02}	3.83	1.2 e^{-02}	1.79	4.9 e^{-03}
AT1G61440	S-locus lectin protein kinase family protein	5.40	1.5 e^{-28}	1.72	4.4 e^{-02}	4.52	3.5 e^{-02}
AT3G28600	P-loop containing nucleoside triphosphate hydrolases superfamily protein	4.27	9.0 e^{-04}	3.46	1.9 e^{-02}	4.66	1.9 e^{-06}
AT3G02810	Protein kinase superfamily protein	4.23	2.5 e^{-10}	4.74	3.1 e^{-13}	1.84	3.4 e^{-02}
AT4G08093	Pseudogene of unknown protein	7.18	8.7 e^{-46}	3.24	1.7 e^{-06}	2.75	4.3 e^{-04}

#Indicates gene is also up-regulated in sca3.
**Indicates gene is also up-regulated in var2.*
Color indicates predicted subcellular localization: blue, cell wall; yellow, nucleus; red, mitochondria; and orange, plasma membrane. No color indicates no prediction.

TABLE 3 | Four genes were down-regulated in *msl2msl3, crl,* and *ggps1.*

Gene	Description	*msl2msl3*		*Crl*		*ggps1*	
		LFC	FDR	LFC	FDR	LFC	FDR
AT3G45940	*XYL2*, Glycosyl hydrolases family 31 protein	−2.97	5.1 e^{-09}	−3.25	3.72 e^{-04}	−2.28	2.5 e^{-03}
AT1G67260	*TCP1*, TCP family transcription factor	−6.83	9.8 e^{-04}	−3.16	9.6 e^{-07}	−3.21	1.1 e^{-08}
AT1G35730	*APUM9*, Encodes a member of the Arabidopsis Pumilio (APUM) proteins containing PUF domain. PUF proteins regulate both mRNA stability and translation through sequence-specific binding to the 3′ UTR of target mRNA transcripts	−3.23	2.1 e^{-05}	−2.99	4.4 e^{-04}	−3.10	1.3 e^{-04}
AT1G12010	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	−2.99	1.5 e^{-17}	−3.19	3.3 e^{-11}	−7.67	1.2 e^{-25}

Shading indicates predicted subcellular localization: blue, cell wall; yellow, nucleus; brown, cytoplasm.

for signaling in response to plastid dysfunction to produce the notched-leaf phenotype, it would be expected that other mutants with wavy leaf margins would also show altered expression of these genes. To test this, we compared our RNA-seq data for *msl2msl3, crl,* and *ggps1* (with LFC $\geq$ 1.65 and FDR $<$ 0.05) to previously published microarray expression data comparing

sca3 to wild type (Hricová et al., 2006) and *var2* to wild type (Miura et al., 2010). The differentially expressed gene list for *var2* was generated by comparing raw microarray data from *var2* white leaf tissue to wild-type leaf tissue (both from Miura et al., 2010), and filtering for LFC $\geq$ 1.65 and an adjusted $p < 0.05$. The differentially expressed gene list for *sca3* leaf tissue was obtained from the supplemental information in Hricová et al. (2006) which contained a list of differentially expressed genes with a fold-change of 1.5 or higher, and $p < 0.05$.

Lists of up-regulated or down-regulated genes from *var2* and *sca3* were compared with those from *msl2msl3, crl,* and *ggps1*. When we used the LFC 1.65 cutoff for all but *sca3* (for which the only data available was a cutoff of 1.5 or greater), there were no genes over-expressed or under-expressed in all five mutants (**Figures 3A,C**). In fact, most of the genes differentially expressed in *sca3* (83.5% of up-regulated, and 77% of down-regulated genes) do not overlap with any other mutant. Conversely, the genes differentially expressed in *var2* overlap substantially with the other mutants—only 33.4% of up-regulated and 51.5% of down-regulated genes are only detected in the *var2* dataset.

Of the original 26 genes identified as over-expressed in *msl2msl3, crl,* and *ggps1,* seven (indicated with asterisks in **Table 2**) are also differentially expressed in *var2*. These include three cell wall-localized proteins and four potential transcription factors. At1g65500, the only gene over-expressed in *msl2msl3, crl, ggps1,* and *sca3* (indicated with a pound sign in **Table 2**), encodes an unknown protein. Individual comparisons between *var2* and other single mutants show the most overlap with *ggps1*, and this likely reflects the larger number of up-regulated genes found in that genotype (**Figure 3**). Two of the four genes identified as down-regulated in *msl2msl3, crl,* and *ggps1* are also down-regulated in the *var2* mutant. These include the cell wall-localized glycosyl hydrolyase encoded by At345940 and the oxidase encoded by At1g12010 (**Table 3**).

To ensure that relevant pathway genes were not excluded due to overly stringent fold-change cutoffs, this entire analysis was repeated using an absolute LFC of $\pm$1.17 or greater and an FDR below 0.05 (**Figures 3B,D**). While this expanded the set of genes included in the comparisons, changing these parameters still did not result in the identification of any genes that were up-regulated or down-regulated in all five mutants. Thus, the absence of a common set of differentially expressed genes cannot be attributed solely to overly stringent LFC requirements.

The List of Differentially Expressed Genes Common to *msl2msl3*, *crl*, and *ggps1* Mutants Does Not Overlap with a Previously Identified Proposed Retrograde Signaling Pathway

Mayer and colleagues performed a meta-analysis of microarray experiments on tissue from plants with genetically or pharmacologically altered chloroplast performance (Glaßer et al., 2014). They identified 39 genes as part of a core plastid retrograde signaling pathway that involves auxin, reactive oxygen species, the phytohormone abscisic acid, and sugar signaling. To determine if any of the mutants in our study showed a similar transcript profile, we examined the 39 genes in the proposed core retrograde signaling pathway in our lists of differentially expressed genes from *msl2msl3*, *crl*, and *ggps1* tissue. Of the 39 genes, 29 were differentially expressed (FDR less than 0.05) in *ggps1* white tissue, 14 in *msl2msl3* double mutants, and 13 in *crl* (Supplementary Table 1). However, only four of these genes, (At5g19120, At2g17880, At3g62950, and At2g18050), were differentially expressed in all three mutant backgrounds we analyzed, and none of the four were differentially expressed in the same direction. An aspartyl protease, a chaperone DnaJ domain protein and a thioredoxin were up-regulated in *ggps1,* but down-regulated in *crl* and *msl2msl3*, while histone H1-3 showed the opposite pattern. None of these genes has been previously identified as a participant in ion transport or plastid homeostasis. It is therefore unlikely that the 39 genes identified as part of a plastid retrograde signaling pathway are involved in the leaf architecture phenotypes seen in these mutants.

DISCUSSION

Understanding the Underlying Source of Pleiotropic Phenotypes in Plastid Dysfunction Mutants

In an effort to identify genes that may be responsible for the leaf architecture phenotypes in the *msl2msl3* double mutant, we performed RNA-seq on three mutants exhibiting similar leaf architecture defects, and then compared those results with each other and with publically available microarray data from two additional mutants. For our analysis we chose genes representing three physiologically distinct pathways to defective leaf morphology: *msl2msl3*, implicated in plastid ion homeostasis; *crl,* involved in chloroplast division; and *ggps1,* required for chlorophyll synthesis (**Figure 1**). We then compared the transcript profile of each mutant with the goal of identifying a core set of co-regulated genes (**Figure 2** and **Tables 1–3**). Because of the biochemical differences underlying the secondary leaf phenotypes, any genes found to show similar gene expression patterns in all mutants would make intriguing candidates for participation in a plastid dysfunction-initiated response pathway. Given that MSL2 and MSL3 are likely to function as ion channels, this approach also could identify potential plastid ion homeostasis signaling pathways.

Thirty Differentially Expressed Genes are Found in Common Between the *msl2msl3*, *crl*, and *ggps1* Mutants

Comparison of differentially expressed genes in these mutants revealed that 26 genes were up-regulated in *msl2msl3, crl* and *ggps1*, while four were down-regulated (**Figure 2**). The presence of an individual gene in all three differentially expressed gene lists is evidence that it is involved in the structural leaf phenotypes observed in these mutants. If a retrograde signal from a plastid to the nucleus were responsible for changes in leaf architecture, an early response to this signal would necessarily involve the activation/deactivation of transcription factors. Indeed, 7 of the 30 genes differentially expressed in

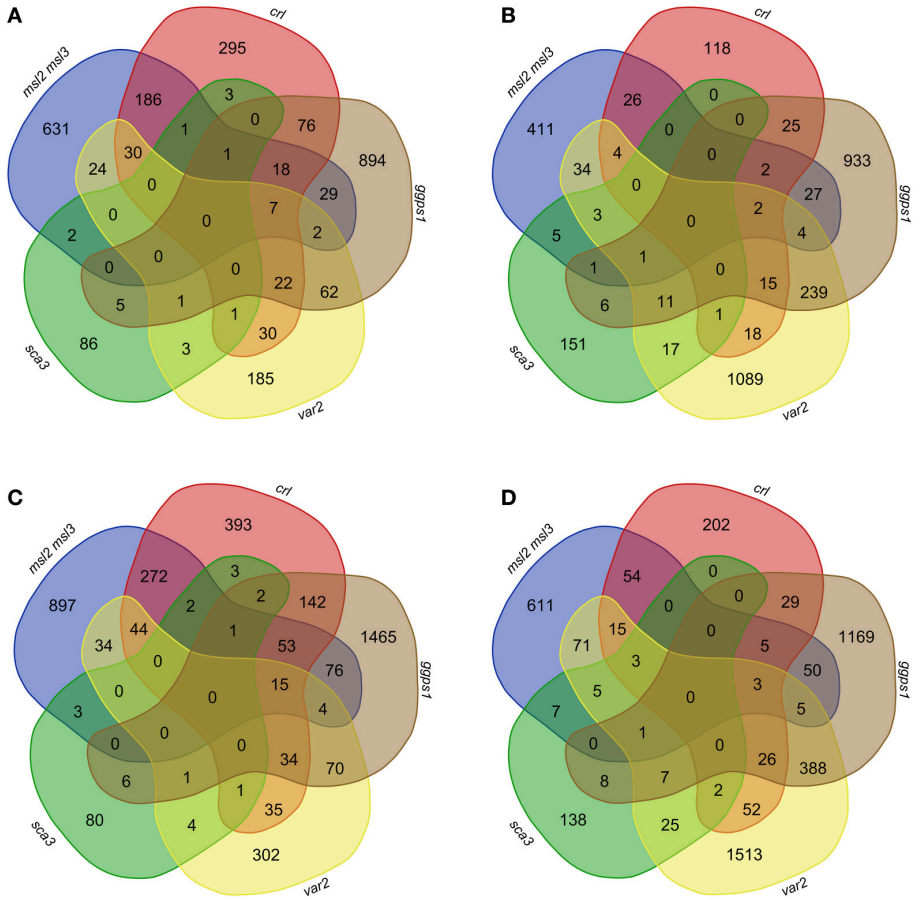

FIGURE 3 | Overlap of differentially expressed genes in *msl2msl3, crl, ggps1, sca3,* and *var2* mutants. Gene lists for each mutant were filtered by significance, removing anything with an FDR > 0.05. Up-regulated genes with an LFC ≥ 1.65 **(A)** and 1.17 **(C)** were compared in *msl2msl3, crl, ggps1, sca3,* and *var2*. Comparisons were also made with genes down-regulated with an absolute LFC ≥ −1.65 **(B)** and −1.17 **(D)**. No genes were found to overlap in all samples.

msl2msl3, crl and *ggps1* are predicted transcription factors (**Table 2**). Two Ethylene Response Factor (ERF) family members are attractive candidates for components of a retrograde plastid pathway, due to their previously documented involvement in plant stress (reviewed in Wang et al., 2013). We also identified three uncharacterized members of the plant-specific NAC domain-containing transcription factor family. This large family of over 100 genes has been implicated in many developmental and abiotic stress responses, including dehydration (Tran et al., 2004), salt tolerance (Hu et al., 2006), hypoxia (Christianson et al., 2009), secondary cell wall synthesis (Zhong et al., 2006), and separation of adjacent organs (Mallory et al., 2004). These three NAC transcription factors could be involved in response to plastid dysfunction or the developmental alterations producing the lobed leaves. Another candidate gene that could impact the expression of other proteins is the down-regulated APUM9. This conserved family of proteins has been shown to repress translation of mRNA by binding to a conserved sequence within the 3′ UTR (reviewed in Abbasi et al., 2011). Related proteins in Arabidopsis have been shown to regulate the translation of proteins required for stem cell population maintenance, such as WUSCHEL, CLAVATA-1, and ZWILLE (Francischini and Quaggio, 2009). Disrupting a similar pathway within the leaf primordia could potentially lead to architectural anomalies such as rumpled or scalloped margins.

Another intriguing group of genes identified by the analysis shown in **Figure 2** are those predicted to be localized to the cell wall. Because the direction and magnitude of plant cell expansion is determined by cellulose patterning and rigidity of the cell wall (Sugimoto et al., 2000), it is possible that this group of proteins could be responsible for wavy leaves or irregular leaf margins. Two of these genes are putative hydrolases, and two are putative lipases; their upregulation could alter cell wall extensibility, leading to structural defects during leaf expansion.

Lack of Evidence for a Global Plastid Dysfunction Response Pathway

As alluring as these ideas are, it would be possible to construct seemingly logical connections for almost any set of genes.

GO terms associated with the genes differentially expressed in *msl2msl3, crl,* and *ggps1* were analyzed for statistically over-representation but no significant enrichment for any GO term was identified. This indicates that these genes are not likely to be working together in a previously identified, coordinated biological process. It does not eliminate the possibility that some are involved in a potential retrograde signaling pathway, but their presence on this list is not sufficient to draw conclusions. We additionally note that it would require a high amount of coordinated activity within the sample to identify significant pathways using GO term analysis with such a small number of genes.

If any of the 30 co-regulated genes we identified through RNA-seq are part of a plastid dysfunction-induced response pathway, they would be expected to show similar patterns of regulation in all mutants that display altered leaf morphology. However, comparison of our RNA-seq datasets to publically available microarray data sets from the *var2* and *sca3* mutants did not support this hypothesis (**Tables 2, 3**, **Figure 3**). The *sca3* differentially expressed gene dataset contained one of the up-regulated genes and none of the down-regulated genes. The *var2* differentially expressed gene dataset contained seven of the up-regulated genes and two of the down-regulated genes from the set of 30 we identified by RNA-seq. However, no genes were found in all five datasets, even after dropping cutoffs to LFC of 1.7, corresponding to a fold-change of approximately 2.25. This suggests that there is no single molecular pathway that is responsible for the common leaf morphology phenotypes observed in our mutants.

There are several possible explanations as to why we were unable to isolate a plastid-initiated signaling pathway. First, it is possible that a single pathway does not exist. While it is appealing to propose that the similar set of pleiotropic phenotypes in the *msl2msl3*, *crl1*, and *ggps1* mutants should trace back to a single pathway, different types of organelle dysfunction may lead to distinct patterns of gene regulation and signaling cascades that produce phenotypic effects that appear superficially similar. In fact, retrograde signaling pathways have already been divided into those that exert "biogenic" or developmental control, and those that exert "operational" control to optimize plastid function in response to changing environmental conditions (Pogson et al., 2008). The disruption of the isoprenoid biosynthesis pathway leading to impaired chlorophyll biosynthesis in the *ggps1* mutant would likely fall into the first category. The inability of *msl2msl3* plastids to maintain an osmotic balance across the plastid membrane is more likely to trigger operational control pathways. The specific biochemical function of CRL has yet to be determined, allowing us to speculate that a lesion in this gene could lead to plastid dysfunctions that activate both biogenic and operational retrograde signaling pathways. For example, it is possible that *crl* and *msl2msl3* share a defect in plastid division while *crl* and *ggps1* might share the presence of cells completely devoid of plastids. This framework may explain the observed overlap in differential gene expression between *crl* and *msl2msl3* and *crl* and *ggps1* as well as the lack of overlap between *msl2msl3* and *ggps1*.

The proposal that no global transcriptional response to plastid dysfunction exists is further supported by comparison of our results with the 39 plastid retrograde signaling module genes reported by Glaßer et al. (2014). None of these genes showed up-regulation or down-regulation in all three of the mutants in our analysis (Supplementary Table 1). The *ggps1* mutant was the best match, with hits on 29 of the 39 genes. This is unsurprising because the interrupted molecular pathway in *ggps1*, isoprenoid biosynthesis—and by extension, chlorophyll production—is the closest match to the chloroplast biogenesis and maintenance perturbations previously examined (inhibition of tetrapyrrole synthesis, transition of low light-adapted plants to high light, inhibition of photoassimilate export, and inhibition of redox potential). The *msl2msl3* double mutant and *crl* had 14 and 13 overlapping differentially expressed genes, respectively, but no gene was differentially expressed in the same direction in all datasets. These results are indicative of a distinct mechanism for individual types of plastid dysfunction, rather than a shared molecular pathway.

Alternatively, it is possible that a common retrograde plastid dysfunction response pathway does indeed exist, but operates in a manner that does not lend itself to discovery via transcriptome analysis. Cellular components required for the leaf morphology phenotypes could be activated through post-translational modifications, either directly or through degradation of an inhibitor. Translation of pathway components may also be regulated, with little change in the amount of mRNA present. Alternatively, the temporal regulation of these signals may make them difficult to identify, with transient spikes in mRNA followed by stable proteins. It is also possible that the secondary leaf phenotypes are a result of signaling occurring in the leaf primordia. This type of system could lead to modified leaf architecture without related transcriptional changes in mature leaves.

It is also possible that microarray analysis has insufficient resolution to identify components of this pathway. While the transcriptomic data generated here utilized RNA-seq, we relied on previously published microarray results for *var2* and *sca3* to complete our analysis. The fundamental output from these two techniques is similar (a fold-change value with an indicator of statistical confidence for each differentially regulated gene) and allows easy comparison between experiments. This approach has been used previously (Kapushesky et al., 2012; Bhargava et al., 2013; Toepel et al., 2013). A drawback of this method is that the final conclusions are only as strong as the weakest dataset. In this case, it is possible that the microarray results incorporated into our meta-analysis did not contain the necessary resolution.

CONCLUSIONS

Here we analyzed a molecular phenotype—global mRNA sequence—in several mutant lines with superficially similar leaf and morphological defects. We hoped both to understand the basis of these phenotypes and to gain insight into the genetic pathways leading from disrupted ion homeostasis, chloroplast division, or chlorophyll biosynthesis to disrupted

leaf development. We were unable to find any commonalities between the genes differentially expressed in these mutants and in other mutant transcriptomes, and conclude that either there is no common plastid-to-nucleus signaling pathway in these mutants, or it is subtle and cannot be detected through transcriptomic methods. These data show that the interaction between organelle dysfunction and resulting defects in plant leaf shape is complex and may occur through several routes, and establishes that the link between defective plastid ion homeostasis and altered leaf development shares only a few common components with other plastid dysfunction response pathways. The availability of these transcriptomic data will provide a valuable tool for the future study of organelle dysfunction, ion homeostasis, and variegation.

RNA SEQUENCING DATA

Raw data from the analyses presented here have been deposited into the NCBI SRA under the accession SRP065605.

AUTHOR CONTRIBUTIONS

DL, MW, and EH designed and interpreted experiments; DL and MW conducted experiments and analyzed the data; DL, MW, and EH wrote and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01148

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The *Arabidopsis* Thylakoid Chloride Channel AtCLCe Functions in Chloride Homeostasis and Regulation of Photosynthetic Electron Transport

Andrei Herdean[1], Hugues Nziengui[1†], Ottó Zsiros[2†], Katalin Solymosi[3†], Győző Garab[2], Björn Lundin[1] and Cornelia Spetea[1]*

[1] Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden, [2] Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary, [3] Department of Plant Anatomy, Eötvös Loránd University, Budapest, Hungary

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****Correspondence:***
Cornelia Spetea
cornelia.spetea.wiklund@bioenv.gu.se

† These authors have contributed equally to this work.

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Chloride ions can be translocated across cell membranes through Cl^- channels or Cl^-/H^+ exchangers. The thylakoid-located member of the Cl^- channel CLC family in *Arabidopsis thaliana* (AtCLCe) was hypothesized to play a role in photosynthetic regulation based on the initial photosynthetic characterization of *clce* mutant lines. The reduced nitrate content of *Arabidopsis clce* mutants suggested a role in regulation of plant nitrate homeostasis. In this study, we aimed to further investigate the role of AtCLCe in the regulation of ion homeostasis and photosynthetic processes in the thylakoid membrane. We report that the size and composition of proton motive force were mildly altered in two independent *Arabidopsis clce* mutant lines. Most pronounced effects in the *clce* mutants were observed on the photosynthetic electron transport of dark-adapted plants, based on the altered shape and associated parameters of the polyphasic *OJIP* kinetics of chlorophyll *a* fluorescence induction. Other alterations were found in the kinetics of state transition and in the macro-organization of photosystem II supercomplexes, as indicated by circular dichroism measurements. Pre-treatment with KCl but not with KNO_3 restored the wild-type photosynthetic phenotype. Analyses by transmission electron microscopy revealed a bow-like arrangement of the thylakoid network and a large thylakoid-free stromal region in chloroplast sections from the dark-adapted *clce* plants. Based on these data, we propose that AtCLCe functions in Cl^- homeostasis after transition from light to dark, which affects chloroplast ultrastructure and regulation of photosynthetic electron transport.

Keywords: *Arabidopsis thaliana*, CLC channel, chlorophyll fluorescence, electron microscopy, photosynthetic electron transport, proton motive force, state transition, thylakoid membrane

Abbreviations: CD, circular dichroism; Chl, chlorophyll; CLC, chloride channel; Cyt, cytochrome; F_v/F_m, maximum quantum yield of photosystem II photochemistry; FR, far red; LHC, light harvesting complex; $\Delta\Psi$, membrane potential; NPQ, non-photochemical quenching; P700, primary donor of photosystem I; PC, plastocyanin; PI, performance index; ΔpH, pH gradient; $g_H{}^+$, proton conductivity through ATP synthase; $v_H{}^+$, proton flux; PMF, proton motive force; PS, photosystem; Φ(II), PSII efficiency; ST, state transition; TEM, transmission electron microscopy.

INTRODUCTION

Photosynthesis is essential for life on Earth. A key element of photosynthesis is conversion of sunlight energy into organic carbon via the generation of a membrane electrochemical potential gradient for protons (H^+), also known as the proton motive force (PMF). To generate PMF, pigments (chlorophylls and carotenoids) bound to proteins in light harvesting complexes (LHCs) absorb photons and transfer their excitation energy to the reaction centers of photosystems (PS). Here the excitation is converted into charge separation, which drives electron transport from PSII to PSI via the cytochrome b_6f (Cyt b_6f) complex. The net result of this process is the oxidation of water molecules (oxygen evolution) and the reduction of $NADP^+$, which is associated with translocation of H^+ into the thylakoid lumen. A light-driven cyclic electron transport around PSI and Cyt b_6f is also operative, which does not evolve oxygen, nor induce $NADP^+$ reduction but only contributes to H^+ translocation. It is the water oxidation and photosynthetic electron transport-coupled H^+ translocation into the lumen that generates PMF, which is composed of transmembrane H^+ concentration (ΔpH) and electrical potential ($\Delta\Psi$) gradients. Both PMF components can drive ATP synthesis, whereas it is thought that only the ΔpH component can activate the photoprotective PsbS- and xanthophyll cycle-dependent components of non-photochemical quenching (*NPQ*) while down-regulating the electron transport during the step of plastoquinol oxidation at Cyt b_6f complex (Kramer et al., 2003). The role of $\Delta\Psi$ in regulation of photosynthesis is less clear, but there is recent evidence in *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) that ion channels, such as the thylakoid K^+ channel TPK3, partially dissipate $\Delta\Psi$ to allow more H^+ to enter the lumen and thus a significant ΔpH to be formed, balancing photoprotection and photochemical efficiency (Carraretto et al., 2013).

Potassium, chloride, magnesium, and calcium are the major ions in thylakoids, and changes in their homeostasis are expected to impact membrane architecture, protein conformation, and electron transport rates (Anderson et al., 2012; Pribil et al., 2014; Finazzi et al., 2015; Pottosin and Shabala, 2015). Evidence for fluxes of these ions across the spinach thylakoid membrane accompanying the inward movement of H^+ during electron transport reactions was provided already by Hind et al. (1974). The proteins involved in K^+ fluxes have been recently characterized in *Arabidopsis* (Carraretto et al., 2013; Armbruster et al., 2014; Kunz et al., 2014). Voltage-dependent chloride channel activities in the thylakoid membrane have been reported in *Peperomia metallica* (Schönknecht et al., 1988) and in the alga *Nitellopsis obtusa* (Pottosin and Schönknecht, 1995), but thus far the proteins responsible for those activities have not been identified.

In plants, three gene families for Cl^- transport have been described thus far, namely slow-anion channels (SLAC), aluminum-activated malate transporters (ALMT), and Cl^- channels (CLCs; Barbier-Brygoo et al., 2011). The *Arabidopsis* genome codes for seven CLC members (AtCLCa to AtCLCg), localized in various intracellular membrane compartments, and thought to be either Cl^- channels or Cl^-/H^+ exchangers. Nevertheless, several of them have been also reviewed as nitrate transporters (Krapp et al., 2014). More specifically, AtCLCa and AtCLCb are tonoplast-located $2NO_3^-/1H^+$ antiporters (De Angeli et al., 2006; von der Fecht-Bartenbach et al., 2010). AtCLCc and AtCLCg are also located in the tonoplast, AtCLCd, and AtCLCf in the Golgi apparatus, and AtCLCe in the thylakoid membrane (Teardo et al., 2005; Marmagne et al., 2007; von der Fecht-Bartenbach et al., 2007; Lv et al., 2009). The selectivity and mechanism of anion transport for these five AtCLCs are unknown (Barbier-Brygoo et al., 2011).

Various physiological functions have been proposed for AtCLCs, based on the phenotypic characterization of corresponding *Arabidopsis* knockout mutants. AtCLCa, AtCLCb, and AtCLCe are required to maintain normal cellular NO_3^- levels (De Angeli et al., 2007; von der Fecht-Bartenbach et al., 2010), and in addition AtCLCe may regulate the photosynthetic activity of thylakoids (Marmagne et al., 2007). AtCLCc participates in both NO_3^- and Cl^- homeostasis, and regulates stomatal movement and salt tolerance (Jossier et al., 2010). AtCLCg is also involved in salt tolerance by altering Cl^- homeostasis in mesophyll cells (Nguyen et al., 2015). AtCLCd has been proposed to regulate lumenal pH in the trans-Golgi network (von der Fecht-Bartenbach et al., 2007), and to act as a negative regulator of plant innate immunity (Guo et al., 2014). The physiological function of AtCLCf is still unknown.

In this study, we addressed the question about the physiological role of AtCLCe in the thylakoid membrane. We show that AtCLCe loss-of-function mutation modifies the arrangement of thylakoid network in chloroplasts and alters photosynthetic electron transport following transfer from light to dark.

MATERIALS AND METHODS

Plant Growth Conditions

A. thaliana cv. Columbia (Col-0) plants and two *clce* mutants in the same background were grown in soil for 7–8 weeks in a growth chamber (CLF PlantMaster, Plant Climatics, Wertingen, Germany) using 8-h-light (120 μmol photons m^{-2} s^{-1})/16-h-dark cycles at 22°C and 70% relative humidity. The SALK_010237 *(clce-2)* and SALK_21945C (*clce-3)* mutants were obtained from the Arabidopsis Biological Resource Center (ABRC, https://www.arabidopsis.org/abrc/). The *clce-2* line was previously characterized by Marmagne et al. (2007).

RNA Isolation and RT-PCR

Total RNA was isolated from rosette leaves of 7-8-week old plants using TriZol reagent (Invitrogen), treated with RNase-free DNAse (Thermo Scientific) to prevent DNA contamination and then purified using HiBind RNA mini columns (Omega Bio-Tek) according to the manufacturer's instructions. cDNA was synthesized using 1 μg of total RNA through iScript cDNA synthesis Kit (Bio-Rad). Finally, 2 μL of reverse transcription reaction were used as template to amplify *AtCLCe* and *β-ATPase* cDNA fragments using Dream Taq DNA Polymerase Kit (Thermo Scientific). The following primers were used for

the *AtCLCe* (*At4g35440*): *forward* TCCAAGTGTTGAAATTGG AGC and *reverse* AGGTGTAACAGTCCATGGCAC, and for mitochondrial ATP synthase β-subunit *(At5g08680)* selected as reference gene: *forward* GATCATGACATCTCTCGAGG and *reverse* TGGTAAGGAGCAAGGAGATC.

Determination of Leaf Chlorophyll (Chl) Content

Chl content was determined from leaf discs after extraction in 96% (*v/v*) ethanol at 65°C for 10 min followed by spectrophotometry (Lichtenthaler and Wellburn, 1983). The Chl content was expressed per leaf area and per fresh weight.

Electrochromic Band Shift (ECS) Measurements

ECS measurements were carried out using a Pulse Amplitude Modulated Chl fluorometer (Dual PAM-100, Walz, Effeltrich, Germany) equipped with a P515/535 module (Schreiber and Klughammer, 2008). Leaves of 30 min dark-adapted plants were illuminated with actinic red light at 100 or 650 μmol photons m^{-2} s^{-1} for 2, 5, or 10 min. After each illumination period, the light was switched off and the dark interval relaxation kinetics (DIRK) of the ECS signal were recorded for 60 s according to Cruz et al. (2001) to estimate PMF size (ECS_t) and relative contribution of ΔpH and ΔΨ to PMF. Before each PMF measurement, a saturating single turnover 5-μs flash of 200,000 μmol photons m^{-2} s^{-1} was applied to determine ECS_{ST}, which was used to normalize ECS_t.

For determination of H^+ conductivity of the thylakoid membrane mainly through ATP synthase ($g_H{}^+$), the leaves were exposed to light at 650 μmol photons m^{-2} s^{-1} for 10 min. At specific time points, the light was switched off to record the ECS signal decay during 600-ms dark intervals. The $g_H{}^+$ parameter was calculated as 1/time constant for decay derived from single exponential fittings of the ECS decay during the first 100 ms (Cruz et al., 2005). The steady state proton flux ($v_H{}^+$) was calculated as $g_H{}^+ * ECS_t/ECS_{ST}$ (Cruz et al., 2005).

Kinetics of Chl *a* Fluorescence Induction

Fast Chl *a* fluorescence induction (*OJIP*) kinetics were recorded using a Plant Efficiency Analyser (Handy-PEA, Hansatech, King's Lynn, Norfolk, UK) by applying saturating red actinic light (635 nm, 3500 μmol photons m^{-2} s^{-1}, 1 s) on plants during dark adaptation intervals of 1–15 min. Where indicated, recorded data points were double normalized to minimum (F_0) and maximum (F_m) fluorescence. The time to reach F_m (t_{Fm} in ms), the maximum quantum yield of PSII (F_v/F_m), the performance index (*PI*), variable fluorescence yield at *J* step (V_J), variable fluorescence yield at *I* step (V_I), and the turnover number of Q_A (*N*) were calculated using Hansatech PEA Plus v1.10 software according to Strasser et al. (2004). Where indicated, detached leaves were incubated in 150 mM KCl or KNO_3 for 30 min in growth light (120 μmol photons m^{-2} s^{-1}) followed by adaptation in darkness for 15 min before the *OJIP* measurements.

Slow Chl *a* fluorescence induction and recovery kinetics were recorded using the Dual PAM-100 instrument (Walz) on 30-min dark-adapted plants using red actinic light of 100 or 650 μmol photons m^{-2} s^{-1} for 10 min followed by 5 min in darkness. The non-photochemical quenching (*NPQ*) and quantum yield of PSII [Φ (*II*)] were calculated using the following equations: $\Phi(II)=(F_m' - F)/F_m'$, where F_m' is defined as the fluorescence value at the plateau level reached during application of a saturating pulse, and *F* is defined as the fluorescence level during illumination averaged for 0.2 s before applying the saturating pulse; $NPQ=(F_m - F_m')/F_m'$, where F_m is the fluorescence value at the plateau level reached during application of a saturating pulse on the 30 min dark-adapted leaf before the onset of illumination.

P700 Oxidation-Reduction Kinetics

To monitor the oxidation-reduction kinetics of PSI, absorbance changes at 830 nm (reflecting the redox state of P700, i.e., PSI primary donor) were recorded using the Dual PAM-100 (Walz) instrument. The 830-nm transmittance was subtracted from the simultaneous recording at 875 nm, and calibrated according to the Dual PAM-100 built-in routine, and finally displayed as P700 $\Delta I/I^*10^3$. Before the measurement, plants were adapted to light (120 μmol photons m^{-2} s^{-1}, 1 h). Oxidation-reduction kinetics were recorded by applying saturating red actinic light pulses (635 nm, 20,000 μmol photons m^{-2} s^{-1}, 200 ms) together with far red (FR) light (730 nm) during the illumination period and after 1, 2, 3, 4, and 5 min of dark adaptation. Half of each dark-adaptation interval (30 s) was done in the presence of FR light to fully oxidize P700 before applying the saturation pulse.

State Transition (ST) Kinetics

ST measurements were carried out according to Lunde et al. (2000) on 30 min dark-adapted plants using the Dual PAM-100 (Walz) instrument. For determination of F_m values in the dark and in either state 1 or state 2, a saturating pulse of red actinic light (5000 μmol photons m^{-2} s^{-1}, 800 ms) was applied. To induce state 2, leaves were illuminated for 15 min with red actinic light (100 μmol photons m^{-2} s^{-1}; "state 2 light"). For transition to state 1, leaves were exposed for 15 min to red light supplemented with FR light ("state 1 light"). The *qT* parameter was calculated as $(F_{m2} - F_{m3})/F_{m2}$, where F_{m2} is the fluorescence value at the plateau level reached during application of the saturating pulse after 15 min illumination with "state 1 light" and F_{m3} after 15 min illumination with "state 2 light." The *qS* parameter was calculated as $[(F_{I'}-F_I)-(F_{II'}-F_{II})]/(F_{I'}-F_I)$ according to Damkjaer et al. (2009), where F_I and F_{II} are the steady-state fluorescence levels in the presence of FR light in state 1 and state 2, respectively, and $F_{I'}$ and $F_{II'}$ are the steady-state fluorescence levels in the absence of FR light in state 1 and state 2, respectively.

Circular Dichroism (CD) Spectroscopy

CD measurements were carried out on a J-815 spectropolarimeter (JASCO, Tokyo, Japan). Detached, water-infiltrated leaves were placed between two glass slides in an optical cell. Spectra were recorded at room temperature between 400 and 800 nm at a scan speed of 100 nm min^{-1}, band-pass of 3 nm and step size of 1 nm. For each sample, 3–4 scans

were averaged. Spectra were normalized to the absorption of the red-most peak of the spectra recorded at the same time as the CD spectra and were corrected for baseline distortions. Measurements were repeated on three different leaves for each genotype. Amplitudes of psi-type CD bands, at around (+)505, (−)675, and (+)690 nm, were determined using the reference wavelengths of 550, 600, and 750 nm, respectively.

Transmission Electron Microscopy (TEM)

Sections (∼2 × 2 mm) cut from the central parts of leaf blades of 7-week-old plants were fixed in 2.5% (*v/v*) glutaraldehyde for 4 days, then post-fixed in 1% OsO_4 (*w/v*) for 2 h. Fixatives were buffered with 70 mM Na_2HPO_4-KH_2PO_4 (pH 7.2). After fixation, samples were rinsed in the same buffer. After dehydration in an alcohol series, samples were embedded in Durcupan ACM resin (Fluka, Buchs, Switzerland). Ultrathin sections (thickness 70 nm) were cut with a Reichert Jung Ultracut E microtome (Reichert-Jung AG, Vienna, Austria), mounted on copper grids and contrasted with 5% uranyl acetate and Reynolds' lead citrate solution. The sections were visualized with a Hitachi 7100 TEM microscope at 75 kV accelerating voltage.

ImageJ software was used to measure granum diameter (at the middle of perpendicular granum sections) on the micrographs. Calculations were done on 210-350 randomly chosen grana originating from 35 different chloroplasts taken randomly from 35 different mesophyll cells per treatment.

Statistical Analyses

Each mean is given ± SEM for at least 5 plants. The data sets were compared between genotypes by one-way ANOVA using OriginPro 8 for Windows. Significant differences were considered at $P < 0.05$.

RESULTS

Phenotype of *clce* Mutants

To investigate the role of AtCLCe in the thylakoid membrane, we have characterized the phenotype of two *Arabidopsis* T-DNA insertion lines in Col-0 background. The *clce-2* mutant was initially characterized by Marmagne et al. (2007). We have identified an additional T-DNA insertion line (*clce-3*) in the same background (**Figure 1A**). Both genotypes lacked the *AtCLCe* transcript, as revealed by RT-PCR analyses (**Figure 1B**). Neither line displayed obvious growth differences from wildtype (**Figure 1C**) and no statistically significant differences in Chl content, Chl *a/b* ratio or specific weight of leaves were obtained (**Table 1**).

Next we compared the photosynthetic performance of dark-adapted plants based on Chl *a* fluorescence induction parameters. The F_v/F_m parameter used as an indicator of the maximum quantum yield of PSII was found slightly but significantly lower in 15 min dark-adapted *clce* mutants than in wildtype due to a significantly lower F_m (**Table 1**). Another Chl fluorescence parameter used as indicator of the overall plant vitality, is the performance index (*PI*) of dark-adapted plants (Strasser et al., 2000). *PI* was found significantly reduced in the *clce* mutants, suggesting that they experience stress during dark-adaptation. These data indicate that AtCLCe loss-of-function mutation lowers photosynthetic performance in dark-adapted plants.

Proton Motive Force and Proton Flux

To determine if PMF partitioning into ΔpH and ΔΨ has been altered in the *clce* mutants as compared to wildtype, leaves were illuminated for 10 min at either 100 or 650 μmol photons m^{-2} s^{-1} and the DIRK of ECS were recorded according to Cruz et al. (2001). In the mutants, a mild but significant increase in ΔΨ was observed at both light intensities, and consequently a decrease in ΔpH occurred (**Figure 2** and Supplementary Figure 1A). This suggests a minor alteration by the AtCLCe loss-of-function in the ion distribution across the thylakoid membrane during illumination. It is of note that differences in PMF partitioning were also observed at 5 min but not after shorter illumination at 650 μmol photons m^{-2} s^{-1} (Supplementary Figure 1B).

Next we investigated the effect of AtCLCe loss-of-function mutation on ATP synthase activity by measuring the thylakoid membrane total PMF (ECS_t), conductivity to H^+ ($g_H{}^+$), and the H^+ flux through ATP synthase ($\nu_H{}^+$) during exposure for 10 min to 650 μmol photons m^{-2} s^{-1}. Both mutant lines displayed a mild but significant increase in all three parameters

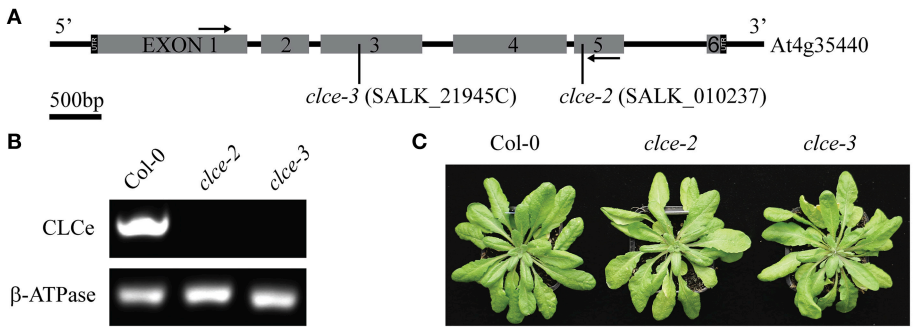

FIGURE 1 | Genotyping of *clce* mutants used in this study. (A) *AtCLCe* gene structure with indicated location of T-DNA insert in each of the two *clce* lines. Black arrows indicate location of primers used for RT-PCR. **(B)** RT-PCR of total RNA from wild-type (Col-0) and *clce* leaves with primers specific for the *AtCLCe* gene and primers for a reference gene as the positive control. **(C)** Representative photos of 7-week-old plants.

TABLE 1 | Chlorophyll (Chl) content and photosynthetic performance of dark-adapted plants.

Genotype	Col-0	*clce-2*	*clce-3*
mg Chl cm^{-2}	0.024 ± 0.001	0.022 ± 0.001	0.022 ± 0.001
mg Chl g^{-1} fresh weight	1.335 ± 0.075	1.232 ± 0.073	1.248 ± 0.043
mg fresh weight cm^{-2}	18.100 ± 0.693	17.670 ± 0.403	17.490 ± 0.429
Chl *a/b*	4.077 ± 0.042	4.065 ± 0.051	4.027 ± 0.029
F_0	486 ± 5	500 ± 10	518 ± 6
F_m	2624 ± 21	2442 ± 2*	2504 ± 18*
F_v/F_m	0.814 ± 0.001	0.795 ± 0.001*	0.795 ± 0.001*
PI	2.503 ± 0.044	2.037 ± 0.042*	2.105 ± 0.036*
V_J	0.368 ± 0.002	0.341 ± 0.002*	0.337 ± 0.001*
V_I	0.750 ± 0.007	0.598 ± 0.003*	0.610 ± 0.007*
t_{Fm} (ms)	338 ± 43	600 ± 49*	580 ± 43*
N	59.493 ± 1.742	127.314 ± 1.633*	122.545 ± 3.991*

The wild-type (Col-0) plants and clce mutants were grown for 7 weeks using a 16 h dark/8 h light (120 μmol photons m^{-2} s^{-1}) photoperiod. Leaf Chl content and a/b ratio were determined from leaf discs of 16-h dark-adapted plants following extraction in ethanol and spectrophotometry. Fast kinetics of Chl a fluorescence were recorded on 15 min dark-adapted plants with Handy-PEA. The following parameters were calculated with the JIP test: the minimum chlorophyll fluorescence (F_0), maximum fluorescence (F_m), the maximum quantum yield (F_v/F_m), the performance index (PI), the relative variable fluorescence yield at 2 ms (V_J) and at 30 ms (V_I), the time to reach F_m (t_{Fm}), and the turnover number of Q_A(N). The data are means ± SEM (n = 5 plants). Asterisks indicate significant difference in the studied parameters between Col-0 and the clce mutants (ANOVA, $P < 0.05$).

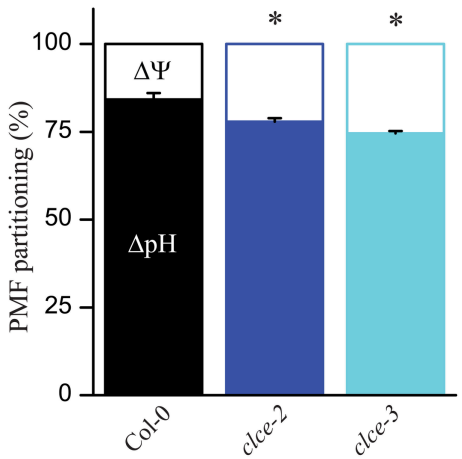

FIGURE 2 | Composition of the proton motive force (PMF). Wild-type and mutant plants were dark-adapted for 30 min and then illuminated for 10 min with 650 μmol photons m^{-2} s^{-1}. Following illumination, kinetics of dark relaxation of the electrochromic shift (ECS) signal were recorded and deconvoluted to determine membrane pH gradient (ΔpH) and membrane potential (ΔΨ) components. The plotted data are means ± SEM ($n = 8$–9 plants). Asterisks indicate statistically significant differences according to ANOVA ($P < 0.05$).

throughout the illumination time (**Figures 3A–C**), suggesting enhanced thylakoid membrane H^+ conductivity. Since the H^+ flux is known to linearly correlate with the rate of linear electron flow (Takizawa et al., 2008), the absence of AtCLCe appeared to have a beneficial effect on electron transfer rate in thylakoids during illumination.

Photoprotection and PSII Efficiency

The ΔpH component of PMF regulates PSII efficiency [Φ (*II*)] and *NPQ* induction with respect to its fast energy-dependent and slow zeaxanthin-dependent quenching components (Nilkens et al., 2010; Ruban et al., 2012). Based on the lower contribution of ΔpH to PMF (**Figure 2**), we expected a higher Φ (*II*) and lower *NPQ* in the mutants as compared to wildtype. Instead, we observed a significantly higher steady-state *NPQ* level in *clce* after about 10 min of illumination at 650 and 100 μmol photons m^{-2} s^{-1} (**Figures 4A,B**). The Φ (*II*) parameter did not significantly differ between wildtype and mutants during the same set of measurements (**Figures 4C,D**). *NPQ* relaxation during the subsequent dark phase after either 650 or 100 μmol photons m^{-2} s^{-1} was significantly slower, whereas Φ (*II*) was consistently lower without reaching wild-type levels (**Figures 4A–D**). The observed effects of AtCLCe loss-of-function mutation on *NPQ* during illumination may have other cause than the altered relative contribution of ΔpH to the PMF. The lower Φ (*II*) after transition from light to dark is in line with the lower F_v/F_m and *PI* of dark-adapted plants (**Table 1**).

Fast Chl Fluorescence Induction and P700 Oxidation-Reduction Kinetics

The fast kinetics of Chl *a* fluorescence induction (*OJIP*) display a polyphasic shape and provide information about the electron transport reactions in the thylakoid membrane (Strasser et al., 2000). The shape of the recorded *OJIP* kinetics was found altered in 15-min dark-adapted *clce* mutants (**Figure 5A**), confirming previous observations (Marmagne et al., 2007). The mutants displayed lower F_v/F_m values due to lower fluorescence level at the *P* step (F_m, **Table 1**). The differences in fluorescence levels appeared already at the *J* step and become greater at the *I* step (**Figure 5A**), corresponding to V_J and V_I parameters (**Table 1**). The curves were double normalized to *J* and *P*, and the curve difference (ΔF_{JP}) between wildtype and *clce* mutants was plotted (**Figure 5A** inset). The resulting peak corresponds to the *I* step, which has been associated with the redox state of the donor and acceptor side of PSI (Schansker et al., 2005).

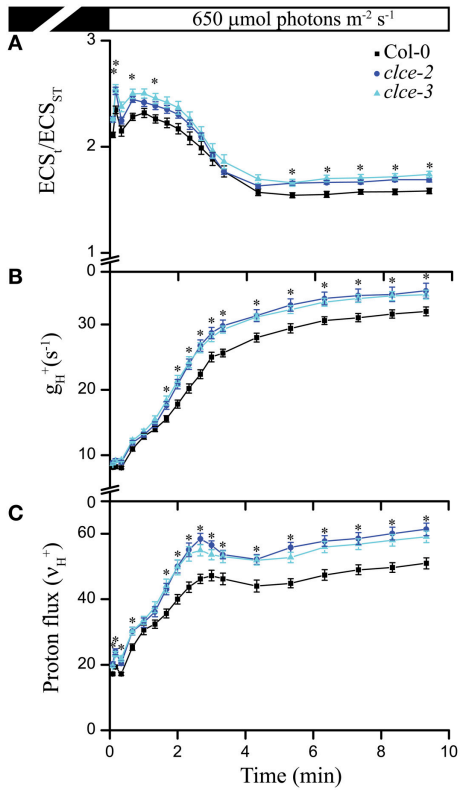

FIGURE 3 | Proton motive force, H^+ conductivity through ATP synthase, and H^+ flux. Electrochromic shift measurements (ECS) were performed on 30 min dark-adapted wild-type and mutant plants. **(A)** Total proton motive force (ECS_t normalized to ECS_{ST}) induction kinetics during illumination at the given light intensity. **(B)** ATP synthase conductivity ($g_H{}^+$), and **(C)** proton flux ($\nu_H{}^+$) were calculated from ECS decay kinetics as described in Section Materials and Methods. The plotted data are means ± SEM (n = 8–9 plants). Asterisks indicate statistically significant differences according to ANOVA ($P < 0.05$).

Marmagne et al. (2007) proposed that the alteration of the *I* peak is due to the disappearance of a rate limiting step in electron transport between the acceptor side of PSII and of PSI. We also found that the time to reach F_m (t_{Fm}) was twice longer in the *clce* mutants than in wildtype (**Table 1**). The *N* parameter for the number of turnovers of PSII primary electron acceptor (Q_A), i.e., number of times Q_A has been reduced in the interval between 0 and t_{Fm}, was twice as high in the mutants (**Table 1**). This is in line with the lower fluorescence at the *J* step, which is also related to Q_A reduction (Strasser et al., 2000).

Interestingly, pre-treatment of leaves with 150 mM KCl in the light followed by 15 min dark-adaptation resulted in similar *OJIP* kinetics in wildtype and mutants (**Figure 5B** and inset). Moreover, pre-treatment with KNO_3 did not rescue the phenotype observed in the *clce* mutants (**Figure 5C**). Instead, the treatment induced a shoulder after the *I* peak (**Figure 5C** inset) of unclear origin. The restoration of *OJIP* wild-type shape in the *clce* mutants by KCl treatment suggests that the untreated samples had altered kinetics due to a disturbed Cl^- distribution across thylakoids in dark-adapted plants.

Next we investigated if the formation of the *I* step in wildtype depends on the duration of dark adaptation interval preceding recording. **Figure 6A** shows the complete absence of the *I* peak after 1 min of dark adaptation with only the *J-P* peaks visible. After 2 min in darkness, the *I* step started to become visible, and after 5 min the complete *OJIP* kinetics could be observed (**Figure 6A**). The plotted ΔF_{JP} curve differences relative to 15-min dark adaptation (**Figure 6A** inset) indicate that 5 min in darkness is enough for complete formation of the *I* step in wildtype. The *clce-2* did not develop the *I* step after either 1 min or longer dark adaptation intervals (**Figure 6B** and inset). Pre-treatment with KCl delayed the appearance of the *I* step from 2 (**Figure 6A**) to 5 min in wildtype (**Figure 6C**), without reaching complete formation of the kinetics within the first 5 min (**Figure 6C** inset) relative to the 15 min dark-adapted leaves. These data suggest that formation of the *I* peak requires at least 5 min of dark adaptation in wildtype, possibly to allow for re-arrangement of electron transport components between PSII and PSI. KCl treatment appears to delay the formation of the *I* peak, since it possibly alters Cl^- distribution across thylakoids influencing electron transport.

To test if PSI electron transfer was affected in the *clce-2* mutant, we used the same dark adaptation intervals as in **Figure 6**, and recorded P700 oxidation-reduction kinetics (**Figure 7A**). The reduction of $P700^+$ was found more pronounced in the *clce* mutant as compared to wildtype after 3 min or longer period in darkness (**Figure 7B**). This suggests an accelerated electron transfer at PSI, which strengthens the possibility that this caused the lower fluorescence at the *I* step in **Figure 6A**. Taken together, the *OJIP* and P700 kinetics data indicate that in dark-adapted plants CLCe activity is important for Cl^- homeostasis of chloroplasts, which in turn influences optimal electron transport between PSII and PSI.

State Transition Kinetics

We further investigated the consequences of AtCLCe loss-of-function on the plant ability to adapt to changes in light quality, and distribution of excitation energy between PSII and PSI (i.e., state transition) (Tikkanen et al., 2006). Steady-state Chl fluorescence levels of *clce-2* in "state 2 light" were found almost identical to the levels recorded in "state 1 light" (**Figure 8A**), whereas wild-type plants had a significant difference in fluorescence levels before the change of light from state 2 to state 1. These differences were no longer visible after treatment with KCl (**Figure 8B**). During illumination with "state 1 light," steady-state fluorescence levels reached the same values in wildtype and *clce-2* mutant, however F_m (F_{m2}) was significantly higher in *clce-2*, indicating a relatively larger PSII antenna size under state 1. Nevertheless, the state transition parameter *qT*, indicating the extent of changes in PSII antenna size, was not found significantly different between wildtype (0.116 ± 0.011) and *clce-2* (0.108 ± 0.003) or *clce-3* (0.114 ± 0.007). We additionally analyzed the kinetics for the transition from state 1 to state 2 (**Figures 8A,B** insets), which is known to result in decreased PSII antennae size (Bellafiore et al., 2005). The halftime ($t_{1/2}$) of PSII antenna detachment from PSII during S1 to S2 transition was found significantly lower in the mutant even after

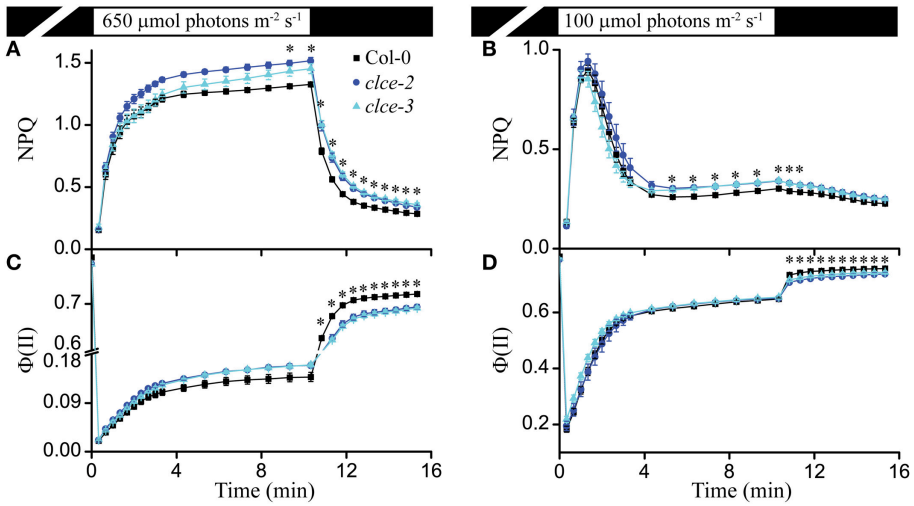

FIGURE 4 | Non-photochemical quenching (*NPQ*) and PSII efficiency [*Φ(II)*]. *NPQ* induction kinetics of wild-type and *clce* plants were recorded during illumination for 10 min at 650 μmol photons m^{-2} s^{-1} **(A)** or 100 μmol photons m^{-2} s^{-1} **(B)** followed by 5 min relaxation in darkness. *Φ* (*II*) from the same set of measurements is plotted in **(C,D)**. The plotted data are means ± SEM ($n = 9$ plants). Asterisks indicate statistically significant differences according to ANOVA ($P < 0.05$).

pre-treatment of the leaves with KCl (**Figure 8C**). Surprisingly, untreated wildtype leaves displayed the same $t_{1/2}$ as KCl-treated *clce-2*. Plant ability to adjust electron transfer to changes in light quality (*qS*) was found 20% higher in *clce-2* relative to wildtype in untreated leaves, whereas after treatment with KCl no significant difference was found between mutant and wildtype leaves, but remained 20% higher than untreated wildtype (**Figure 8D**).

Circular Dichroism Analysis

We recorded CD spectra as a non-invasive method of studying the macro-organization of complexes in the thylakoid membrane (Garab and van Amerongen, 2009). In general, both wildtype and the *clce-2* mutant displayed typical CD spectra, suggesting no major differences in macro-organization of the complexes. Relatively lower values in the +red psi-type (PSI) and +blue PSI CD (**Figure 9**) were observed in the *clce-2* mutant as compared to wildtype, indicating a minor perturbation in the organization of PSII-LHCII macrodomains and/or a smaller domain-size compared to wildtype (Garab et al., 1991; Barzda et al., 1994).

Chloroplast Ultrastructure

Representative TEM images (**Figure 10**) show that in leaves from the 16-h dark-adapted wild-type plants, chloroplasts were half-lens shaped with higher convexity than the elongated and flat chloroplasts observed in the 3-h light-adapted wild-type and *clce* plants. Dark-adapted *clce* plants had a more round shape and peculiar ultrastructural features: often a large thylakoid-free stromal zone was located next to the cell wall, and thylakoids with a bow-like arrangement were situated at the vacuolar side of the chloroplast (**Figure 10**). These features were prominent for ∼75% of *clce* chloroplast sections, while they were also observed on ∼50% of the wild-type chloroplast sections in the dark-adapted samples. However, in the latter the thylakoids had much less distorted, and less typical bow-like appearance, and the thylakoid-free stroma region was also smaller. The chloroplasts of 3-h light-adapted plants showed regular ultrastructure: their thylakoids were flat and the thylakoid network was arranged parallel to the cell wall, and only small thylakoid-free stroma regions were observed in ∼30 and 10% of *clce* and wild-type chloroplast sections, respectively (**Figure 10**).

Detailed analyses of granum structure revealed no statistically significant differences between *clce* and wild-type plants either in the dark- or light-adapted states. The average granum diameters were around 450 and 400 nm in the dark- and in the light-adapted states, respectively. Similarly, the average number of appressed thylakoids per grana (i.e., granum height) was seven in both genotypes, and irrespectively from the light conditions.

There were no apparent differences in the starch contents of *clce* and wild-type chloroplasts in the light. However, assimilatory starch produced in the light almost completely disappeared during the dark-adaptation period in the *clce* mutant since only 50% of the analyzed chloroplast sections contained one small and very thin starch grain. Among the analyzed wild-type chloroplast sections from dark-adapted plants, 95% contained larger and often several starch grains. Taken together, the AtCLCe loss-of-function mutation influences the chloroplast ultrastructure in dark- but not in light-adapted plants.

DISCUSSION

Role of AtCLCe in Partial Depolarization of the Thylakoid Membrane

Cl^- has been long considered to be the major counter-anion during electron transport-coupled H^+ translocation, whose import into the lumen is expected to result in rapid

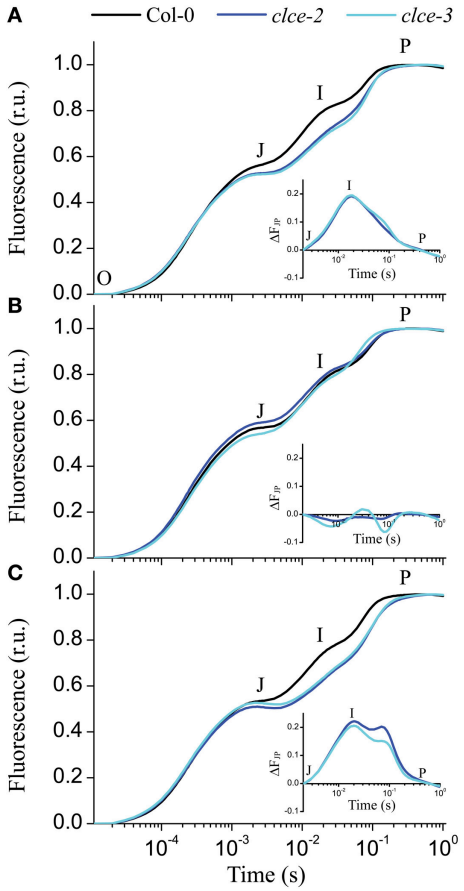

FIGURE 5 | Fast chlorophyll *a* fluorescence *OJIP* transients of untreated and salt-treated leaves. Detached wild-type and mutant leaves that have been incubated in the light for 30 min in water **(A)**, 150 mM KCl **(B)**, or 150 mM KNO_3 **(C)** were removed from the solution and were dark adapted for 15 min before *OJIP* transients were recorded. The transients were double normalized to F_0 and F_m. The time points for the calculation of the *OJIP* parameters are marked: *O*—the fluorescence intensity at 50 μs, *J*—at 2 ms, *I*—at 30 ms, and *P*—the maximum fluorescence intensity. Insets in each plot show the curve difference of double normalized data at *J* and *P* (ΔF_{JP}) between wildtype and each of the *clce* mutants. The plotted data are means ± SEM ($n = 5$ plants).

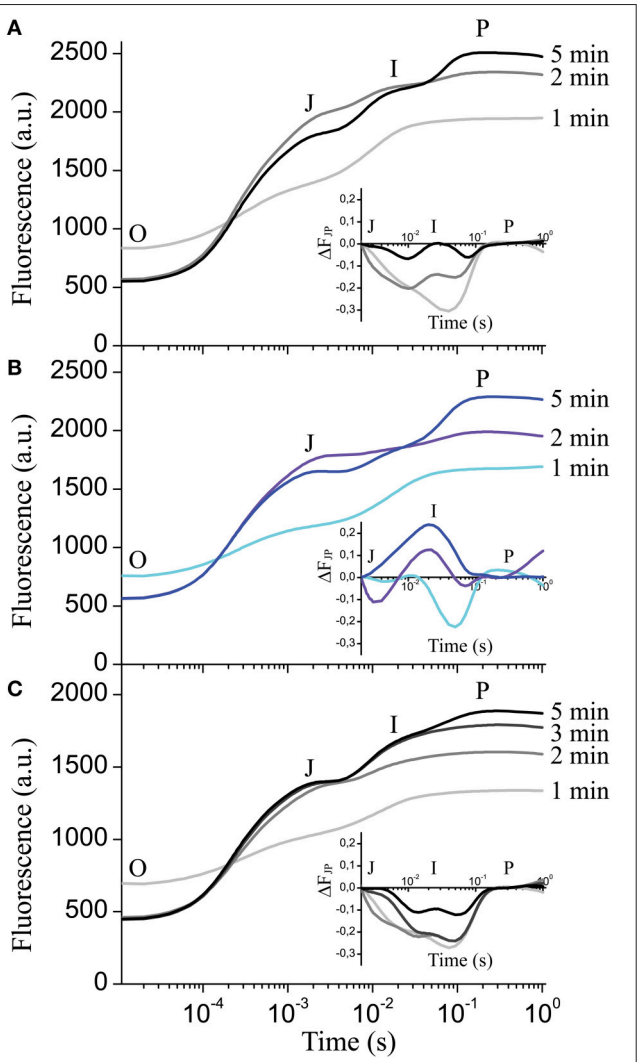

FIGURE 6 | Effect of dark adaptation interval on the fast chlorophyll *a* fluorescence *OJIP* transients. *OJIP* transients were recorded on leaves of wild-type **(A)** and mutant plants **(B)**, following the indicated dark adaptation intervals. Insets show the curve difference (ΔF_{JP}) of double normalized data at *J* (2 ms) and P, between 15 min dark adapted wildtype and 1, 2, and 5 min dark adapted wildtype **(A)** or *clce-2* **(B)**. **(C)** *OJIP* transients were recorded on wild-type leaves pre-incubated with 150 mM KCl in the light followed by the indicated dark adaptation intervals. Inset shows the curve difference (ΔF_{JP}) of double normalized data at *J* and P between 15-min dark-adapted KCl-treated wildtype and 1, 2, 3, and 5-min dark-adapted KCl-treated wildtype. The plotted transients are means ± SEM ($n = 5$ plants).

partial depolarization of the thylakoid membrane (Hind et al., 1974). Cl^- is thought to be imported in the thylakoid lumen immediately after onset of illumination, and exported to the chloroplast stroma during transitions to dark (Hind et al., 1974). Malfunction of either the import or the export mechanism would result in altered Cl^- distribution within the chloroplast, hence altered $\Delta\Psi$ during steady-state photosynthesis. As the only anion channel so far localized to thylakoids, AtCLCe has been hypothesized to be responsible for the partial depolarization of the thylakoid membrane in the light (Finazzi et al., 2015; Pottosin and Dobrovinskaya, 2015). Our data show small but significant increase in $\Delta\Psi$ and total PMF in the *clce* mutants that occurred only at longer illumination time (≥5 min, **Figures 2**, **3A** and Supplementary Figure 1). This supports the hypothesized role of AtCLCe in the partial depolarization of thylakoids, and in addition suggests that activation of AtCLCe may require factors dependent on the light exposure time. H^+ conductivity through ATP synthase ($g_H{}^+$) and proton flux ($\nu_H{}^+$) during illumination were also slightly increased (**Figures 3B,C**). Our data are in agreement with those of Kramer et al. (2003), who proposed that ATP synthase activity is driven by the amplitude of total PMF. Since the effects of the AtCLCe loss-of-function mutation on membrane depolarization and ATP synthase activity are only minor, additional, yet unidentified anion channels must

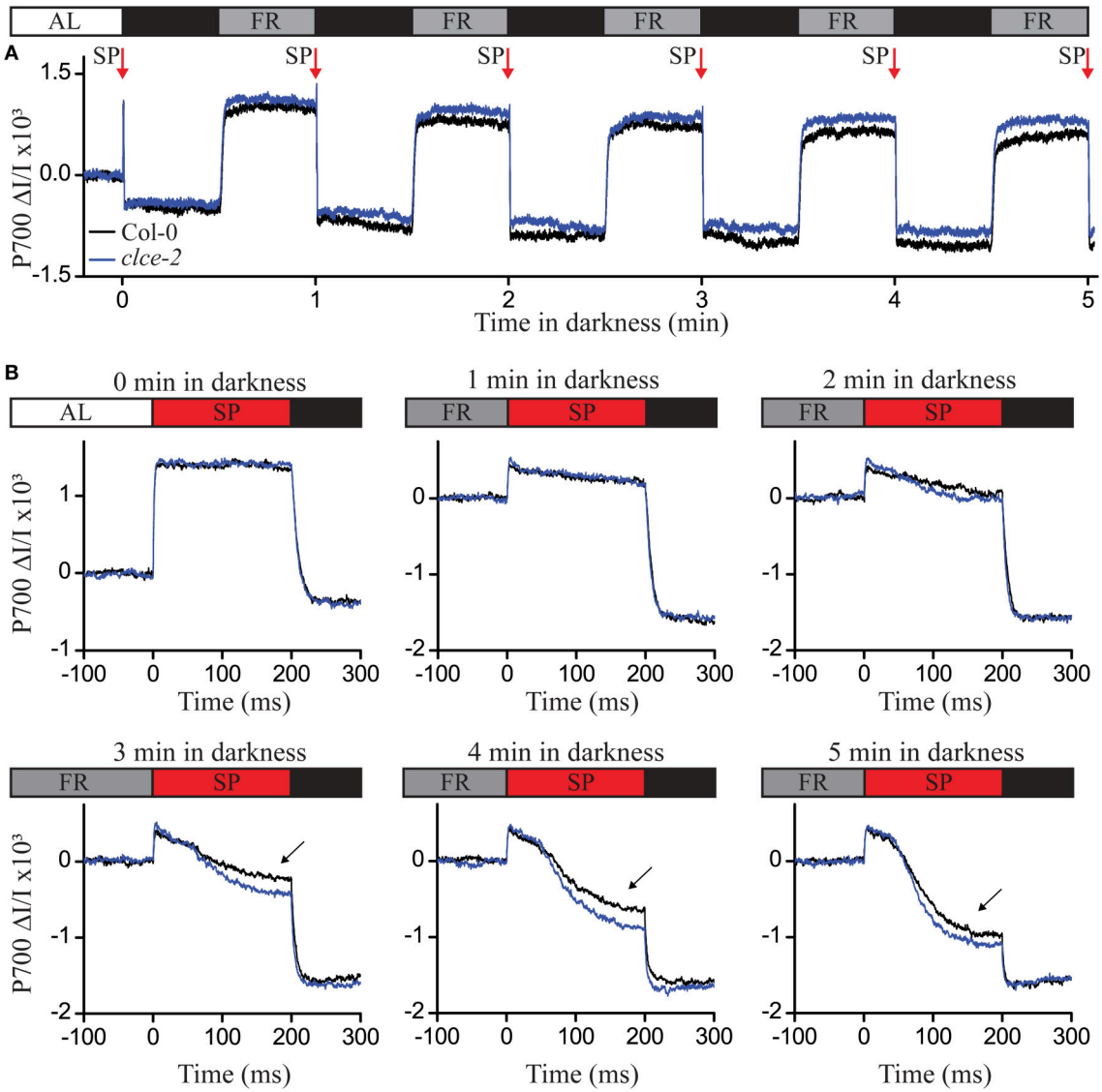

FIGURE 7 | P700 oxidation-reduction kinetics following different dark adaptation intervals. Wild-type and mutant plants have been illuminated with actinic light (AL) of 120 μmol photons m^{-2} s^{-1} for 1 h before recording the P700 signal as difference in transmittance at 875 nm and 830 nm. The steady state P700 signal in the light was recorded for 15 s, followed by 5 cycles of 30 s (indicating the P700 reduced state) and 30 s of far-red (FR) illumination in darkness (indicating the P700 oxidized state) **(A)**. Before AL was switched off, and after each cycle of darkness and FR, a saturation pulse of 200 ms at 20,000 μmol photons m^{-2} s^{-1} (SP red arrow) was applied to record the P700 reduction kinetics **(B)**. The curves were normalized to the initial P700 values in light (**A**,**B**—0 min in darkness) or after the FR illumination **(B)**. Black arrows indicate differences in P700 reduction kinetics after 3, 4, and 5 min in darkness. The plotted data are means of measurements from 5 plants.

contribute to the ion compensation of H^+ uptake into the lumen during illumination.

Our data showing that AtCLCe can regulate $\Delta\Psi$ suggest an electrogenic transport activity, i.e., the protein could work either as a channel or as an exchanger of anion and H^+ at a stoichiometry of at least 2:1, which is the experimentally determined ratio for the algal CLC (Feng et al., 2010). If AtCLCe worked as an anion/H^+ exchanger, then it was expected to play an active role in mediating pH homeostasis or to act as a H^+ leak to reduce lumenal pH. The mechanism of transport is unknown as well as whether there is coordination between the ATP synthase and AtCLCe to generate the required transmembrane pH gradient. Such coordination might be indirect via the same protein regulator (e.g., Batelli et al., 2007).

Role of AtCLCe in Regulation of PSII Efficiency and Photoprotection

Cl^- ions are thought to be important for channeling H^+ from the oxygen-evolving complex of PSII to the thylakoid lumen (Guskov et al., 2009; Umena et al., 2011). Additionally,

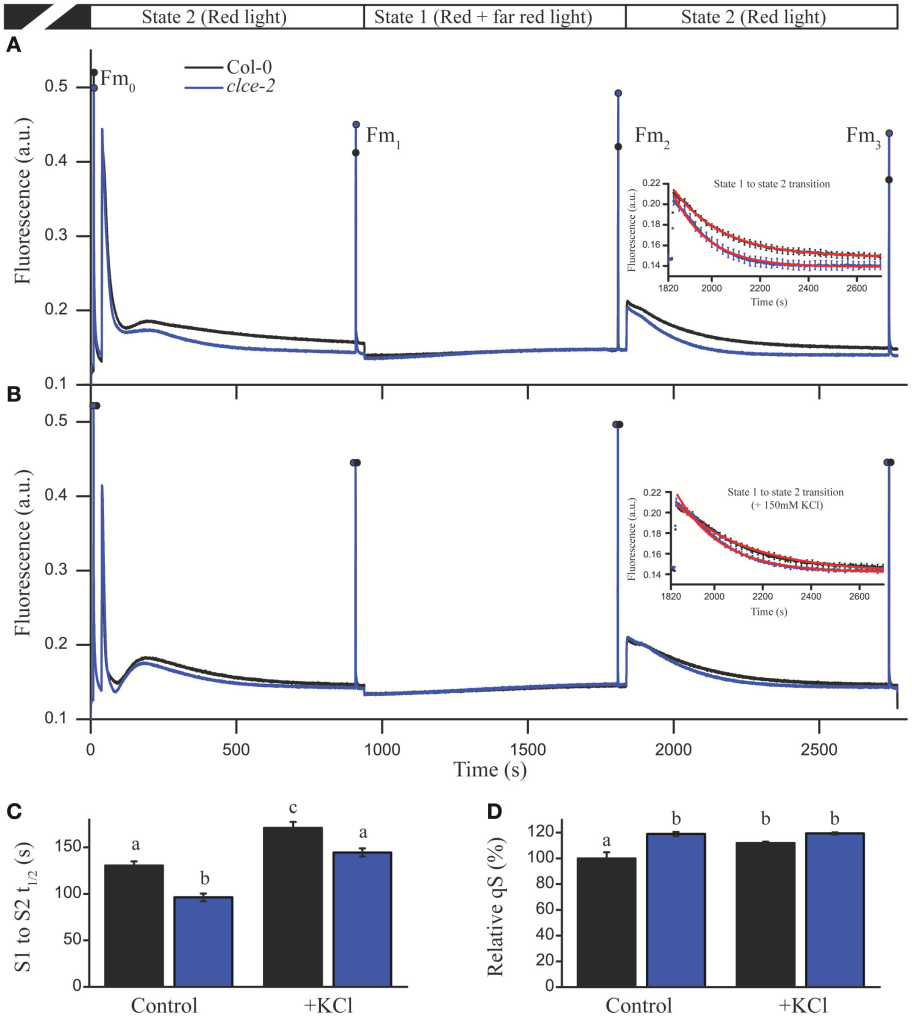

FIGURE 8 | State transition and light harvesting antenna changes in leaves. State transition measurements were performed in untreated **(A)** and KCl-treated **(B)** wild-type and mutant leaves. Insets represent state 1 to state 2 transition kinetics. **(C)** The halftime ($t_{1/2}$) of fluorescence relaxation from S1 to S2 was calculated by fitting an exponential decay function on the fluorescence signal shown in insets. **(D)** Relative values of *qS* parameter indicate the ability of the chloroplast to adjust electron transfer to changes in light quality. The plotted data are means ±SEM ($n = 4$–5 plants). Different letters in panels **(C,D)** indicate statistically significant differences (ANOVA, $P < 0.05$).

in vitro experiments in media depleted of Cl^- showed that PSII particles harbor unstable oxygen-evolving complex (Nash et al., 1985). Under our experimental conditions, PSII efficiency of the *clce* mutants in the light [Φ (*II*)] was found unchanged, and only during the subsequent dark recovery phase was lower relative to wildtype (**Figures 4C,D**). These observations suggest that AtCLCe loss-of-function mutation affects PSII activity during dark adaptation rather than during illumination. The cause behind the observed changes in PSII activity could be the thylakoid ultrastructural changes in dark-adapted plants discussed below. Moreover, the lower F_v/F_m and *PI* parameters in the *clce* mutants (**Table 1**), further indicate an unfavorable organization of the electron transport components in darkness, since these parameters can only be determined correctly after subsequent dark adaptation. The higher steady-state *NPQ* in *clce* (**Figures 4A,B**) cannot be easily explained by the lower ΔpH contribution to PMF partitioning (**Figure 2**), however, other unknown factors related to the ΔΨ component may play a role.

Role of AtCLCe in Regulation of Electron Transport via Re-arrangement of Thylakoid Network

Fast chlorophyll fluorescence (*OJIP*) kinetics is a useful tool to obtain information about functioning of the electron transport chain. The observed decrease in fluorescence levels at the *I* step seen in the *OJIP* kinetics of *clce* mutants (**Figure 5A**, **Table 1**) together with the more pronounced $P700^+$ reduction (**Figure 7**) indicate accelerated electron transfer at PSI (Schansker et al.,

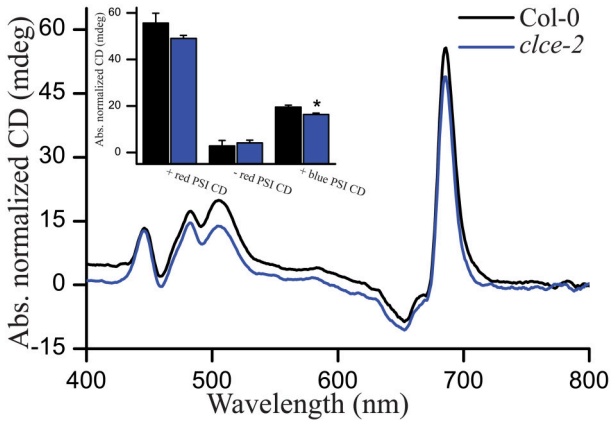

FIGURE 9 | Representative circular dichroism (CD) spectra of H_2O-infiltrated detached leaves. Insets are amplitudes of psi-type CD bands at around (+)505 nm (+blue), (−)675 nm (−red). and (+)690 nm (+red), with reference wavelengths at 550, 600. and 750 nm, respectively. Data are the means ± SEM ($n = 3$ plants). Asterisks indicate significant differences (ANOVA, $P < 0.05$).

2005). The extended time to reach F_m suggests that it takes twice as long in the mutants as compared to wildtype to completely close all PSII centers (**Figure 5A** inset). Moreover, the reduced variable fluorescence at the J (V_J) and I (V_I) steps, as well as the double number of Q_A reduction events (N) (**Table 1**) in the *clce* mutants further suggest an accelerated electron transfer between PSII and PSI as compared to wildtype.

The chloroplast thylakoid lumen undergoes swelling during illumination and shrinkage in darkness (Kirchhoff et al., 2011; Yoshioka-Nishimura et al., 2014). This is thought to be driven mainly by Cl^- influx into the thylakoid lumen in the light and efflux in darkness (Kirchhoff, 2013). Expansion of the lumen is necessary for efficient electron transport between Cyt b_6f and PSI via the soluble protein plastocyanin (PC), whose diffusion was proposed to depend on physical space. Additionally, lumen swelling is important for PSII repair (Kirchhoff et al., 2011) and state transition (Chuartzman et al., 2008). Based on this knowledge and our data, we hypothesized that the *clce* mutants may retain partially swollen thylakoids even after dark adaptation. A swollen lumen would allow for increased mobility of PC, facilitating electron transport from Cyt b_6f to PSI (Kirchhoff et al., 2011). However, no differences in shrinkage/swelling of the thylakoid lumen or significant alterations in granum height and diameter could be resolved by TEM between *clce* and wild-type plants (**Figure 10**). The grana and stroma thylakoid network of wildtype and mutant chloroplasts was flat and arranged parallel to the cell walls to maximize photosynthetic energy capture under relatively low light conditions (120 μmol photons m^{-2} s^{-1}). Nevertheless, dark-adapted *clce* chloroplasts had a more pronounced, special bow-like arrangement of the thylakoid network. Similar arrangement of the thylakoid system was reported for the chloroplasts of other photosynthetic species under different stress conditions, including excess of heavy metals that cause disturbances in the ion homeostasis of chloroplasts (e.g., reviewed by Solymosi and Bertrand, 2012). We suggest that the peculiar chloroplast shape and thylakoid arrangement in the *clce* mutant in darkness could be a mechanism to overcome altered Cl^- homeostasis within the chloroplast. The disturbances in chloroplast ultrastructure may in turn alter the functioning of the photosynthetic electron-transport chain. The *clce* mutant can adapt to light similarly to wildtype as indicated by similar thylakoid ultrastructural changes. This suggests that additional Cl^- transport mechanism must exist in thylakoids in the light. This unknown mechanism may be also involved in the restoration of wildtype *OJIP* kinetics by KCl pre-treatment (**Figure 5B**). Similar treatment with KNO_3 did not restore the wild-type kinetics (**Figure 5C**), suggesting that either NO_3^- homeostasis was not altered in the *clce* mutant or that changes in NO_3^- homeostasis did not affect photosynthesis in the *clce* mutants.

The altered *OJIP* kinetics observed in *clce* leaves could also be reproduced in wildtype following dark adaptation for 1 min (**Figures 6A,B**). More specifically, this short dark adaptation resulted in a complete absence of the *I* step in both wildtype and mutant, and only the *OJP* kinetics were visible. In leaves treated with KCl, we observed a slower formation of the *I* step, since it became visible only after 5 min (**Figure 6C** and inset), and even after 15 min the amplitude was still lower relative to untreated leaves (**Figures 5A,B**). The observation of a delay in formation of the *I* step upon KCl treatment suggests that ion homeostasis in darkness is important for a proper re-arrangement of the electron transport chain components. We also found a faster transition of *clce-2* from state 1 to state 2 (**Figures 8A–C**) as well as the higher *qS* values (**Figure 8D**). A possible explanation is that Cl^- ions may directly influence the electrostatic interactions of LHCII with PSII or PSI, which in turn affect their ability to migrate, also leading to an unfavorable arrangement in darkness. The macro-organization of complexes and the structural stability of the chiral macro-domains have been shown to depend on the ionic strength of the medium (Garab et al., 1991; Cseh et al., 2000). Alternatively, a long-lived membrane potential in darkness due to sustained Cl^- ions trapped in the thylakoid lumen may also have a negative effect on the dark relaxation of the electron transport chain.

To conclude, our findings suggest that AtCLCe functions in Cl^- homeostasis within the chloroplast leading to re-arrangement of the electron transport chain in thylakoids after transition from light to dark. Changed Cl^- distribution across thylakoids may be one of the strategies to ensure maximum quantum yields and balance photochemical utilization with photoprotection by *NPQ* upon light-to-dark and dark-to-light transitions. We propose a minor role for AtCLCe in light-driven Cl^- import into the thylakoid lumen, and a major role in Cl^- export to the chloroplast stroma upon dark adaptation. The major Cl^- import mechanism driven by membrane potential produced during illumination remains to be identified. Cl^- export by AtCLCe in darkness would hence be driven by the inversed membrane potential across the thylakoid membrane, which occurs when light is switched off, and would facilitate

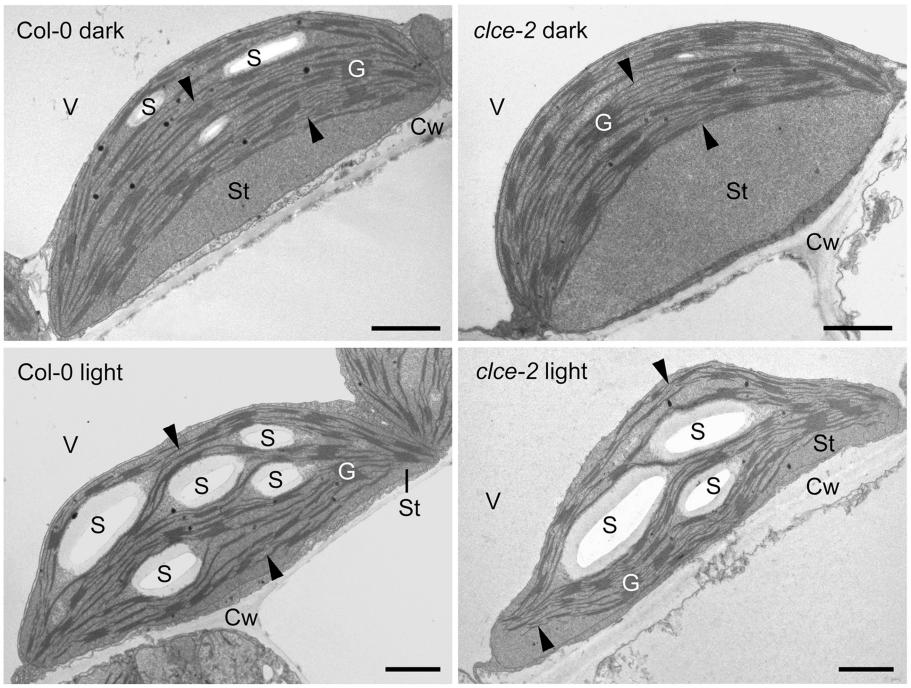

FIGURE 10 | Representative transmission electron micrographs of chloroplasts. Leaves from 7-week-old wild-type and mutant plants that were dark-adapted for 16 h ("dark") or further illuminated for 3 h at 120 μmol photons m^{-2} s^{-1} ("light") were fixed in dim green light for electron microscopy. Arrowhead, stroma thylakoids; Cw, cell wall; G, granum; S, starch; St, chloroplast stroma; V, vacuole. Scale bar: 1 μm.

re-arrangements of the electron transport chain components in thylakoids.

AtCLCe—a Cl^- Channel or an NO_3^-/H^+ Exchanger?

AtClCe has in general a modest homology with the other plant CLCs, and forms a distinct family branch together with AtCLCf (Barbier-Brygoo et al., 2011). Phylogenetic analyses indicated the presence of homologs in green algae, brown algae, diatoms and cyanobacteria, sharing 25–35% identity with AtCLCe, but thus far none have been characterized (Pfeil et al., 2014).

CLCs were initially thought to be involved in Cl^- transport after the first CLC was cloned, which was the voltage-dependent Cl^- channel of torpedo fish (CLC-0; Jentsch et al., 1990). Crystal structures revealed bacterial CLCs as secondary active transporters that exchange Cl^- and H^+ with a 2:1 stoichiometry (Accardi and Miller, 2004). Animal CLCs were found to function as Cl^- channels at the plasma membrane or as $2Cl^-/1H^+$ exchangers in organellar membranes (Jentsch, 2015). Even though they catalyze distinct transport reactions, they share the basic protein architecture. More specifically, CLC proteins are homodimers with separate ion pathways within each monomer (Dutzler et al., 2002). Each monomer consists of a transmembrane component, which forms the ion transportation pathway, and in the case of eukaryotic members, also of a cytosolic cystathionine beta-synthase (CBS) domain component, which binds nucleotides and regulates the transmembrane component.

Multiple sequence alignment of AtCLCe with three CLCs from prokaryotes and eukaryotes indicated 17.9% identity with AtCLCa (De Angeli et al., 2006), 20.2% with CLC from *Cyanidioschyzon merolae* (Feng et al., 2010), and 23.3% with the CLCa from *Escherichia coli* (Dutzler et al., 2002; Supplementary Figure 2). AtCLCe sequence lacks many residues in the anion selectivity filter conserved in most CLCs (Dutzler et al., 2002; Feng et al., 2010). It does not harbor either the conserved serine in the Cl^- binding site of crystallized CLCs or the proline residue, shown to be crucial for preference of NO_3^- vs. Cl^- in AtCLCa (Wege et al., 2010). Nevertheless, the positively charged residue (lysine) at this position may still be able to coordinate Cl^- in the transportation pathway, but reduces the chances of a preference for transport of NO_3^-. Thus the previous observation that *clce* mutants displayed altered nitrate accumulation (Monachello et al., 2009) could be an indirect effect due to possible alteration in the expression of NO_3^-/H^+ exchangers from the AtCLC family. In addition, our data showing similar *OJIP* phenotype in untreated and KNO_3-treated *clce* mutants (**Figure 5**) indicates that even if AtCLCe would play a role in nitrate homeostasis, this did not affect photosynthetic electron transport in thylakoids. Instead, AtCLCe appears to function in Cl^- homeostasis, which affects electron transport via thylakoid re-arrangements.

AtCLCe sequence contains the so-called "gating glutamate" (Supplementary Figure 2), which is conserved in almost all CLCs and which protonation opens the Cl^- transportation pathway (Feng et al., 2010). However, AtCLCe sequence does not contain the "proton glutamate" important for H^+ translocation,

which could indicate that this CLC member is not an anion H^+ exchanger. Instead, at the "proton glutamate" position, a serine residue is present in AtCLCe and a threonine in algal CLC, which was shown to still function as an exchanger (Feng et al., 2010). In addition to serine and the gating glutamate, a tyrosine residue is involved in coordination of the Cl^- ion in the translocation pathway. This residue is replaced by a threonine in AtCLCe, which is also polar and harbors a hydroxyl group important for coordination. Finally, the aspartate residue involved in ATP binding in the CBS domain (De Angeli et al., 2009) is replaced by a cysteine in AtCLCe. As reviewed by Pottosin and Dobrovinskaya (2015), electrophysiological studies of AtCLCe are required to determine the Cl^- vs. NO_3^- selectivity and channel vs. exchanger mechanism of transport for this protein. This will also allow to establish whether AtCLCe is responsible for the channel activity previously reported in thylakoids (Schönknecht et al., 1988).

AUTHOR CONTRIBUTIONS

AH, BL, and CS conceived the study and designed the experiments. AH carried out chlorophyll fluorescence, P700, ST, and ECS measurements. HN conducted screening of the mutants and RT-PCR. OZ carried out CD analyses. KS carried out TEM analyses. AH and CS wrote the manuscript. All authors helped to edit the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00115

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MINI REVIEW
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Proton Gradients and Proton-Dependent Transport Processes in the Chloroplast

Ricarda Höhner[1], Ali Aboukila[2], Hans-Henning Kunz[1] and Kees Venema[2]**

[1] Plant Physiology, School of Biological Sciences, Washington State University, Pullman, WA, USA, [2] Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estacion Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain

Proton gradients are fundamental to chloroplast function. Across thylakoid membranes, the light induced -proton gradient is essential for ATP synthesis. As a result of proton pumping into the thylakoid lumen, an alkaline stromal pH develops, which is required for full activation of pH-dependent Calvin Benson cycle enzymes. This implies that a pH gradient between the cytosol (pH 7) and the stroma (pH 8) is established upon illumination. To maintain this pH gradient chloroplasts actively extrude protons. More than 30 years ago it was already established that these proton fluxes are electrically counterbalanced by Mg^{2+}, K^+, or Cl^- fluxes, but only recently the first transport systems that regulate the pH gradient were identified. Notably several $(Na^+,K^+)/H^+$ antiporter systems where identified, that play a role in pH gradient regulation, ion homeostasis, osmoregulation, or coupling of secondary active transport. The established pH gradients are important to drive uptake of essential ions and solutes, but not many transporters involved have been identified to date. In this mini review we summarize the current status in the field and the open questions that need to be addressed in order to understand how pH gradients are maintained, how this is interconnected with other transport processes and what this means for chloroplast function.

Keywords: proton gradient, chloroplast, thylakoid, envelope, cation/H+ exchanger

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Consejo Superior de Investigaciones Científicas, Spain

****Correspondence:***
Hans-Henning Kunz
henning.kunz@wsu.edu
Kees Venema
kev@eez.csic.es

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INTRODUCTION

Proton (H^+) gradients across biomembranes represent strong driving forces vital for cellular and cell organellar function. In plant cells, several compartments with different pH exist in parallel. While the apoplast and the vacuole maintain fairly acidic pH levels generally between pH 5 and 7 (Grignon and Sentenac, 1991; Martiniere et al., 2013; Shen et al., 2013). The cytosol has to stay neutral (pH 7.2-7.4) to ensure proper biochemical reactions (Schumacher, 2014). Only mitochondria, the chloroplast stroma and some peroxisomes offer alkaline reaction conditions with pH values equal to or higher than 8 (Shen et al., 2013). The pH gradients have to be actively maintained. In the chloroplast thylakoid membrane or in mitochondria this is achieved by H^+-transporting electron transfer chains while other organelles depend on ATP fueled H^+-pumps (ATPases). Although H^+-pumping activities have been detected in several organellar membranes, the molecular identity of all proteins involved is not known. The established pH-gradients are critical to drive secondary active transport by for instance organellar ion/H^+

exchange. In recent decades, most research has focused on H^+-gradient dependent transporters in endomembrane organelles, but knowledge on mitochondria and chloroplasts lags behind (Bassil et al., 2012; Pittman, 2012). Given the central role of mitochondria and chloroplasts for plant energy metabolism, these systems are urgently awaiting more targeted research activity.

This review focuses on the chloroplast, which harbors the pathway of arguably the most important biochemical process for life on earth, photosynthesis. Eukaryotic photosynthesis depends on the H^+ and ion-gradients established between the sub-organellar chloroplast compartments, the thylakoid lumen and the stroma. We will discuss current knowledge on H^+-gradients and H^+-coupled transport across the chloroplast membranes, and how pH-homeostasis in chloroplast compartments is achieved and maintained.

THE H^+-GRADIENT ACROSS THE THYLAKOID MEMBRANE

Light-induced electron transport across the thylakoid membrane gives rise to H^+-pumping into the thylakoid lumen. The resulting H^+-electrochemical potential difference (pmf) is used for ATP synthesis by the thylakoid ATP synthase. It is generally assumed that both components of the pmf, that is the pH gradient (ΔpH) and the electric field ($\Delta\Psi$), generated by the electron transport chain are equally capable of driving ATP synthesis (Kramer et al., 2003). Although the luminal pH was suggested to drop below pH 5 in some circumstances, recent studies show more modest values of 5.7–7.8 while the stromal pH rises to about 7.8–8.0 (Takizawa et al., 2007). Early reports indicated that the H^+-accumulation is electrically compensated by K^+ or Mg^{2+} efflux, or Cl^- influx (Enz et al., 1993), but only recently, first transporter genes involved in pH- and osmoregulation in the thylakoid were discovered.

First, the two-pore potassium channel TPK3 was confirmed by immunodetection to localize to the stroma lamellae (Carraretto et al., 2013; **Figure 1**). TPK3-RNAi-silenced *Arabidopsis* plants revealed stunted growth phenotypes along with anthocyanin accumulation under ambient light conditions (90 μE). TPK3 mutants are compromised in generating ΔpH while increasing $\Delta\Psi$ by 20% compared to wild-type controls (Carraretto et al., 2013). The channel is *in vitro* activated by Ca^{2+} and acidification and releases K^+ from the thylakoid membrane to regulate the pmf (Carraretto et al., 2013). Additionally, light-induced Mg^{2+} release into the stroma (Krause, 1977) and luminal Cl^- uptake (Enz et al., 1993) was demonstrated. However, the identity of the Mg^{2+} transporter at the thylakoid membrane remains hypothetical (**Figure 1**). T-DNA mutants of the thylakoid membrane channel AtCLCe reveal only subtle effects on photosynthesis (Marmagne et al., 2007), and a role in charge equilibration or Cl^- import into the lumen still remains hypothetical as indicated in **Figure 1**. Thus, K^+ appears to be the fundamental coupling ion.

Recently, an electro-neutral K^+ efflux antiporter (KEA3) from the Cation Proton Antiporter (CPA2) family was identified in the thylakoid membrane, predominantly in stroma lamellae (**Figure 1**), of *Arabidopsis* (Armbruster et al., 2014; Kunz et al., 2014). Independent *kea3* T-DNA insertion mutants reveal an increased ΔpH component of pmf partitioning with a 20% diminished $\Delta\Psi$, suggesting a role in pmf regulation via K^+/H^+ exchange across the thylakoid membrane (Kunz et al., 2014). An increase in conductivity to counter ions, for instance by opening of the TPK3 channel, will promote the development of the ΔpH component, but short-circuit the membrane potential, while the activity of KEA3 would only dissipate the ΔpH component, leaving the $\Delta\Psi$ intact. Armbruster et al. (2014) showed a crucial role for KEA3 in altering the dynamics of the non-photochemical quenching (NPQ) component qE under fluctuating light conditions. qE dissipates excessive excitation energy into heat and is triggered by low lumen pH (Muller et al., 2001). KEA3 was shown to increase photosystem II (PSII) quantum yield [φ(II)] during the transition from high light to low light by accelerating the downregulation of qE via H^+-efflux from the lumen (Armbruster et al., 2014). At the same time, reduced luminal acidification in plants with impaired TPK3 function causes increased sensitivity to ambient light intensities, as the mechanisms to dissipate excess light energy cannot be activated (Carraretto et al., 2013). A similar observation was made for cyanobacterial TPK3 counterpart SynK (Checchetto et al., 2012). In a SynK-deficient mutant, the decreased ΔpH component also leads to the inability to trigger NPQ. Thus, it appears that the coordinated activation and deactivation of TPK3 and KEA3 enables the interconversion of $\Delta\Psi$ and ΔpH in response to environmental stimuli, in order to optimize photosynthesis (Kramer et al., 2003; Armbruster et al., 2014).

Genes with high sequence similarity to KEA3 exist in green algae, moss and higher plants, with one gene copy appearing in both *Chlamydomonas* and rice, and two gene copies in *Physcomitrella* (Chanroj et al., 2012). The cyanobacterium *Synechocystis* sp. expresses a CPA2 gene NhaS3, with some sequence similarity to CHX members and KEA3 from *Arabidopsis* (Chanroj et al., 2012). NhaS3, as a potential Na^+ (K^+)/H^+ antiporter was shown to be located in the thylakoid membrane and to enhance K^+ uptake when expressed in *Escherichia coli*. The authors therefore concluded that NhaS3 is involved in thylakoid lumen ion-homeostasis (Tsunekawa et al., 2009).

It was reported that the thylakoid ΔpH also energizes Ca^{2+} import across the thylakoid membrane (Ettinger et al., 1999). Ettinger et al. (1999) suggested that Ca^{2+} import is driven by a hypothetical Ca^{2+}/H^+ antiporter (**Figure 1**), the gene of which remains elusive.

In summary, the ΔpH component of the pmf contributes to the ATP production, import of proteins, and the uptake of essential ions such as Ca^{2+}. Further, the partitioning of pmf in ΔpH and $\Delta\Psi$ is regulated via transport activities of KEA3 and TPK3, which has fundamental impact on photosynthetic efficiency and other pH-dependent processes. Finally, thylakoid H^+-transport establishes the physiological pH in lumen and stroma, thereby ensuring the activity of the light-dependent photosynthetic reactions and the Calvin Benson cycle enzymes.

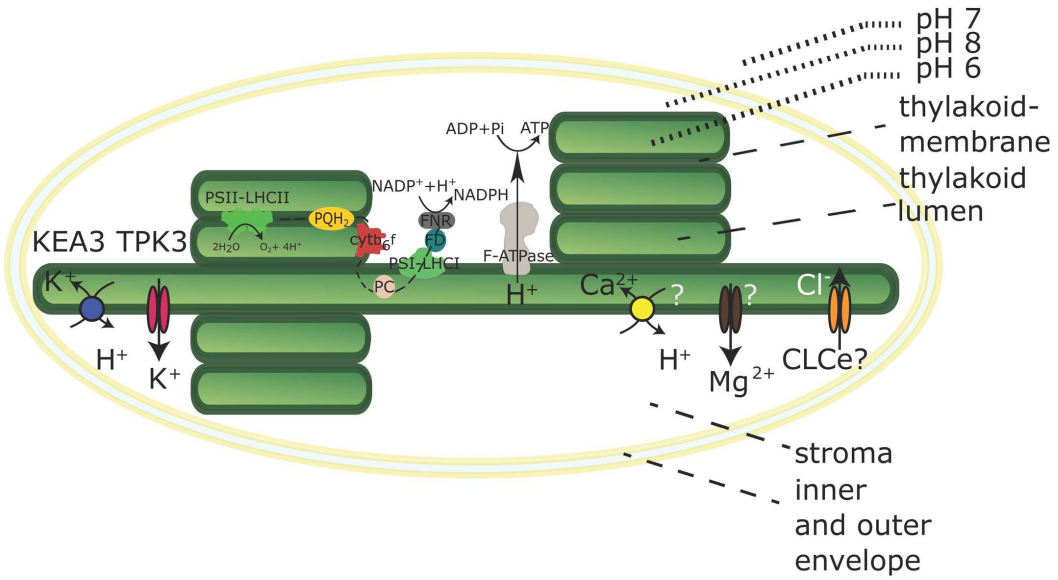

FIGURE 1 | Chloroplast with its electron transport chain and selected transporters in the thylakoid membrane. Transport directions are shown for chloroplasts in the light. The K^+/H^+ antiporter KEA3 and the Potassium Channel TPK3 are confirmed thylakoid membrane K^+ transporters. The molecular identity of Ca^{2+} and Mg^{2+} transporters has not been determined and the localization and transport activity of the CLCe channel is uncertain. Both is indicated by question marks. The black dashed line indicates linear electron transport in the thylakoid membrane. Ratios and dimensions of the protein complexes involved in the electron transport chain are schematic and do not reflect physiological conditions.

THE H^+-GRADIENT ACROSS THE CHLOROPLAST ENVELOPE MEMBRANE

In the dark the cytoplasmic and stromal pH are close to 7, but upon illumination the stroma becomes alkaline, as a consequence of H^+-pumping across the thylakoid membrane. An alkaline stroma pH is a prerequisite for the full activation of pH-dependent Calvin Benson cycle enzymes (Heldt et al., 1973; Werdan et al., 1975). This implies that a pH gradient between the cytosol (pH 7) and the stroma (pH 8) is established upon illumination. It is assumed, that in order to maintain this pH gradient, an active H^+-export mechanism exists in the envelope membrane to compensate for passive H^+-diffusion from cytosol to stroma. Evidence for an active H^+-extrusion is based on the observation that illumination of isolated chloroplasts induces a small transitory acidification in slightly buffered solutions (Heber and Krause, 1971; Heldt et al., 1973; Heber and Heldt, 1981; Demmig and Gimmler, 1983). Experiments on equilibration of lipophilic cations and tracer studies in isolated chloroplasts indicate that a membrane potential of around −100 mV (negative inside, **Figure 2**) exists across the envelope membrane due to a Gibbs Donan equilibrium, in low salt solutions. At higher KCl concentrations, similar to the situation inside the cell, this membrane potential becomes very small (Demmig and Gimmler, 1983). In illuminated chloroplasts the membrane potential increases by about 10 mV, concomitant with light induced H^+-extrusion and coupled K^+ uptake (Demmig and Gimmler, 1983; Wu et al., 1991). The K^+/H^+ exchange is not abolished by the K^+ ionophore valinomycin, indicating that K^+ and H^+-fluxes are electrically and thus indirectly coupled (Demmig and Gimmler, 1983; Wu and Berkowitz, 1992; Wang et al., 1993). Several mechanisms have been proposed to explain the H^+-efflux, but the molecular identities of the transporters involved are still elusive.

H^+-extrusion was suggested to depend on a K^+-stimulated vanadate sensitive envelope P-type H^+-ATPase (Scherer et al., 1986; Berkowitz and Peters, 1993; Mi et al., 1994; Shingles and McCarty, 1994) but the *Arabidopsis* genome does not encode for chloroplast predicted P-type H^+-pumps (Axelsen and Palmgren, 2001). Other reports found that the envelope ATPase activity is sensitive to oligomycin, which is not a P-type ATPase inhibitor but a F_o subunit inhibitor of the F_1F_o ATP Synthase. However, no ATPases were identified in chloroplast proteomics studies of envelope membranes. In conclusion the existence of an envelope H^+-ATPase is hypothetical. Alternatively, an electron-transfer chain may be responsible for H^+-extrusion. Although proteins that could fulfill such functions are found in the envelope membrane, their function in H^+-extrusion has never been demonstrated (Jäger-Vottero et al., 1997; Murata and Takahashi, 1999; Kovacs-Bogdan et al., 2010). In algae, the plastome-encoded, putative heme-binding, inner envelope protein YCF10 from *Chlamydomonas* is essential for enhancing carbon fixation and inorganic carbon uptake (Rolland et al., 1997). The YCF10 protein was suggested to be involved in plastid pH regulation and to be part of a redox chain in the envelope membrane (Jäger-Vottero et al., 1997; Rolland et al., 1997). In *ycf10* deficient mutants of *Chlamydomonas* the CO_2 or HCO_3^- uptake was compromised and cells failed to grow photoautotrophically (Rolland et al., 1997). It was hypothesized that YCF10 is crucial for acidification of the intermembrane

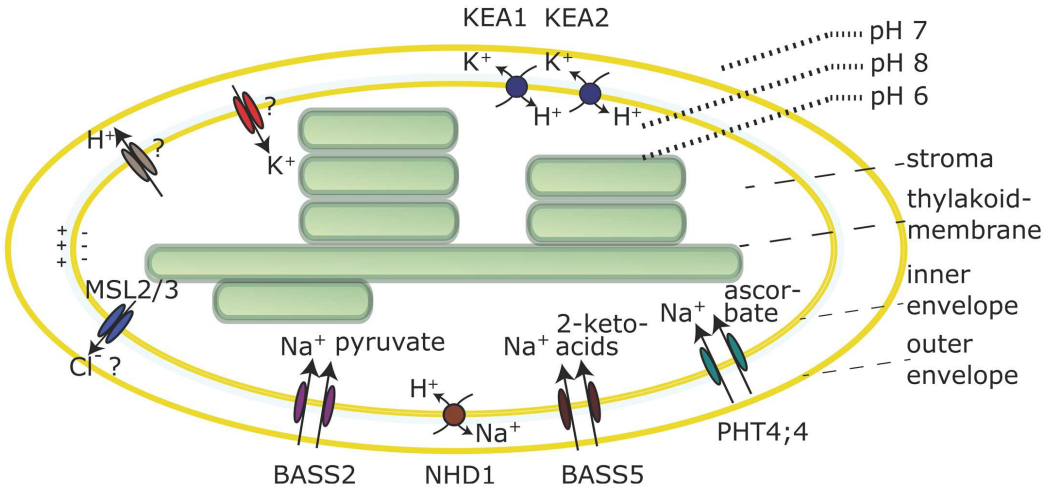

FIGURE 2 | Selected transporters in the chloroplast envelope membrane. Transporter activities are shown for chloroplasts in the light. The nature of the H^+ efflux mechanism and the molecular identity of K^+ channels is still unknown and the Cl^- substrate specificity of the MSL2/3 channels is not confirmed. Both is indicated by question marks.

space by H^+-translocation to allow for the conversion of HCO_3^- to CO_2, which in turn could diffuse into the chloroplast (Rolland et al., 1997). A similar observation was made for the cyanobacterial homolog PcxA (CotA) that was shown to be involved in light-induced Na^+-dependent H^+-extrusion. The *cotA* insertion mutant MA29 of the cyanobacterium *Synechocystis* sp. shows no H^+-export leading to a diminished CO_2 uptake (Katoh et al., 1996).

Lastly, it was speculated that an alkaline stromal pH could be maintained by direct H^+-export from the thylakoid lumen to the cytosol by some unknown temporary or spatial connection (Heber and Heldt, 1981). In summary, the mechanism by which H^+ move across the envelope membrane and if this involves an ATP-dependent H^+-pump or electron transport coupled H^+-export into the intermembrane space or some other mechanism remains unknown (**Figure 2**).

H^+-DEPENDENT TRANSPORT MECHANISMS ACROSS THE CHLOROPLAST ENVELOPE MEMBRANE

Although the mechanism by which the envelope pH-gradient is generated and maintained during the day remains unclear, it represents the driving force for secondary active H^+-coupled transport processes across the inner envelope membrane. Thus, it is worthwhile to focus at the characterized and putative H^+-dependent transport mechanisms and their transport direction across the inner envelope membrane.

Electrogenic coupling of K^+ uptake to H^+-extrusion in chloroplasts has been demonstrated many times, and the presence of a K^+ channel in the envelope membrane was hypothesized (**Figure 2**) based on biochemical studies with membrane vesicles (Wang et al., 1993) as well as patch clamp studies (Heibert et al., 1995). The chloroplast inner envelope is also reported to contain non-selective cation channel activities (Pottosin et al., 2005). However, up to today the molecular identity of these channels is unknown. Instead several, most likely electroneutral, K^+/H^+ or Na^+/H^+ antiporters were identified. The envelope pH-gradient dictates antiport activity via such antiporters toward K^+-efflux, which can be an important feature for plastid osmoregulation (Shabala and Pottosin, 2010). Light induced shrinkage of chloroplasts was observed more than 40 years ago (Nobel et al., 1969). K^+/H^+ antiporters could be involved in this response, together with mechanosensitive channels MSL2 and MSL3 for which Cl^- efflux activity was hypothesized (Veley et al., 2012; **Figure 2**).

Two members of the K^+/H^+ antiporter family KEA, KEA1 and KEA2 were confirmed to reside in the envelope membrane of *Arabidopsis* (Kunz et al., 2014). A short version of the KEA2 protein comprising the antiport domain and the regulatory C-terminal KTN domain, but not the long N-terminal domain of unknown function (Chanroj et al., 2012) was purified and reconstituted in liposomes containing the pH indicator pyranine to demonstrate ΔpH-dependent K^+/H^+ antiport (Aranda-Sicilia et al., 2012). KEA1 and KEA2 represent very close homologs, most likely due to a gene-duplication event and thus are expected to facilitate the same transport function. Moreover, single T-DNA insertion *kea1* or *kea2* mutants do not show visible phenotypes indicating functional redundancy in *Arabidopsis* (Kunz et al., 2014). *kea1kea2* double mutant plants revealed stunted growth, pale green leaves and low photosynthetic efficiency (Kunz et al., 2014). Chloroplasts from double mutant plants have a swollen appearance, suggesting they suffer from excessive K^+ accumulation. Interestingly, this phenotype depends on the leaf developmental stage, i.e., young leaves were found to be most strongly compromised (Kunz et al., 2014). Additionally, since the pmf across the thylakoid membrane depends not only on light but also on the stromal pH (Hauser et al., 1995), the lack of K^+/H^+-exchange across the envelope membrane in *kea1kea2*

mutants probably causes downstream effects that trigger changes in pmf partitioning with lower ΔpH and in turn lower qE (Kunz et al., 2014). This further emphasizes the linkage between plastid ion and pH homeostasis, and their importance for efficient photosynthesis.

The rice chloroplast envelope KEA homolog is encoded by a single locus named *AM1*, (*albino midrib mutant1)*. The *am*1 mutant was isolated in an EMS screen and displays green- and white leaf variegations (Sheng et al., 2014). Surprisingly, no photosynthetic alterations were found in *am1* mutants. However, mutants did reveal altered chloroplast ultrastructure along with increased drought tolerance (Sheng et al., 2014). The authors attribute *AM1* function in rice to chloroplast development and drought stress (Sheng et al., 2014). Interestingly, the growth and photosynthesis defects in *Arabidopsis kea1kea2* double mutant plants could be rescued partially in high NaCl concentrations. Salt or drought stress are known to increase cytoplasmic and vacuolar osmotic values due to accumulation of salt or synthesis of osmolytes. Possibly, this counteracts the increased osmotic values of the chloroplast stroma in absence of these KEA antiporters (Kunz et al., 2014).

In addition to the KEA K^+/H^+ antiporters, another cation exchanger was identified in the plastid envelope membrane of *Arabidopsis*, the Na^+/H^+ exchanger NHD1 (Furumoto et al., 2011; Muller et al., 2014; **Figure 2**). NHD1 is an NHAD type carrier that is also found in algae, mosses and other plant species (Barrero-Gil et al., 2007; Cosentino et al., 2010). AtNHD1 and homologs from *Mesembryanthemum crystallinum* and *Physcomitrella* were shown to be localized in the envelope membrane (Barrero-Gil et al., 2007; Cosentino et al., 2010; Muller et al., 2014). Investigation of the *Arabidopsis* T-DNA insertion mutant *nhd1-1* showed compromised φ(II) and higher NPQ in response to a NaCl shock treatment (Muller et al., 2014). Proteomics studies on isolated *Arabidopsis* thylakoid membrane fractions also detected NHD1 in the stroma lamellae (Tomizioli et al., 2014). Therefore, a dual localization of the protein within the chloroplast cannot be excluded at the moment.

H^+-gradient driven chloroplast Na^+-efflux by NHD1 could prevent Na^+-accumulation in the organelle during salt stress. Alternatively, this activity could be coupled to the action of Na^+-dependent transporters in the envelope membrane. As such, AtNHD1 was suggested to operate as a two-translocator system together with the sodium:pyruvate cotransporter BASS2 (Furumoto et al., 2011; **Figure 2**). Several more Na^+-dependent carriers in the envelope membrane have been described that facilitate ascorbate import (PHT4;4; Miyaji et al., 2015) or 2-keto acids transport (BASS5; Gigolashvili et al., 2009; Sawada et al., 2009; **Figure 2**). For PHT4;4 and BASS2 experimental evidence from *in vitro* transport studies have revealed the Na^+-dependency. Furthermore, transporters of the PHT2 and PHT4 family, mediate H^+ and/or Na^+-dependent transport of phosphate into chloroplasts and are localized in the envelope membrane (Ferro et al., 2002; Versaw and Harrison, 2002; Roth et al., 2004; Guo et al., 2008).

In diatoms that evolved by secondary endosymbiosis, plastids possess four envelope membranes. To supply the plastid gene expression machinery with nucleotides and provide energy-rich components during the absence of photosynthesis, diatoms utilize nucleotide carriers that efficiently shuttle the compounds from the cytosol, where they are synthesized, into the stroma across the four membranes. Unlike the plastid nucleotide transporters (NTTs) from plant and algae, NTT1 from the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* is suggested to enter the innermost plastid envelope and function as H^+-dependent symporter, which implies a vital function for an envelope pH-gradient in the diatoms (Ast et al., 2009).

Biochemical studies on intact isolated chloroplasts have suggested further H^+-dependent transport processes across the envelope membrane. Measurements on inner envelope membrane vesicles revealed that Ca^{2+}-uptake was stimulated by an electrochemical H^+-gradient across the membrane. Further, Ca^{2+}-movement across the membrane could be shown in the presence of a K^+-diffusion potential gradient (Roh et al., 1998). Two potential candidates were suggested as membrane potential driven Ca^{2+}-transporters in the chloroplast envelope. However, for neither of them the Ca^{2+} transport activity or the substrate specificity could be proven and thus the genetic loci encoding for an H^+/Ca^{2+} uptake mechanism remains unknown. In summary, there is little doubt that Ca^{2+} is crucial for photosynthesis *per se* and therefore fine-tuned delivery systems into stroma and thylakoid lumen have to exist, reviewed in (Hochmal et al., 2015). Because of the significance of Ca^{2+} transporters for chloroplast function this issue needs more detailed research in the future.

It is of note that further transporters functionally important for chloroplast ion homeostasis exist in the envelope membrane. However, since these transporters are not driven by ΔpH, they are not listed here, but are reviewed comprehensively in a recent article (Finazzi et al., 2015).

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Plant Science

REVIEW
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Role of Ions in the Regulation of Light-Harvesting

Radek Kaňa[1,2]* *and Govindjee*[3]

[1] Institute of Microbiology, Academy of Sciences of the Czechia, Třeboň, Czechia, [2] Faculty of Science, Institute of Chemistry and Biochemistry, University of South Bohemia, České Budějovice, Czechia, [3] Center of Biophysics and Quantitative Biology, Department of Biochemistry, Department of Plant Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

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***Correspondence:**
Radek Kaňa
kana@alga.cz

Dedication:

Dedicated to the pioneering research of James (Jim) Barber on many aspects of photosynthesis including the electrostatic properties of the chloroplast thylakoid membrane, and who received the 2016 Communication Award of the International Society of Photosynthesis Research (ISPR).

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Regulation of photosynthetic light harvesting in the thylakoids is one of the major key factors affecting the efficiency of photosynthesis. Thylakoid membrane is negatively charged and influences both the structure and the function of the primarily photosynthetic reactions through its electrical double layer (EDL). Further, there is a heterogeneous organization of soluble ions (K^+, Mg^{2+}, Cl^-) attached to the thylakoid membrane that, together with fixed charges (negatively charged amino acids, lipids), provides an electrical field. The EDL is affected by the valence of the ions and interferes with the regulation of "state transitions," protein interactions, and excitation energy "spillover" from Photosystem II to Photosystem I. These effects are reflected in changes in the intensity of chlorophyll *a* fluorescence, which is also a measure of photoprotective non-photochemical quenching (NPQ) of the excited state of chlorophyll *a*. A triggering of NPQ proceeds via lumen acidification that is coupled to the export of positive counter-ions (Mg^{2+}, K^+) to the stroma or/and negative ions (e.g., Cl^-) into the lumen. The effect of protons and anions in the lumen and of the cations (Mg^{2+}, K^+) in the stroma are, thus, functionally tightly interconnected. In this review, we discuss the consequences of the model of EDL, proposed by Barber (1980b) Biochim Biophys Acta **594**:253–308) in light of light-harvesting regulation. Further, we explain differences between electrostatic screening and neutralization, and we emphasize the opposite effect of monovalent (K^+) and divalent (Mg^{2+}) ions on light-harvesting and on "screening" of the negative charges on the thylakoid membrane; this effect needs to be incorporated in all future models of photosynthetic regulation by ion channels and transporters.

Keywords: ions, non-photochemical quenching, state transitions, light-harvesting protein complexes, photosynthesis, photoprotection

INTRODUCTION

Thylakoids and other energy transducing membranes produce ATP employing transmembrane electrochemical gradient of protons; see original papers proving this concept (Junge and Witt, 1968; Junge et al., 1968, 1970; Schliephake et al., 1968) as well as selected reviews (Junge, 2004, 2013; Junge and Nelson, 2015). The proton gradient is formed by proton pumps and is coupled with

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDL, electrical double layer; PS I (II), photosystems I (II); TM, thylakoid membrane

light-driven electron transport. Peter Mitchell explained the entire process by his chemiosmotic theory (Mitchell, 1961, 1966), for which he received the 1978 Nobel Prize in Chemistry. In this theory, the unidirectional electrochemical gradient of protons across the membranes, the "proton motive force" (*pmf*), is used for ATP synthesis; this *pmf* (Equation 1) consists of two components: electrical ($\Delta\psi$—the membrane potential) and chemical (ΔpH—proton gradient across membrane), both of which can be used for ATP synthesis (cf. **Figure 1**):

$$pmf(mV) = \Delta\psi + \frac{2.3RT}{F}\Delta pH$$

where, R is the universal gas constant, F the Faraday constant, and T the temperature, in Kelvin (equivalent to temperature in Celsius plus 273.15). In mitochondria, $\Delta\psi$ predominates (Mitchell, 1966), whereas in chloroplasts, ΔpH is the major component (Ort and Melandri, 1982). The ΔpH across the membranes is used not only for making ATP from ADP and Pi, but is also known to control the efficiency of light harvesting and photoprotection (Wraight and Crofts, 1970; Briantais et al., 1979) and regulates electron transport and ATP synthesis (Tikhonov, 2013).

There is still no clear consensus about the precise division of steady-state thylakoid proton motive force between the ΔpH and the electric field gradient ($\Delta\psi$). Based on measurements of electrochromic shift, there are irreconcilable findings indicating a partial (Cruz et al., 2001), or a total collapse of $\Delta\psi$ (Johnson and Ruban, 2014) under steady state conditions. To resolve this discrepancy, we need to know the extent of particular counter-ion transport (i.e., K^+, Cl^-, Mg^{2+}) in the steady state (see discussion Kramer et al., 1999; Cruz et al., 2001) as the transport of both protons and ions affect the partitioning of *pmf* into $\Delta\psi$ and ΔpH (Kramer et al., 2004; Lyu and Lazár, 2017). The *pmf* partitioning is an important factor indirectly regulating the light-harvesting: (1) by lumen acidification that triggers non-photochemical quenching (see Horton, 2014); (2) by changes in ion (mostly Mg^{2+}) concentration in the stroma, which affects excitation energy redistribution between the two photosystems, during state transitions (see Barber, 1980b). Importantly, the concentration of ions and the presence of electrical fields across

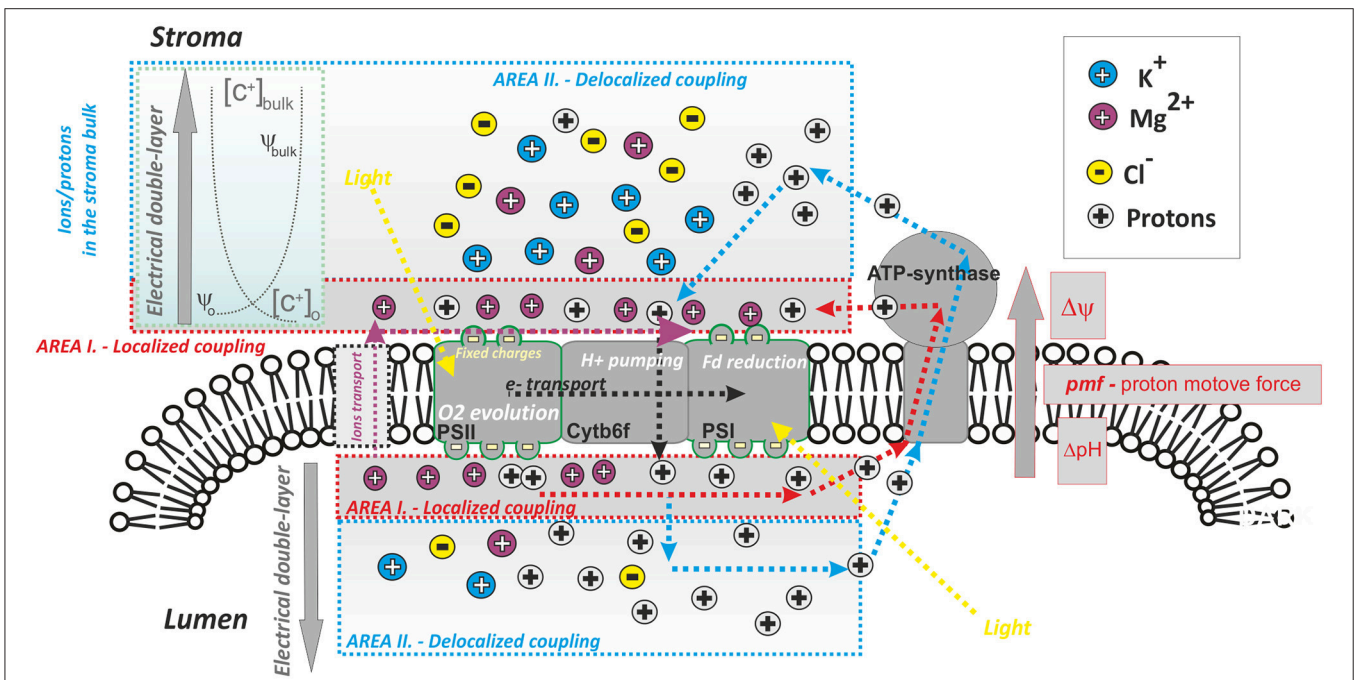

FIGURE 1 | A simplified scheme of photophosphorylation in photosynthesis: role of proton and ion heterogeneity. ATP synthase uses proton gradient across the thylakoid membrane; the proton transport across the thylakoid is coupled to light-driven electron transport (dotted black lines). The proton gradient forms electrochemical gradient of protons [*pmf*—proton motive force, which includes $\Delta\psi$ (the membrane potential component) and ΔpH (proton gradient component; see Equation 1)]; *pmf* is the driving force for ATP synthesis. The most accepted view of photophosphorylation is: "delocalized phosphorylation"—equilibrated concentration of protons in the bulk (in the stroma/lumen) is used (original Mitchell theory—dashed blue lines, see Mitchell, 1966). A second less accepted view is that of "localized phosphorylation" (dashed red lines, see Mulkidjanian et al., 2006): protons from the local domains in lumen/stroma that are in the close vicinity of membrane (see Area I) are involved in ATP synthesis (Dilley, 2004). Localized protons interact with ions attached to fixed membrane charges, mostly Mg^{2+} (note: K^+ and Cl^- are more abundant in the thylakoid stroma/lumen bulk, see Barber, 1980b). The distribution of the dominant ions (K^+, Mg^{2+}, Cl^-) in the local (Area I) and bulk domain (Area II) areas is controlled by properties of the electrical double layer—EDL (see Cevc, 1990); EDL is characterized by the ion profile around the membrane, as well as the electrical field around the membrane, between ψ_o and ψ_{bulk}. (cf. **Figure 2**). Photosynthetic proton pumping into the lumen is accompanied by counter-ion transport from lumen into stroma. The process of electron transport and the use of protons by ATPase can be uncoupled by the addition of various ionophores (nigericin—electroneutral antiporter H^+/K^+; valinomycin—K^+ ionophore; A23187—Mg^{2+}) that can disrupt the membrane potential and ion/proton gradients. The efficient activity of the particular ionophore in uncoupling requires the presence of appropriate cationic species at the membrane surface (Barber, 1980b); therefore, their uncoupling ability differs between high & low screening modes when more Mg^{2+} and K^+ are attached to thylakoid membranes (see **Figure 5**).

the membrane ($\Delta\psi$) are tightly interconnected as they both form an "electrical double layer"—EDL (see **Figures 1**, **2**). Here, we have a typical heterogeneous structure defined by charges and by electrical fields formed close to the negatively charged membranes (Cevc, 1990). In chloroplasts, both monovalent and divalent cations electrostatically interact with negatively charged surface of the thylakoid membrane proteins (see e.g., Barber, 1980a,b, 1982, 1986, 1989), and both the free ions and the fixed charges of membrane proteins form the EDL of the thylakoid membrane (**Figure 2**). Any change in heterogeneous EDL affects reactions in photosynthesis (Barber, 1980b).

The original chemiosmotic theory did not include any heterogeneity in ions or in proton concentrations (Mitchell, 1961, 1966). It had suggested that ATP synthesis is driven by equilibrated proton gradient between two homogenous cellular compartments in the bulk aqueous phases (see **Figure 1**); this type of photophosphorylation is considered to be delocalized. Even though the theory was experimentally

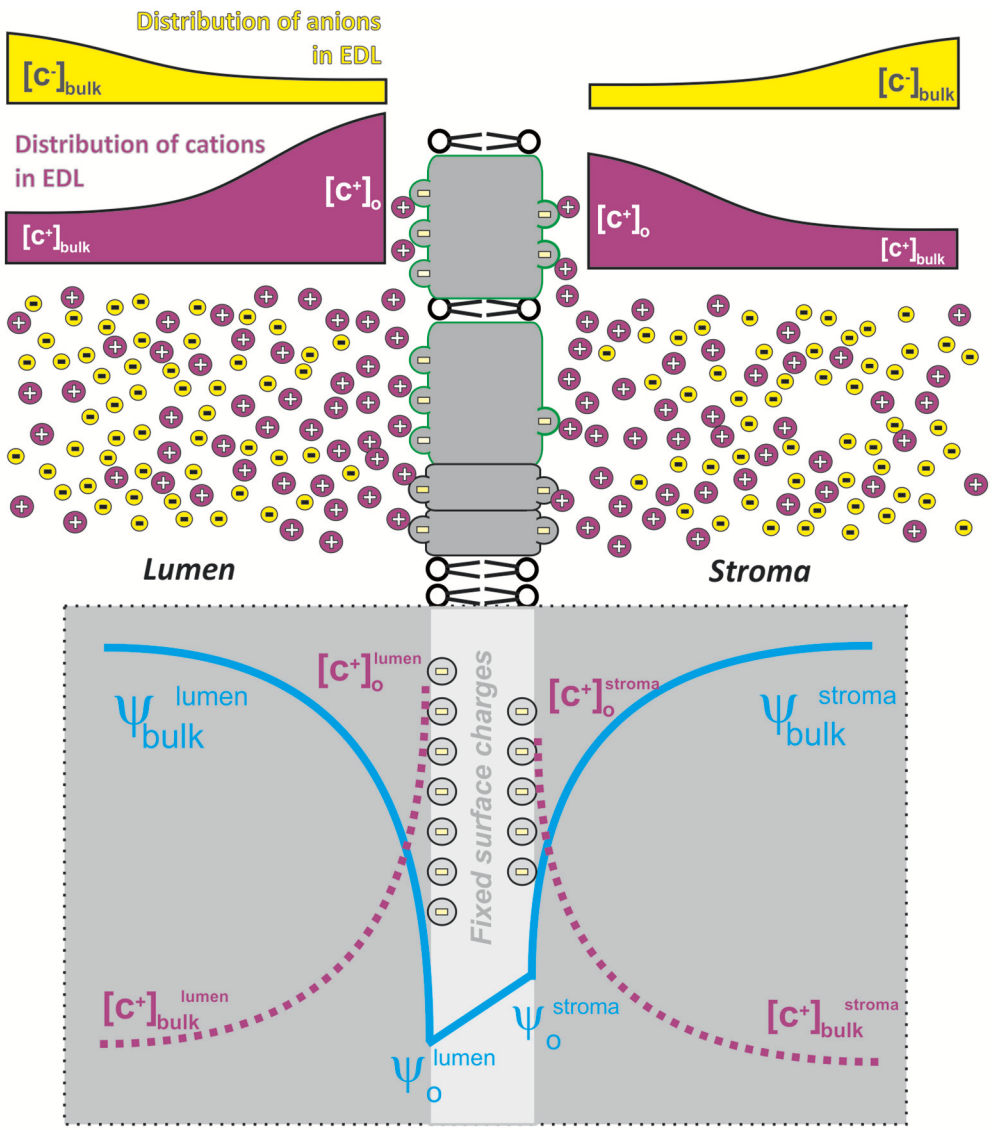

FIGURE 2 | A schematic representation of charge/electrostatic field distribution around thylakoid membranes that forms electric double layer (EDL). Negatively charged amino acid residues form fixed charges on the thylakoid membrane surface that is screened by positive ions (mostly Mg^{2+}) in the lumen as well as in the stroma. The concentration of screening cations decreases with distance from the membrane surface in contrast to that of the anions that are more abundant farther from the negative charges of the membrane. This charge distribution then forms the electric double bilayer (EDL) where the electrical field (see blue line) on the luminal/stromal surface of thylakoid membrane (ψ_o-lumen, ψ_o-stroma) is higher than the electrical field measured in the bulk (ψ_{bulk}^{lumen}; ψ_{bulk}^{stroma}); there is also a characteristic distribution (see dotted magenta lines) in the concentration of cations at the membrane $[C^+]_o$ and in the bulk $[C^+]_{bulk}$. EDL then represents asymmetric charge distribution of both the anions (e.g., Cl^-) and the cations (K^+; Mg^{2+}) caused by fixed charges on the thylakoid membrane; the concentration of the anions/cations progressively increases/decreases with the distance from the charged thylakoid membrane surface (Barber et al., 1977). The difference between ψ_{bulk}-ψ_o is characterized by the extent of electrostatic screening—the higher the electrostatic screening by the ions the lower is the observed difference.

confirmed (see e.g., Junge et al., 1968) some experimental data indicated that proton transfer can sometimes proceed much faster between localized proton domains, situated close to the negatively charged membrane surfaces (**Figure 1**) by the so—called "localized photophosphorylation" (Dilley, 2004; Mulkidjanian et al., 2006). Importance of localized and/or delocalized photophosphorylation at particular physiological conditions needs to be reconsidered (Dilley, 2004). Therefore, it is obvious that the pmf partitioning must be studied with consideration of the heterogeneous EDL membrane (**Figures 1**, **2**). Moreover, theoretical model has shown an effect of H^+/ATP stoichiometry, ionic strength, and buffering capacity on pmf partitioning (Lyu and Lazár, 2017). There are also some other, alternative models of photophosphorylation proposing higher importance of ions; for example there is a mechano-chemiosmotic photosphorylation model, where ATP synthase is considered as a *"Ca^{2+} /H^+–K^+ pump-pore enzyme"* (Kasumov et al., 2015). Last but not the least, there is not only heterogeneity in the distribution of protons (localized/delocalized), but also in the cations (monovalent K^+ and divalent Mg^{2+}) that seems to be distributed asymmetrically—K^+ being mostly in the bulk of the stroma and Mg^{2+} ions being associated closely with the thylakoid membranes due to their divalent charges (see e.g., Barber, 1980b, 1982; references therein).

In summary, protons and ions are distributed heterogeneously in close vicinity of negatively charged thylakoid membrane surface (see **Figures 1**, **2**). The attraction of ions close to the membrane surface results in screening of the electric field generated by the membrane surface charge. We note that ion screening of membrane charges represents an electrostatic interaction, and it differs from direct neutralization of membrane charges by ions (e.g., H^+ binding to amino acid residues, see Barber, 1980a). The extent of this screening affects photosynthesis in various ways; effects have been observed on, e.g., variable chlorophyll (Chl) *a* fluorescence (Murata, 1969a), through changes in chlorophyll-proteins, in thylakoid membrane (TM) stacking (Barber, 1980a), and in excitation energy redistribution during light—induced state transitions (see e.g., Barber, 1982; Staehelin and Arntzen, 1983; Telfer et al., 1983). The direct binding of some ions (e.g., Zn^{2+} or Li^{3+}) or protons at low pH represents a different effect when membrane stacking is not connected with fluorescence changes (Barber, 1980a). In the following, we describe the role of EDL (i.e., membrane screening), charge neutralization on the membrane (i.e., direct ions interaction with the membrane) on variable Chl *a* fluorescence and on the regulation of light-harvesting in state transitions and during non-photochemical quenching.

VARIABLE CHLOROPHYLL *A* FLUORESCENCE OF PHOTOSYSTEM II

The light-harvesting efficiency and the photochemistry in the photosystems (especially Photosystem II, PSII) are often inferred from variable Chl *a* fluorescence measurements (see e.g., chapters in Papageorgiou and Govindjee, 2004). At room temperature, the ratio of variable to minimal chlorophyll *a* fluorescence ($F_v/F_m = (F_m - F_o)/F_m$, where F_m is the maximal fluorescence for totally closed reaction centers of PSII, F_o is the minimal fluorescence at very low excitation intensity, and F_v is the variable fluorescence, see **Figure 3**) has been extensively used as a measure of the efficiency of PSII photochemistry (Krause and Weis, 1991; Govindjee, 2004) since PSI fluorescence is very low and constant (see Giovagnetti et al., 2015; references therein). The kinetic changes in variable chlorophyll *a* fluorescence reflect several processes affected by electron transport, protonation, phosphorylation, NPQ in the antenna, and in PSII reaction centers, as well as in excitation energy redistribution between the two photosystems during state transitions.

Time dependent variable Chl *a* fluorescence intensity changes (fluorescence induction, or fluorescence transient) is measured when dark-adapted cells are exposed to high light; the first measured point is the "O" level, the F_o, the initial fluorescence (**Figure 3**). This is followed by an increase to the "P" level (in ms range), due mainly to the reduction of Q_A to Q_A^-. An effect of other processes including electric field, and conformation of proteins is a matter of discussion for the first second after the sample is exposed to light (see e.g., Schansker et al., 2014 for a review). For instance, the non-photochemical thermal (JIP, see Samson et al., 1999) phase can be attributed to the release of quenching of fluorescence associated with light-driven conformational changes in PSII (Schansker et al., 2011), or of *"photoelectrochemical quenching"* controlled by trans-thylakoid proton pump, powered by light-driven Q cycle (see Vredenberg et al., 2009, 2012). The fast Chl *a* fluorescence rise, during the first second of illumination (**Figure 3B**) is labeled as a O-J-I-P transient (O for the minimum fluorescence, J, and I for inflections, and P for the peak (Strasser et al., 1995; Lazár, 1999, 2006; Stirbet and Govindjee, 2012; Schansker et al., 2014). However, the slower changes in fluorescence (in tens of seconds, to minutes, see **Figure 3A**) reflect changes in other physiological processes, including state transitions, non-photochemical quenching, and even photoinhibition.

Chlorophyll *a* fluorescence transients, during both fast (∼in seconds) and slow (in minutes) time range, have different characteristics in plants, and in cyanobacteria (Ruban and Johnson, 2009; Papageorgiou and Govindjee, 2011; Kaňa et al., 2012a; Kirilovsky et al., 2014). These transients are affected by changes in several factors including: **(a)** the efficiency of PSII photochemistry (reflected by the OJIP phase); (**b**) state transitions (Ruban and Johnson, 2009); **(c)** the coupling and uncoupling of antenna from PS I and or PS II (Kaňa et al., 2009; Kirilovsky et al., 2014); **(d)** photoinhibition of PSII in high light (Prášil et al., 1992); **(e)** lumen acidification during NPQ (e.g., Ruban et al., 2012; Zaks et al., 2013; also see Demmig-Adams et al., 2014); **(f)** the efficiency of carbon cycle reactions; and **(g)** divalent and monovalent ion concentrations that affect EDL, as well as the electric properties of thylakoid membranes (Barber and Mills, 1976; Barber, 1980b, 1982).

The effect of ionic composition of the suspension medium on variable Chl *a* fluorescence of chloroplasts and thylakoid membranes has been intensively studied during the 1970s—1980s (Vandermeulen and Govindjee, 1974; Barber and Mills, 1976). Ion dependent NPQ of Chl *a* fluorescence at room

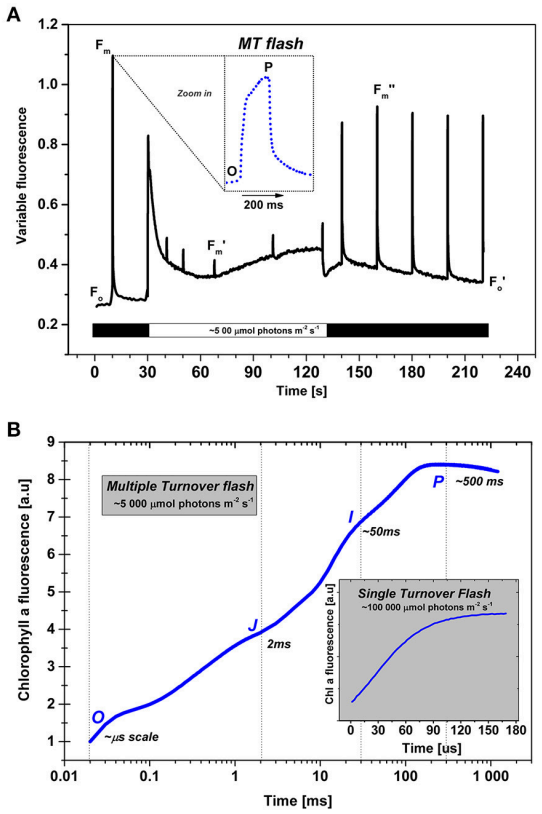

FIGURE 3 | Chlorophyll a fluorescence measurements in photosynthetic organisms. (A) (top): A protocol used for the detection of PSII photochemistry upon exposure with ~500 µmol photons $m^{-2}s^{-1}$ light (Krause and Weis, 1991). The minimal level of Chl *a* fluorescence of open PSII reaction centers plus that from PSI is F_o; it is measured in a dark adapted sample at very low ("measuring") light (~5 µmol photons $m^{-2}s^{-1}$). The maximal fluorescence in dark-adapted sample is F_m, but its changed value during actinic light is referred to as $F_{m'}$, and after the actinic light period light, it is labeled as $F_{m''}$. In the experiment shown, it was measured with high intensity multiple turnover (MT) flashes (~1500 µmol photons $m^{-2}s^{-1}$, flash duration 200 ms), given for a short period (~200 ms). (Note: MT flash induces multiple events of charge separation in PSII.) The fluorescence increase during the MT flash has a characteristic polyphasic rise to a plateau or a peak (see inserts in both panels A and B). The F_m and $F_{m'}$ (as well as $F_{m''}$) values are used for the calculation of PSII photochemistry as well as for non-photochemical quenching (NPQ) of the excited state of Chl *a*, the latter equals (Fm-Fm′)/Fm. Black bars (near the abscissa) represent periods without actinic irradiation (i.e., darkness), whereas during the open (clear) bar, actinic light is on. The sample used in the experiment shown here was *Rhodomnas salina* cells (from Kaňa et al., 2012b). **(B)** Chl *a* fluorescence transient measured at high intensity (~5000 µmol photons $m^{-2}s^{-1}$) MT flash (from Kaňa et al., 2008). The inset shows Chl *a* fluorescence transient in a short, 100 µs long, single turnover (ST) flash, at very high irradiation (~100 000 µmol photons $m^{-2}s^{-1}$). Using the MT flash at high light intensity, we observe a polyphasic O-J-I-P fluorescence transient, where the O–J rise is due to primary photochemical reactions, the subsequent J–I–P transient being the thermal phase (cf. Stirbet and Govindjee, 2012). The fluorescence rise in single turnover (ST) flash (see inset in **A**) is the fast O-J-I-P fluorescence change during the single charge separation event induced by the ST flash that closes all the PSII reaction centers in a very short period (in about 30–100 µs) due to the extremely high intensity of light (~100,000 µmol photons $m^{-2}s^{-1}$, duration 100 µs). We note that explanation of experimental differences between ST and MT fluorescence parameters, obtained with single and multiple turnover flashes, requires detailed knowledge of the studied model organism (see e.g., Kolber et al., 1998; Koblížek et al., 2001).

temperature has been examined in green algae (Mohanty et al., 1974), in guard cells of *Vicia faba* (Ogawa et al., 1982), as well as in cyanobacteria (Papageorgiou and Stamatakis, 2004). The effect of Mg^{2+} has also been examined by low temperature (77 K) fluorescence spectra in plants. Further, variable Chl a fluorescence during the J-(I)-P phase (**Figure 3B**) of fluorescence induction may also be affected, in general, by electric field on the thylakoid membrane, as well as by PSI-dependent photoelectric stimulation and transient release of *"photo-electrochemical quenching"* affected by trans-thylakoid proton pump, which, in turn, also involves the Q cycle (Vredenberg and Bulychev, 2002; Vredenberg et al., 2009, 2012). Even though the Vredenberg model has not been generally accepted, mainly, due to several controversial assumptions (see e.g., Stirbet and Govindjee, 2012), the effect of electrical field and ions on total variable Chl *a* fluorescence must be taken into account in light of other experimental data, i.e., the effect of valinomycin, a K^+ ionophore, on the thermal phase of Chl *a* fluorescence induction (Pospíšil and Dau, 2002; Antal et al., 2011), and the effect of ions on variable Chl *a* fluorescence *in vivo* (Mohanty et al., 1973, 1974; Ogawa et al., 1982; Papageorgiou and Stamatakis, 2004; Krupenina and Bulychev, 2007). The effect of cations and or electrical field on fluorescence could also be related to the EDL in thylakoids, formed by ions and fixed membrane charges (see **Figure 2**; Cevc, 1990). Different screening (low/high—i.e., due to electrostatic interactions) of the membrane charges by ions interferes with the light harvesting process in many aspects, and it is clearly seen through changes of variable fluorescence during organization (or reorganization) of thylakoid membranes (Barber, 1980a,b, 1982, 1986, 1989).

THE ELECTRICAL DOUBLE LAYER (EDL) AND THE THYLAKOID MEMBRANE

The electrical double layer (EDL) represents a typical structure formed when (biological) membrane surfaces, with fixed negative charges on them, are in contact with an aqueous medium containing cations (Cevc, 1990); here, then, we have asymmetric charge distribution of ions that progressively increases toward electrically charged surfaces, i.e., thylakoids (**Figure 2**). The definition of EDL is based on changes of electrical field (ψ_o a parameter measurable by electrochromic shift (Cruz et al., 2001) and ion concentration ($[C^+]$ with increasing distance from the membrane; **Figure 2**). In fact, ions located close to the membrane surface result in *screening* of the electric field of the membrane surface charge (i.e., damping of electrostatic field of fixed charges caused by the presence of interacting ions). The term screening in this case describes the ability of ions in the aqueous phase to increase the negative surface potential closer to zero (see ψ_o in **Figure 2**; for details and equations, see e.g., Barber, 1980b) and it is different than direct interaction of ions with the membranes (i.e., charge neutralization, see Barber, 1980a).

The thylakoid membrane is negatively charged at physiological pH (see reviews Barber, 1980b, 1982; references therein). The average area per single electronic charge on the thylakoid membrane has been estimated to be about 10 nm^2

(Barber, 1980b). Based on isoelectric measurements on isolated thylakoids (Åkerlund et al., 1979), the total fixed negative charges on thylakoid membranes have been estimated to be higher on their luminal, than on their stromal, surface. There are also some experiments indicating an increase in the fixed negative charge during irradiation (see discussion in Barber, 1982) and reference therein). These charges can be attributed to the carboxyl groups of glutamic and aspartic acids in the pigment protein complexes that are negatively charged at the physiological pH (cf. Behrens et al., 2013). An involvement of negatively charged lipids (sulfoquinovosyldiacylglycerol and phosphatidylglycerol) in the total charge, on the thylakoid membrane, is still unclear since these lipids represent only about 20% of all the lipids and they are mostly the boundary lipids for the membrane proteins or act as their cofactors (see e.g., Mizusawa and Wada, 2012). However, the effect of anionic lipids and negatively charged domains of chlorophyll-containing proteins on antenna aggregation/dis-aggregation is indeed a reality (Schaller et al., 2011, 2014). Therefore, the involvement of negatively charged lipids in the total thylakoid membrane charges and on the organization of membrane proteins needs to be further explored.

The EDL in photosynthesis was initially examined for changes in variable Chl *a* fluorescence intensity at different ion concentrations (Barber and Mills, 1976; **Figure 4**). This effect is independent of PSII activity since it was measured with PSII inhibited by DCMU (Gross and Hess, 1973). In the simplest model, monovalent (K+, Na+) and divalent (Mg^{2+} Ca^{2+}) ions have antagonistic effects on chlorophyll a fluorescence (Gross and Hess, 1973; Wong and Govindjee, 1979). In the low salt medium, Chl *a* fluorescence is inhibited by monovalent ions (∼5 mM of K^+); however, this inhibition is reversed by the addition of divalent ions (e.g., ∼5 mM of Mg^2 see e.g., **Figure 4B**). The decrease in Chl fluorescence intensity by monovalent cations has been rationalized by the presence of divalent cations on the membrane surface, before the addition of monovalent ions (Nakatani et al., 1978); the addition of monovalent ions causes their exchange with divalent ions, and Chl *a* fluorescence decreases because monovalent ions have lower capacity of electrostatic screening (Barber, 1989). This effect has been found to be even more complex since ∼5 mM K^+ can reduce Chl *a* fluorescence in the presence of low Mg^{2+} (about 0.1 mM, see Mills et al., 1976), but the high concentration (e.g., ∼50 mM) of monovalent K^+ has been shown to enhance variable Chl fluorescence just as low divalent Mg^{2+} does (Barber et al., 1977; Mills and Barber, 1978). Therefore, the conclusion is that the maximal variable fluorescence can be observed only when positive charge density on the thylakoid membrane surface is sufficiently high and above a critical value (Barber and Mills, 1976).

This dependence of fluorescence on ion concentrations is based only on the valence of interacting ions: a similar effect is observed for K^+ and Na^+, which is different for divalent Mg^{2+} and Ca^{2+} (Nakatani et al., 1978); further, there is almost no specificity in ion-protein and in ion-lipid interactions for particular ions (Mills and Barber, 1978). This phenomenon can be thus associated with a delicate interplay of mixed "electrolytes"

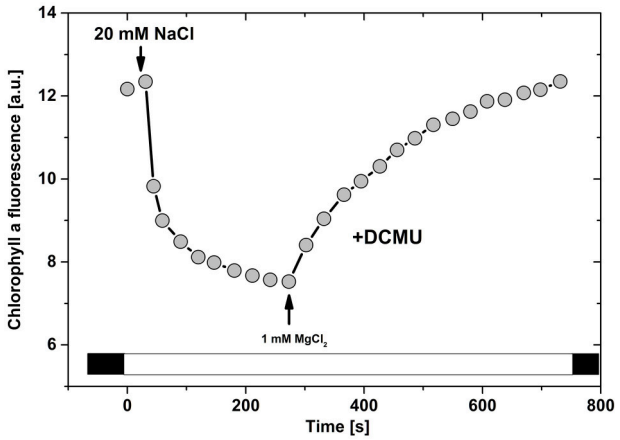

FIGURE 4 | Ion induced changes in variable chlorophyll *a* fluorescence in isolated thylakoids (data adapted from Vandermeulen and Govindjee, 1974). Time course of cation induced changes in chlorophyll *a* fluorescence in oat chloroplast suspension containing 5 μg chlorophyll ml^{-1}, and 5 μM DCMU in low salt buffer. Subsequently, 20 mM NaCl and 1 mM $MgCl_2$ were added (see arrows).

(e.g., in lumen, or stroma) containing a mix of ions (e.g., K^+, Mg^{2+}, Cl^-, and Ca^{2+}) with fixed negative charges of proteins (or lipids) on the thylakoid membranes. These ions can then electrostatically interact with negative charges on thylakoid membranes, thus influencing photosynthesis (Barber et al., 1977). The above-described phenomenon has been theoretically explained by James Barber, and his coworkers, using a modified Gouy-Chapman theory, including non-linear Poisson-Boltzmann ion distribution (Barber et al., 1977). This theoretical approach provided an electrochemical model for EDL on thylakoid membranes at different ion concentrations, and simulated further research in this area (Barber et al., 1977; Rubin and Barber, 1980; Barber, 1989). We can now write several conclusions and caveats: **(a)** Much lower concentration of divalent cations is required in the bulk solution compared with that of the monovalent cations to provide the same extent of electrostatic screening, as reflected in the surface potential (ψ_o); **(b)** The way in which positive charges distribute within the diffuse layer is different for divalent and monovalent cations; **(c)** The concentration of anions in the diffuse layer is very low. Thus, the observed different effects of particular ions (K^+, Mg^{2+}, Cl^-, and Ca^{2+}) on variable Chl *a* fluorescence can be explained by a pure electrochemical effect, due to their different electrostatic screening of charges of membrane proteins (Rubin and Barber, 1980). We note that the effect, discussed here, does not include direct cation binding on membranes (Barber, 1980a). The direct and unspecific cation binding to negatively charged residues could not be totally ruled out (Mills and Barber, 1978; Barber, 1980a). However, in the case of the direct ion binding to membranes (e.g., for La^{3+}, Zn^{2+} or for protons at pH 4.3) no fluorescence changes are observed (**Figure 4**), but only membrane stacking (Berg et al., 1974; Barber and Searle, 1978; Mills and Barber, 1978; Gerola et al., 1979; Barber et al., 1980; Chow and Barber, 1980).

Therefore, Mills and Barber (1978) and Barber (1980a) concluded that electrostatic forces (i.e., effect of EDL) are responsible for reversible fluorescence changes connected with membrane re-organization.

Different concentrations of monovalent and divalent ions (and their different mixtures) affect EDL properties and produce not only changes in Chl *a* fluorescence (**Figure 4**), but they also affect many other photosynthetic processes (see **Figure 5** and reviews by: Barber, 1982, 1986, 1989 and Stys, 1995). In the simplest model, we define two extreme states that are characterized by high or poor electrostatic screening (**Figure 5**). These two extreme states are defined not only by their particular ion concentration, but by their combined effect on ψ_o (see e.g., Rubin and Barber, 1980; Barber, 1989). In view of this, the thylakoid membrane seems to be in the same state with "high electrostatic screening" under the following conditions (**Figure 5**): (**i**) Divalent cations in the diffuse layer, with a low concentration of monovalent cation in the bulk medium; (**ii**) Very high concentration of monovalent cations (e.g., >50 mM of K^+) in the bulk medium (Barber, 1986). Further high electrostatic screening is found (see **Figure 5**) under the following conditions: (**1**) In stacked thylakoid membranes at high Mg^{2+} (Izawa and Good, 1966) since membrane surface charges regulate membrane-membrane interaction; (**2**) When there is an increase in Chl *a* fluorescence yield (Gross and Hess, 1973; Vandermeulen and Govindjee, 1974); (**3**) When there is a decrease in the "*spillover*" of excitation energy from Photosystem II (PS II) to Photosystem I (PS I) (Murata, 1969a)—separation of PSI and PSII into domains; (**4**) When there is a transition to high-fluorescence State I (more antenna coupled to PSII); (**5**) When there is high protein aggregation of antennas and photosystems (we note that protein aggregation, based on current model of NPQ, leads to a quenched state of antennas with low fluorescence yield—this discrepancy needs to be solved by future experiments); (**6**) In isolated thylakoids, A23187, a Mg^{2+}/Ca^{2+} ionophore, is a better uncoupler under high, than low, electrostatic screening mode; the uncoupler activity of all the ionophores (including nigericin—H^+/K^+ antiporter, valinomycin—K^+ carrier, gramicidin—cation ionophore, and A23187—Mg^{2+} ionophore) is dependent on adequate supply of appropriate cations at the membrane surface (Barber, 1980b); (**7**) At high-electrostatic screening mode, there is minimal quenching of fluorescence by "spillover" of excitation energy from PSII to PSI; (**8**) There is positive coupling of electron transport with phosphorylation (Walz et al., 1971); (**9**) Higher PSII efficiency at high screening mode is due to a positive effect on water oxidation and reduction of plastoquinone (Dau and Sauer, 1991; Karge et al., 2014; Khan et al., 2015). In conclusion, the switch between the low and the high electrostatic screening is caused by changes in ions and their concentration. This process affects light-harvesting efficiency in photosynthesis, especially during state transitions, or during excitation energy spillover, as monitored by Chl *a* fluorescence. In addition, there is also an effect on the NPQ of the excited state of Chl *a* (Mills et al., 1976). In view of the above observations, we describe below effects of ions on some of these processes.

REDISTRIBUTION OF EXCITATION ENERGY BETWEEN THE PHOTOSYSTEMS, MEMBRANE STACKING AND THE ROLE OF IONS IN STATE TRANSITIONS

The redistribution of absorbed light between the two photosystems is controlled by state transitions, a process described independently by Bonaventura and Myers (1969) in green algae and by Murata (1969b) in red algae, and discussed soon thereafter by Duysens (1972). This phenomenon was also described earlier, without being named as such, by Papageorgiou and Govindjee (1968a,b), as recognized by Krause and Jahns (2004). This process defined as state transitions has been studied in details in cyanobacteria, green algae, and in higher plants (Ruban and Johnson, 2009; Papageorgiou and Govindjee, 2011; Kirilovsky et al., 2014; Nagy et al., 2014; Kodru et al., 2015). In contrast, fluorescence changes usually attributed to state transitions (see e.g., Allen and Mullineaux, 2004) seem to be absent in algae from the red evolutionary clade (excluding the cryptophytes Cheregi et al., 2015) and the red algae (Kaňa et al., 2014). The basic function of state transitions is redistribution of the absorbed light between the two photosystems (PSI and PSII) which optimizes the overall electron transport since the two systems operate in series (Govindjee and Björn, 2012).

In vivo, the two light-induced states have been defined as: (**1**) State I—characterized by an increased effective light absorption cross-section of PSII (i.e., a bigger PSII antenna); it is called State 1 because it is induced by light preferentially absorbed by PSI; (**2**) State II—characterized by an increased effective cross-section of PSI (bigger PSI antenna); it is called State II because it is induced by light preferentially absorbed by PSII antenna (Duysens, 1972; Goldschmidt-Clermont and Bassi, 2015). At room temperature, Chl *a* fluorescence is high in State I, but in State II, it is low since the quantum yield of PS II fluorescence is much higher than that of PSI due, in part, to the higher rate of photochemistry in PSI than in PSII and persistent quenching of PSI fluorescence by P700. The single unmistakable way to differentiate between State I and State II in green plants, green algae and in cyanobacteria is to compare fluorescence spectra at 77 K. In State I in plants, far-red PS I emission at 735 nm (F735) is lower compared to that from PSII at 685 nm (F685), whereas in State II, it is the opposite (Allen and Mullineaux, 2004). We note that in organisms that lack a well-separated far-red emission of PSI [e.g., in red-clade alga *Chromera velia* (Belgio et al., 2017) and in the dinoflagellate Gonyaulax polyedra (Govindjee et al., 1979)], this method cannot be used.

In addition to state transitions described above for intact cells, ions regulate excitation transfer between photosystems (see e.g., Murata, 1969a; Wong et al., 1979). In fact, fluorescence changes typical for State I and State II can also be induced in isolated chloroplasts/thylakoids that are exposed to different ionic composition (see **Figure 4**): (**1**) When exposed to high [Mg^{2+}], thylakoids in higher fluorescence State I not only have higher PSII antenna size, but their membranes are in high electrostatic screening mode (Murata, 1969a), and are highly stacked; (**2**) Low fluorescent State II occurs if the concentration

		ELECTROSTATIC SCREENING	
		(A) LOW	(B) HIGH
Conditions	Monovalent - K^+;Na^+ [mM]	2-40 mM (low [Mg^{2+}])#	High concentration >40 mM#
	Divalent - Mg^{2+}; Ca^{2+} [mM], [μM]	< 210 μM + 0 mM[K^+] (< 440μM+10mM [K+])#	> 210 μM+0 mM[K^+] (< 440μM+10mM [K+])#
	General conditions	Low salt medium	High salt medium
EFFECT ON PHOTOSYNTHESIS	(1) Chlorophyll *a* fluorescence	Low	High
	(2) Membrane interaction	Membrane separation	Membrane stacking
	(3) PSI/PSII interaction	Close connection - High PSII-PSI spillover	Spatial separation - Low PSII-PSI "spillover"
	(4) State transitions	Low fluorescence State II	High fluorescence State I
	(5) Protein repulsion /aggregations	Protein repulsion	Protein aggregation
	(6) Proposed role in NPQ	Sustained quenching, quenching by PSI	qE, minimal quenching by PSI
	(7) Preferreble uncoupler	Nigericin (K^+/H^+)	A 23187 (Mg^{2+})
	(8) Coupling e- transport/ATP phosphosphorylation	Negative - Removal of the soluble coupling factor (CFI) from ATPase	Positive effect
	(9) PSII photochemistry	Inhibition	Positive effect

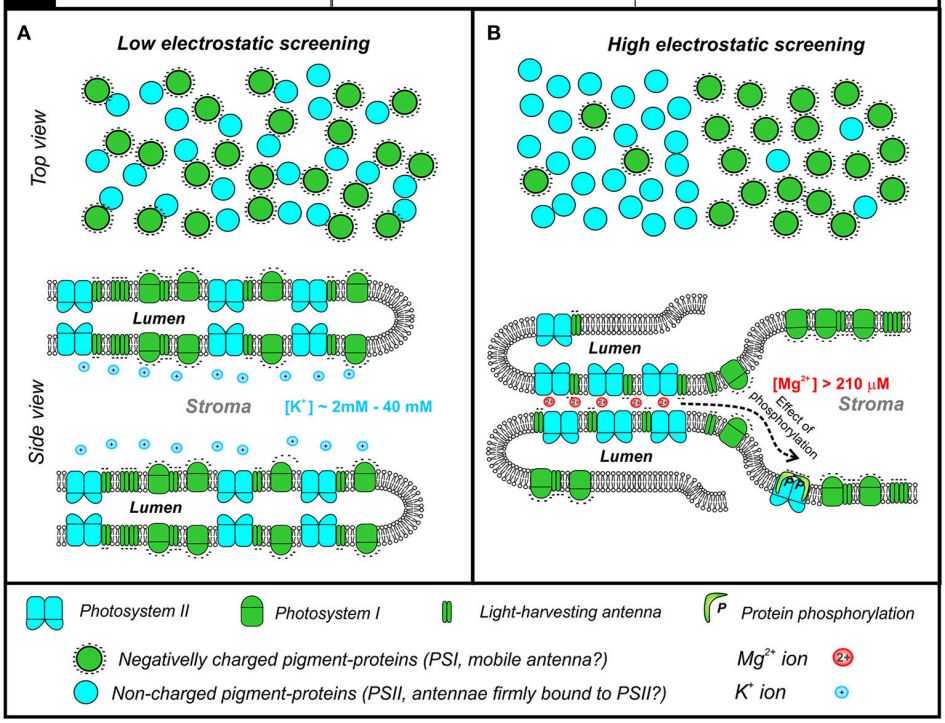

FIGURE 5 | A simplified scheme of changes in protein organization in different states of electrostatic screening (adapted from Barber, 1980b). The table summarizes basic conditions for low or high screening—high screening is induced by high divalent cation content [e.g., >220 μM Mg^{2+} with no (K^+), >440 μM Mg^{2+} with 10 mM (K^+), see (Mills and Barber, 1978) for details]. The switch between low/high screening mode is a complex interplay between monovalent/divalent cation concentrations as it is described by EDL model of thylakoid membrane (Barber et al., 1977). The low/high screening state is then reflected in various physiological processes including intensity of Chl *a* fluorescence (low intensity /high intensity), thylakoid membrane stacking or separation, lower/high excitation spillover between photosystems, State II/State I, protein repulsion/aggregation, proposed role in non-photochemical quenching, preferable uncouplers (Mg^{2+} ionophore A23187 vs. K^+/H^+ antiporter nigericin), effect on electron-proton coupling in photophosphorylation, effect on PSII photochemistry. The screening is caused by ion attachment to membranes that results in screening of the electric-field of the membrane charge (i.e., damping of electrostatic field of fixed charges caused by the presence of interacting ions). **(A)**. Simplified scheme of membrane protein organization in the state of low electrostatic screening. Green cycles represent negatively charged proteins (PSI complex, light harvesting antennas), cyan particles represent less charged PSII. Monovalent cation K^+ is shown as a dominant ion attached to the negatively charged membrane surface. **(B)** A simplified scheme of membrane protein organization in the state of high electrostatic screening, divalent cation, Mg^{2+}, is proposed to be the main ion attached to the negatively charged membrane surface. Role of protein phosphorylation on protein redistribution is indicated (# values were taken from Mills and Barber, 1978).

Mg^{2+} cation is lowered to very low values (below 100 μM, without any other cation like K^+, see Mills and Barber, 1978) or in the presence of monovalent cations (e.g., ∼5 mM K^+) in ion—free medium; in this case, a decrease in PSII fluorescence is considered to be due to State I to State II transition (see **Figure 4**). We note that milimolar [K^+] and [Mg^{2+}], given alone, have opposite effects on fluorescence (K^+ ions decrease, and Mg^{2+} ions increases fluorescence). However, if they are given together, the effect is more complex (for details, see Mills and Barber, 1978). The fluorescence decrease can be also induced by anions; higher effects are observed with increasing valence (Jajoo et al., 1998, 2001). Therefore, it is clear that anions (Jajoo et al., 1998; Jajoo and Bharti, 1999) and monovalent cations can reverse the effect of Mg^{2+} that switches thylakoid membranes into a high electrostatic screening mode (see **Figure 5**). These negative charges act on the outer side of the thylakoid membrane due to the electrostatic interaction with the negative charges of proteins, and this affects the extent of stacking of thylakoid membranes (Barber, 1982).

As a matter of fact, state transitions are triggered by redox shifts of the PQ-pool [the PQ-pool is more reduced in State II (Mullineaux and Allen, 1986)]; in higher plants and in green algae, it is accompanied by phosphorylation of a mobile light-harvesting complex (in State II) (Horton and Black, 1980; Allen et al., 1981; Tikkanen and Aro, 2012). This occurs on the outer surface of thylakoid membranes (for further information, see Vener et al., 2001; Vener, 2007), and contributes to the total negative charge of the outer thylakoid membrane surface. There is a specific STN7 kinase that phosphorylates light harvesting antenna of PSII—LHCII, which is then redistributed to PSI (Bellafiore et al., 2005) during state I to state II transition. On the other hand, another kinase, Stn8, phosphorylates PSII subunits and is involved in PSII repair during photoinhibition (Tikkanen et al., 2008). The current model of state transitions in higher plants involves phosphorylation which leads to addition of negative charges on the mobile LHCIIb phosphate groups provide those charges; this is in agreement with the electrostatic screening model of state transitions that includes a role of negative charges on the pigment proteins (Barber, 1980b, 1982; Staehelin and Arntzen, 1983; Stys, 1995). Indeed, the higher mobility of phosphorylated (mobile) LHCII has been confirmed (see Consoli et al., 2005); see reviews by Mullineaux (2008), Kaňa (2013), Kirchhoff (2014). The connection between electrostatic screening, protein phosphorylation, protein redistribution and membrane organization (stacking/de-stacking) is still not clear; in all likelihood, phosphorylation of PSII in plants exposed to high light enhances the stacking dynamics of the photosynthetic membranes (Barber, 1982; Fristedt et al., 2010).

The molecular mechanism of excitation energy redistribution during state transitions is either due to antenna (LHC II) redistribution between the two photosystems, or a change in PSI/PSII interaction. The latter causes differences in "excitation energy spill over" from one system to another (from the "slower" PSII to the "faster" PSI, see e.g., Mirkovic et al. (2016) where PSI acts as an efficient fluorescence quencher (Barber, 1982; Slavov et al., 2013). In some cases, the antenna uncoupled from one photosystem (e.g., from PSII) is not necessarily coupled to the other photosystem (e.g., PSI) and remain(s) uncoupled in the membrane (Kaňa et al., 2009; Kirilovsky et al., 2014; Unlu et al., 2014; Cheregi et al., 2015). The role of these uncoupled antennas needs to be taken into account in any future model of state transitions (Goldschmidt-Clermont and Bassi, 2015). Based on the correlation between state transitions, membrane stacking and ion composition, and interconnection between state transition mechanism and changes in electrostatic screening (Barber, 1980a), we may very well consider combining the phenomenon of electrostatic screening with the current model of state transitions in higher plants that requires getting a negative charge through phosphorylation (Tikkanen and Aro, 2012). We note that phosphorylation can have a dramatic effect on the membrane surface charge as there are multiple phosphorylation sites on PS II, and each phosphorylation provides two negative charges at a stromal pH of 8.0. Further experiments and analysis are needed to reach this goal.

The molecular mechanism of state transitions representing antenna redistribution and/or change in excitation energy spillover between PSII/PSI is caused by a variation in the balance between electrostatic repulsion (coulombic repulsive forces between negative charges of proteins affected by electrostatic screening) and protein attraction caused by van der Waals forces that are independent of charges on the proteins (see e.g. Barber, 1980b; Stys, 1995). The higher/lower electrostatic screening of negatively charged proteins by ions affects the balance between protein repulsion (low electrostatic screening, **Figure 4**) and attraction (high electrostatic screening). This concept is based on **D**erjaguin-**L**andau-**V**ervey-**O**verbeek (DLVO) theory for the aggregation of colloids—more divalent cations lead to membrane stacking due to reduction in electrical repulsion (Barber, 1982; Stys, 1995). This process occurs *in vitro* when changes in [Mg^{2+}] affect PSII LHCII interactions (see e.g., Kiss et al., 2008). Moreover, membrane stacking can be induced also by lowering of stromal pH to about 5.4 inducing electrostatic neutralization (i.e., by increasing [H^+]), which is closer to the isoelectric point of the thylakoid membrane (Gerola et al., 1979; Jennings et al., 1979). The stimulating effect of low pH on stacking has also been shown in the light-induced spontaneous tendency of thylakoid membranes to stack (Janik et al., 2013). Proteins can lose charges when the pH of the medium is close to the pKs of their negatively charged amino acids (Nakatani and Barber, 1980; Behrens et al., 2013); close to their pKs, proteins become electroneutral. However, in this case membrane stacking is usually caused by charge neutralization, not by charge shielding through EDL. Moreover, this effect is not connected with protein redistribution and with fluorescence changes (Barber, 1980a).

However, when thylakoid membranes stack, Chl *a* fluorescence increases, and State I induced by high Mg^{2+} (high electrostatic screening state, see **Figure 5**) usually appears simultaneously (Barber, 1980a; Barber et al., 1980). In this model, which is based on EDL, ion-dependent effects on antenna proteins are single regulatory events [e.g., re-arrangement of different super complexes at different [Mg^{2+}] inducing stacking of thylakoid membranes leading to changes in Chl *a* fluorescence (Rumak et al., 2010)]. However, there are some special cases when they occur independently of each other, usually when direct

charge neutralization (direct ion/proton binding) occurs (Mills and Barber, 1978; Barber, 1980a). An example is Mg^{2+} induced antenna coupling with PSII, without membrane stacking (Kiss et al., 2008); whether this is because of electronic screening, or neutralization, needs to be ascertained (Mills and Barber, 1978; Barber, 1980a; Scoufflaire et al., 1982). However, we speculate that these ion-induced effects (membrane stacking/fluorescence changes) are controlled by two different mechanisms that usually, but not always, co-occur.

In fact, the two independent processes induced by Mg^{2+} addition have also been shown to exist through fluorescence measurements (Jennings et al., 1982). In one case, an ion effect has been shown to work only on the stromal side (e.g., by the application of impermeable poly L-lysine): there was restacking without fluorescence increase (Berg et al., 1974). Moreover, it has been clearly shown that ion-induced thylakoid stacking/unstacking in the grana region can appear without any change in variable Chl *a* fluorescence, and without any change in the connectivity between different units of antenna (Kirchhoff et al., 2004), again depending on whether electrostatic screening or neutralization is present (Mills and Barber, 1978; Barber, 1980a; Scoufflaire et al., 1982). On the contrary, these phenomena are found to be connected in thylakoids (granal and stromal membranes) indicating the importance of stroma for thylakoid membrane reorganization (Kirchhoff et al., 2004). This indicates a role of a stromal factor, or a requirement of *less-protein-crowded* stromal thylakoids for grana reorganization. Based on the hypothesis proposed by Barber (1980a), the membrane stacking connected with fluorescence changes represents a switch between high/low electrostatic screening (i.e., it is an effect of electrostatic interaction—EDL); the disconnection between fluorescence and membrane stacking is caused by the presence of membrane charge neutralization, which means direct interaction of ions (or protons) with membrane charges. There are also some other experimental data indicating that proton/ion regulation of light-harvesting requires at least two independent regulatory events (Wollman and Diner, 1980; Jennings et al., 1982; Kirchhoff et al., 2003, 2004; Stoitchkova et al., 2006; Kiss et al., 2008); plausible candidates for these effects could be screening or binding of ions (Barber, 1980a). However, to explore conditions, when these processes (i.e., membrane stacking and state transitions) are independent, additional experiments affecting the ratio of ion screening/ion binding are needed to confirm the proposed concepts.

The mechanism of state transition(s) requires a certain reorganization of thylakoid membrane proteins. The positive effect of protein phosphorylation on structural flexibility of the thylakoid membrane architecture has been confirmed (Varkonyi et al., 2009). Thylakoid membrane proteins are also differently organized (random or into domains) for different electrostatic screening (low or high; see **Figure 4**; Barber, 1982). The movement of these differently charged proteins is induced by lateral charge displacement on the outer side of the thylakoid membranes (see e.g., Barber, 1986); this can be induced either by protein phosphorylation higher mobility of phosphorylated (mobile) LHCII (Consoli et al., 2005) or by some unspecific effects of ions causing columbic repulsion between PSII and PSI. We note that only with screening (i.e., electrostatic effect of ions without interaction), we have lateral diffusion of PSI (in stroma lamellae) and of PSII (in grana), as well as changes in spillover from PSII to PSI. With neutralization of surface charges by ion binding (e.g., protonation at low pH, Barber et al., 1980), the membranes simply collapse on each other to give a grana-like appearance with no lateral separation of PSI and PSII. Therefore, increase in the electrostatic screening leads to the formation of heterogeneous domains of low-charge/high-charge, resulting in fluorescence changes (Barber et al., 1980).

REGULATORY ROLE OF PROTONS AND IONS IN TRIGGERING NON-PHOTOCHEMICAL QUENCHING OF THE CHLOROPHYLL EXCITED STATE

Non-photochemical quenching (NPQ) of the excited state of chlorophyll *a* is a process that protects PSII against excess light (Ruban et al., 2012; Zaks et al., 2013; Croce and Van Amerongen, 2014); for further details see Demmig-Adams et al. (2014) by stimulating the dissipation of excessive irradiation into heat (Kaňa and Vass, 2008). NPQ significantly reduces the quantum yield of variable fluorescence (by even 60%, see Ostroumov et al., 2014) and affects the efficiency of energy transfer from the antenna to the reaction centers (see review on the energy-transfer dynamics in photosynthesis Mirkovic et al., 2016). In higher plants, NPQ occurs mainly in the light-harvesting antenna (Gilmore et al., 1995; Horton et al., 1996; Belgio et al., 2012). However, in cold tolerant plants or in certain extremophiles (algae or cyanobacteria), closed PSIIs can also act as quenchers (see e.g., Ivanov et al., 2008; Krupnik et al., 2013). Kinetically, NPQ is divided into at least three major components: (**1**) the "energy dependent" quenching (qE), which is triggered by a faster (<1 min) light-driven proton translocation across the thylakoid membrane (Barber, 1976; Krause et al., 1983); (**2**) a slower, less dominant, quenching component that has been attributed to state transitions (qT—Allen et al., 1981); and (**3**) slowest components, such as zeaxanthin-dependent quenching, qZ (Nilkens et al., 2010; Ocampo-Alvarez et al., 2013), and a photoinhibitory quenching, qI (Krause, 1988), more generally, simply a "sustained quenching" (Ruban and Horton, 1995).

The triggering role of protons in qE activation was proposed for the first time by Wraight and Crofts (1970) for cyclic electron flow in samples where PSII activity was inhibited by DCMU, and diaminodurene (DAD), a mediator of PSI-dependent electron transport stimulating ΔpH, was added (see discussion in Papageorgiou and Govindjee, 2011). The triggering of qE by luminal protons *in vivo* was established directly by pH titration in isolated chloroplasts (Briantais et al., 1979). Finally, a direct protonation of pigment-proteins has been observed *in vitro* (Ruban et al., 1994; Walters et al., 1996; Kaňa et al., 2012b; Xiao et al., 2012; Belgio et al., 2013). The qE sensitivity to luminal protons is controlled by various allosteric regulators (see the details of the concept in Ruban et al., 2012) including xanthophylls (e.g., zeaxanthin, and violaxanthin Niyogi et al.,

1998; Kaňa et al., 2016) and the PsbS protein (see e.g., Li et al., 2000; Johnson and Ruban, 2010).

The co-regulation of low pH-induced qE with ions, and with electrostatic screening of thylakoids, is not clear. Noctor et al. (1993) have shown that a relatively high concentration of Mg^{2+} (about 10 mM) is necessary to obtain maximal qE in isolated thylakoids. This Mg^{2+}-dependent mechanism for NPQ (probably related to quenching by PSI, see below) can be also induced by changes in Mg^{2+} concentration in the dark (Briantais et al., 1979; Krause et al., 1983). This could indicate that the maximal extent of flexible qE requires high electrostatic screening of thylakoid membrane charges, which means a higher content of Mg^{2+} on the thylakoid surface (see **Figure 5**). However, some specific effects of ions on antenna aggregation, and, thus, fluorescence quenching, cannot be totally excluded. Mills et al. (1976) have suggested existence of a cation-sensitive site influencing fluorescence on the stromal surface of thylakoids, based on a similar effect of impermeable choline and K^+ on fluorescence decrease. A possible ionic effect of K^+ has also been shown for the "slow" component of NPQ based on experiments with added valinomycin, a specific K^+ ionophore (Sokolove and Marsho, 1979). However, this data, seemingly, contradicts earlier data showing that qE is sensitive only to K^+/H^+ antiporter—nigericin, but not inhibited by valinomycin (Wraight and Crofts, 1970). Therefore, further experiments that would more carefully consider screening mode (high/low—see **Figure 5**) are needed to resolve the above-mentioned discrepancy.

Ion concentration can also affect Chl *a* fluorescence quenching *in vivo* (Mohanty et al., 1974; Ogawa et al., 1982); however, the mechanism of its effect is not yet clear. Further, we could propose a different mechanism for qE-triggering at low electrostatic screening of thylakoid membrane charges (see **Figure 5**) when spillover of excitation energy from PSII to PSI may be increasing—in this case, PSI may be acting as a fluorescence quencher. A similar mechanism for NPQ has been proposed for desiccating mosses and lichens (Yamakawa et al., 2012; Slavov et al., 2013). This could be a typical photoprotective NPQ mechanism during desiccation (Bilger, 2014) or in certain algae it could be due to a high spillover of energy from PSII to PSI (see e.g., data obtained with algae *C. velia* Quigg et al., 2012; Kotabová et al., 2014). Further, data of Ruban and Horton (1995) indicate that pH-independent fraction of sustained quenching can be inhibited by the addition of nigericin, a K^+/H^+ uncoupler. One could speculate that both, i.e., quenching by PSI (due to spill over of excitation energy from PSII to PSI) and pH independent fraction of sustained quenching are present at low electrostatic screening mode (see **Figure 5**). However, to confirm these hypotheses, further experiments are needed.

We suggest that the co-regulation of NPQ by both pH and ions (e.g., by Mg^{2+}) is indicated by Mg^{2+} counter ion transport upon exposure of photosynthetic samples to light (**Figure 1**) since the accumulation of protons in the lumen is balanced by the efflux of Mg^{2+} ions to the surface of the thylakoid membrane (Hind et al., 1974; Chow et al., 1976; Ishijima et al., 2003). Moreover, there is data showing the presence of Mg^{2+} transporter in the chloroplast (Drummond et al., 2006) and light induced changes in Mg^{2+} concentration (Ishijima et al., 2003). The stimulating role of Mg^{2+} on protein aggregation *in vitro* is well known: higher Mg^{2+} content accelerates reversible quenching of Chl *a* fluorescence in isolated antenna by forming aggregates of LHCIIs *in vitro* (Ruban et al., 1994). This supports the idea that antenna aggregation is a plausible mechanism for qE quenching (Horton et al., 1991). Similar aggregation induced quenching can be also caused by chemicals having strong effects of membrane impermeable cations such as the polyamines (e.g., petruscine, spermidine, and spermine) on Chl *a* fluorescence quenching (Ioannidis and Kotzabasis, 2007). These organic compounds are synthesized by living cells, and they can quench the maximal fluorescence in the dark and stimulate NPQ in light *in vivo* in higher plants (Ioannidis and Kotzabasis, 2007; Ioannidis et al., 2012) and in algae (Ioannidis et al., 2011). This simulation of NPQ seems to be due to antenna aggregation (Tsiavos et al., 2012).

The above—mentioned phenomenon has been confirmed *in vivo*, and the effect, to a great extent, simulates proton-triggered quenching in isolated antenna (Tsiavos et al., 2012; Malliarakis et al., 2015). Further, this is in agreement with the effect of other tertiary amines—e.g., dibucaine, which has been shown to stimulate NPQ (Ruban et al., 1994; Phillip et al., 1996; Gilmore and Yamasaki, 1998) and bind to thylakoid membrane surfaces (Gilmore and Yamasaki, 1998). As Mg^{2+} accumulates on the thylakoid membrane surface when it is exposed to light (Hind et al., 1974), we can speculatethat the cations (polyamines, Mg^{2+}) present on the stromal surface could synergistically stimulate qE (triggered by low lumen pH) by allowing antenna aggregation. This type of mechanism would be in line with the presence of a *"cation sensitive site"* on the stromal side of the thylakoids (Mills et al., 1976). Indeed, one from the subdomain of CP29 antenna from spinach has been proposed to be regulated by chemiosmotic factor (Ioannidis et al., 2016). However, the stimulatory effect of ions on NPQ of fluorescence is, apparently, against the proposed role of Mg^{2+} in EDL theory (see **Figure 4**), where Mg^{2+} induced high screening mode is observed by high Chl *a* fluorescence. It seems that there are two quite different mechanisms for cation effects on Chl *a* fluorescence yield; it could be by direct binding (no fluorescence increase, see **Figure 5**; possibly stimulating NPQ) or by the effect on EDL (no binding connected with fluorescence increase). However, in any case these contradictory conclusions require new experimental approaches to be used.

GENERAL ASPECTS OF LIGHT-HARVESTING AND ION TRANSPORT

Protons have distinct effects on the efficiency of the light-harvesting process at the level of excitation energy dissipation of excess light by NPQ of excited state of Chl *a* molecules (Ruban et al., 2012; Adams et al., 2014; Demmig-Adams et al., 2014; Horton, 2014), whereas, other ions may regulate excitation distribution and redistribution, and stacking of thylakoid membranes (Barber, 1982; Staehelin and Arntzen, 1983; Minagawa, 2011; Papageorgiou and Govindjee, 2011). Proton-induced membrane stacking is a well-known phenomenon

(Gerola et al., 1979; Jennings et al., 1981a,b). It is caused by neutralization of negative charges on proteins at high proton concentration (see e.g., Barber, 1980a). In photosynthetic cells, these processes are interconnected and light-induced proton pumping is intertwined with counter ion transport (Barber et al., 1974; Hind et al., 1974). Light-induced accumulation of protons in the lumen is balanced mostly by the efflux of Mg^{2+} ions (Hind et al., 1974; Chow et al., 1976; Ishijima et al., 2003) or K^+ ions (Chow et al., 1976; Tester and Blatt, 1989; Carraretto et al., 2013) to the stroma and/or uptake of Cl^- ions into the lumen (Hind et al., 1974; Vambutas and Schechter, 1983; Vambutas et al., 1984, 1994). This has been confirmed by the observation of a light-induced increase in K^+ and Mg^{2+} concentration in the stroma (Dilley and Vernon, 1965; Hind et al., 1974; Krause, 1977; Portis, 1981), and the accompanying higher Cl^- concentration in the lumen (Hind et al., 1974; Vambutas and Schechter, 1983).

It is not yet clear which counter ion is dominant in chloroplasts *in vivo* since most of the earlier measurements were done on isolated chloroplasts where proton exchange with the cytoplasm could be under-or over-estimated (see e.g., Hind et al., 1974). However, a different major influence of cations has been suggested (see discussion by Cruz et al., 2001) which leads to shrinking of thylakoids after illumination (Dilley and Vernon, 1965; Nobel, 1968). However, there are irreconcilable findings: thylakoid lumen shrinkage (Posselt et al., 2012), and its opposite, a swelling (Kirchhoff et al., 2011). We need an answer to this dilemma. Generally, in most experiments, Mg^{2+} has been suggested to be the major physiological counterion for H^+ pumping into the chloroplast (Barber et al., 1974; Hind et al., 1974; Barber, 1976; Enz et al., 1993; Cruz et al., 2001; Ishijima et al., 2003) since another mobile cation, K^+, seems to be rather bound or trapped in the chloroplast (**Figure 1**). Inorganic anions induce state changes in spinach thylakoid membranes (Jajoo et al., 1998) that may indicate a role for Cl^- as a counter ion since its transport into the thylakoid lumen could compensate for the H^+ resulting in thylakoid membrane swelling, observed upon exposure of chloroplasts to light (Kirchhoff et al., 2011). It is also plausible that different counterions behave differently under high/low light. Indeed, counterions for H^+ pumping are affected by lumen pH, which would indicate that different ions act as counter-ions at low light (Cl^-) and at high light [Mg^{2+}] (Ben-Hayyim, 1978).

All the available data support the concept that the role of protons and of all other ions in the regulation of photosynthesis is interconnected since proton/cation antiport (proton/anion symport) is required during photophosphorylation as the electrochemical part of pmf (Ort and Melandri, 1982; Cruz et al., 2001) is reduced in the presence of light in higher plant chloroplasts (**Figure 1**). This idea is further supported by the fact that stacking of thylakoid membranes can be induced either by high proton or with high Mg^{2+} concentration (cf. Gerola et al., 1979; Jennings et al., 1979, 1981a,b; Barber, 1980a). Thus, research on the regulation of light-harvesting efficiency requires a new approach when the effects, and mechanisms, of both protons and all other ions will be addressed simultaneously. This is even more important in light of the discovery of several ion channels and transporters in the chloroplast (see, e.g., Carraretto et al., 2013; Armbruster et al., 2014; Hamamoto and Uozumi, 2014; Kunz et al., 2014; Herdean et al., 2016; also see recent reviews by Checchetto et al., 2013; Hanikenne et al., 2014; Pfeil et al., 2014; Finazzi et al., 2015; Xu et al., 2015; Carraretto et al., 2016).

AUTHOR CONTRIBUTIONS

Both the authors have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Chloroplast Iron Transport Proteins – Function and Impact on Plant Physiology

*Ana F. López-Millán[1], Daniela Duy[2] and Katrin Philippar[2]**

[1] Department of Pediatrics, Children's Nutrition Research Center, Baylor College of Medicine, United States Department of Agriculture/Agricultural Research Service, Houston, TX, USA, [2] Plastid Fatty Acid and Iron Transport – Plant Biochemistry and Physiology, Department Biology I, Ludwig-Maximilians-University of Munich, Munich, Germany

Chloroplasts originated about three billion years ago by endosymbiosis of an ancestor of today's cyanobacteria with a mitochondria-containing host cell. During evolution chloroplasts of higher plants established as the site for photosynthesis and thus became the basis for all life dependent on oxygen and carbohydrate supply. To fulfill this task, plastid organelles are loaded with the transition metals iron, copper, and manganese, which due to their redox properties are essential for photosynthetic electron transport. In consequence, chloroplasts for example represent the iron-richest system in plant cells. However, improvement of oxygenic photosynthesis in turn required adaptation of metal transport and homeostasis since metal-catalyzed generation of reactive oxygen species (ROS) causes oxidative damage. This is most acute in chloroplasts, where radicals and transition metals are side by side and ROS-production is a usual feature of photosynthetic electron transport. Thus, on the one hand when bound by proteins, chloroplast-intrinsic metals are a prerequisite for photoautotrophic life, but on the other hand become toxic when present in their highly reactive, radical generating, free ionic forms. In consequence, transport, storage and cofactor-assembly of metal ions in plastids have to be tightly controlled and are crucial throughout plant growth and development. In the recent years, proteins for iron transport have been isolated from chloroplast envelope membranes. Here, we discuss their putative functions and impact on cellular metal homeostasis as well as photosynthetic performance and plant metabolism. We further consider the potential of proteomic analyses to identify new players in the field.

Keywords: chloroplast, iron transport, metal homeostasis, membrane protein, transporter

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****Correspondence:***
Katrin Philippar
philippar@lmu.de

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INTRODUCTION

Chloroplasts, which are unique and highly specialized organelles, originated from the endosymbiosis of an ancestor of today's cyanobacteria with a mitochondria-containing host cell (Gould et al., 2008). In higher plants, chloroplasts perform essential functions of primary and secondary metabolism, but first and foremost are the site of photosynthesis and thus represent the basis for all life dependent on atmospheric oxygen and carbohydrate supply. In addition to mature, autotrophic chloroplast of green leaves, the plastid organelle family includes many specialist types with manifold biosynthetic functions in certain tissues and developmental

stages (e.g., proplastids, etioplasts, elaioplasts, gerontoplasts or chromoplasts). Since, however, due to experimental accessibility most research has been performed on chloroplasts, in the following we will simply refer to plastids, if besides chloroplasts other organelle types are involved. Nevertheless, all metabolic functions of plastids require different selective transport mechanisms across the outer and inner envelope (IE) membranes of the organelle.

In plant cells, the transition metal iron (Fe) plays a major role in redox reactions and as cofactor of many proteins due to its potential for valency changes (Raven et al., 1999). In chloroplasts, Fe is an important component of the photosynthetic apparatus - i.e., found in all electron transfer complexes (PSII, PSI, cytochrome b_6f complex, and ferredoxins) - and is required for biogenesis of cofactors such as heme and Fe–S cluster (for overview see Balk and Schaedler, 2014; Briat et al., 2015). Besides being essential components of the photosynthetic electron transport, Fe-cofactor containing proteins are also involved in protein import and chlorophyll biosynthesis. Chloroplasts represent the Fe-richest organelle in plant cells containing 80–90% of the Fe found in leaf cells (Terry and Low, 1982). However, excess Fe generates ROS, which cause oxidative damage (for overview Briat et al., 2010b). In chloroplasts, this situation is most prominent, since Fe and ROS, like hydrogen peroxide (H_2O_2) produced by the photosynthetic electron chain are in close proximity (Asada, 1999; Mubarakshina et al., 2010). In consequence, free unbound Fe leads to the formation of hydroxyl radicals via the Fenton reaction (Halliwell and Gutteridge, 1992). On the other hand, plastid Fe-deficiency impairs chlorophyll biosynthesis, leads to leaf chlorosis and requires remodeling of the photosynthetic apparatus (Spiller and Terry, 1980; Moseley et al., 2002). Chloroplasts suffering from Fe starvation are specifically impaired in proper function of photosystem I (PSI), which contains 12 Fe atoms per monomer. Thus, to maintain cellular Fe-homeostasis and guarantee proper plant development and growth, Fe transport into and out of plastids as well as Fe storage and buffering of free Fe within these organelles need to be tightly controlled (Abadía et al., 2011; Briat et al., 2015). Furthermore, besides in the plastid family, strict control of Fe homeostasis occurs within the plant cell, other organelles and throughout all organs (see Thomine and Vert, 2013).

In this review we will first present an overview of the characteristics of chloroplast Fe-acquisition known so far. Next we describe the characteristics and function of proteins involved in chloroplast Fe transport. Since this transition metal plays an important role not only in many processes in plastid organelles, but also for plant performance in general, we discuss the impact of plastid Fe-homeostasis and transport on plant physiology.

CHLOROPLAST IRON ACQUISITION

The mechanisms by which Fe is obtained by chloroplasts are not as well-known as the two Fe-acquisition strategies - reduction-based (strategy I) and chelation based (strategy II) - across the plasma membrane of root cells (for overview see Morrissey and Guerinot, 2009; Kobayashi and Nishizawa, 2012; Brumbarova et al., 2015). Nearly two decades ago, uptake experiments with isolated intact chloroplasts and purified IE membrane vesicles described the existence of a light-dependent plastid uptake of ^{59}Fe(III), chelated by epihydrohymugineic acid in barley - a strategy II plant (Bughio et al., 1997). The absorption of Fe by illuminated chloroplasts was inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea, an inhibitor of photosystem II, suggesting that Fe absorption depends upon electron transport or the ATP generated in thylakoids. On the other hand, in the strategy I plant pea, an inward Fe^{2+} transport accross the IE of the chloroplast was described (Shingles et al., 2001, 2002). This Fe^{2+} transport was inhibited by Zn^{2+}, Cu^{2+}, and Mn^{2+} in a competitive manner, and was activated by protons, similar to the reduction based iron (Fe^{2+}) acquisition mechanism in roots. More recently, Fe-uptake experiments using bathophenantroline disulfonate (BPDS) on isolated sugar beet (*Beta vulgaris*) chloroplasts described that ferric (Fe[III]) citrate was preferred over ferrous (Fe[II]) citrate as an iron source (Solti et al., 2012). This Fe uptake was strongly connected to the photosynthetic performance of the chloroplast and subjected to negative feedback regulation. There are evidences of a reduction-based mechanism for chloroplast Fe-acquisition in strategy I and II plants, since the existence of a chloroplast ferric chelate oxidoreductase (FRO) has been demonstrated at the enzymatic activity level (Mikami et al., 2011; Solti et al., 2014). In intact barley (*Hordeum vulgare*) chloroplasts, FRO activity was induced by Fe deficiency under light, whereas it was repressed under dark conditions (Mikami et al., 2011). On isolated *B. vulgaris* chloroplast envelope fractions, Solti et al. (2014) showed that similar to the strategy I Fe-uptake in roots, a FRO enzyme, which clearly localized at the IE, and not the OE membrane, was responsible for complexed Fe(III) reduction and production of free Fe^{2+} for Fe uptake. This enzymatic activity was higher with NADPH than NADH, again suggesting the dependence of the Fe-acquisition mechanism on photosynthesis. The biphasic kinetics and its modification under Fe-deficient conditions also indicated the existence of high and low affinity mechanisms for Fe reduction. Since in the model plant *Arabidopsis thaliana* most likely only one member of the FRO family is localized in chloroplasts, this kinetics could be related to post translational modifications, differential splicing or to the existence of a yet unknown Fe-reduction mechanism.

In *Arabidopsis,* the GFP-tagged FRO7 protein localizes to the chloroplast envelope (Jeong et al., 2008; **Figure 1**). Here, At-FRO7 is required for survival under Fe-limiting conditions, for efficient photosynthesis, and for proper chloroplast Fe acquisition in young seedlings (Jeong et al., 2008). The *fro7* loss-of-function mutants accumulate approximately 33% less iron per microgram of chlorophyll, show less Fe(III)-chelate reductase activity in isolated chloroplasts than the wild-type and have a chlorotic appearance when grown under Fe-limiting conditions. However, FRO7 is not Fe regulated (Mukherjee et al., 2006), the *fro7* loss-of-function mutants do not show visible growth phenotypes under standard conditions and their phenotype under Fe deficiency can be rescued by Fe addition, therefore suggesting the existence of redundant Fe uptake systems in chloroplasts and/or specialized plastids. In addition, staining of

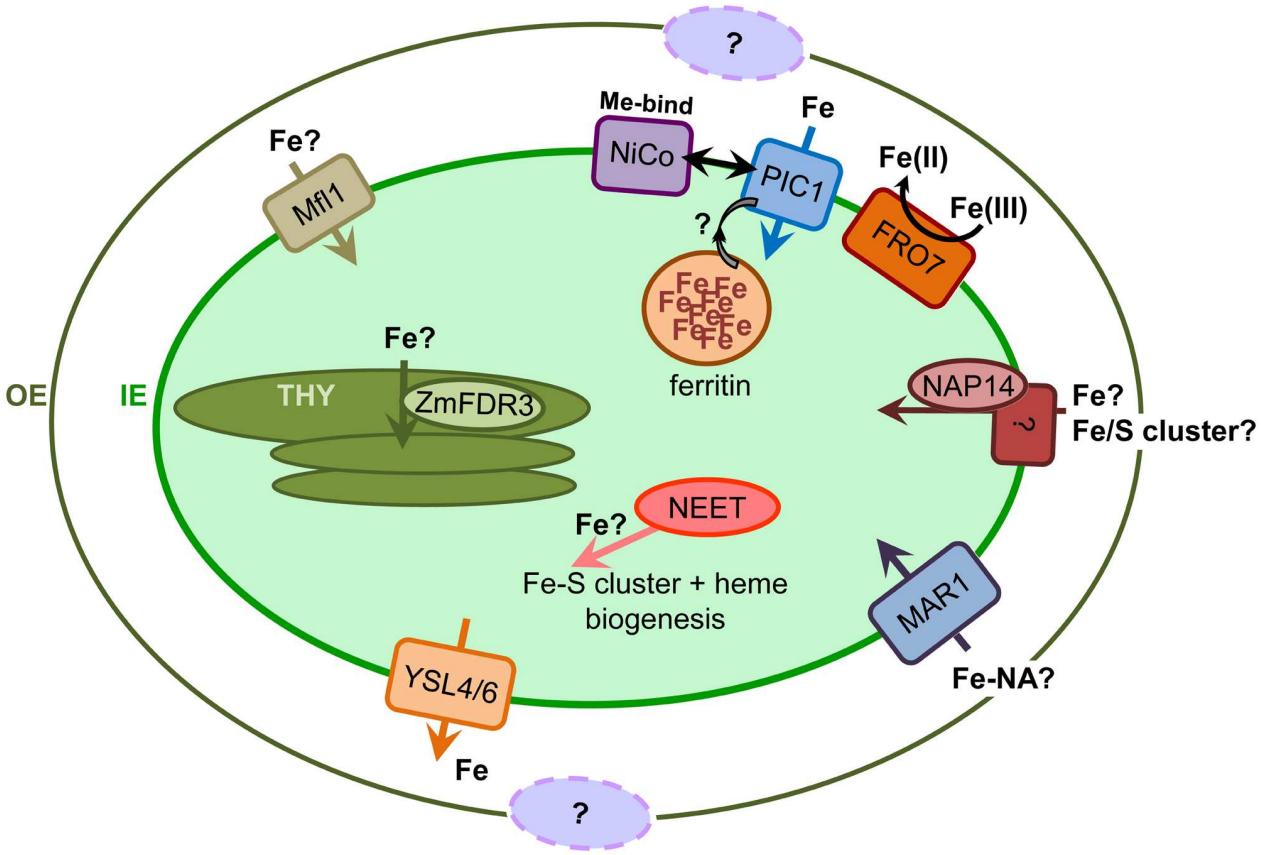

FIGURE 1 | Iron transport and homeostasis in chloroplasts. Proteins and their possible functions in plastid Fe-transport and homeostasis are described throughout the text. Please note that for the sake of convenience, all transport proteins are depicted at the inner envelope membrane (IE), although only PIC1 could be localized unequivocally in the IE. Fe-transport proteins in the outer envelope (OE) are still unknown. For details on localization and structure of proteins compare **Table 1**. Me-bind, metal binding domain; THY, thylakoid membranes.

FRO7 promoter-GUS reporter lines revealed low expression in mature leaves (Jeong et al., 2008), supporting that its role may be ascribed to certain early developmental stages. The latter findings might also explain why - although containing several membrane-spanning domains (**Table 1**) - FRO7 is not found in envelope membranes within the AT_CHLORO database, which represents a comprehensive summary of proteomic analyses on chloroplast proteins with sub-plastidial annotation (Ferro et al., 2010).

In summary, these observations indicate that chloroplast Fe-transport across the outer envelope (OE) membrane occurs most likely via Fe(III) chelates, preferably Fe(III) citrate. Uptake across the IE, however, seems to occur mainly in the form of free Fe^{2+} and is accomplished by a reduction based and proton-driven mechanism similar to strategy I Fe-uptake in roots. However, other Fe-acquisition mechanisms cannot be excluded and also might depend on plant species, which prefer either strategy I or II mechanisms, different plastid types and/or developmental stages of tissues and organs. A certain diversification for Fe uptake has already been described in Gram-negative cyanobacteria - the evolutionary progenitors of chloroplasts - that import Fe(III) chelates or oxides across the outer membrane but at their plasma membrane either use a reduction based uptake of Fe^{2+} by the FeoB transporter or directly transport oxidized Fe^{3+} bound to periplasmic binding proteins *via* the Fut ABC transporter complex (see Kranzler et al., 2013, 2014).

Chloroplast Iron Transport Proteins

Due to their endosymbiotic origin, chloroplasts as well as mitochondria are unique since they are surrounded by two membranes similar to their Gram-negative prokaryotic ancestors. Solute transporters in the IE membrane of chloroplasts were mainly derived from endomembranes of the eukaryotic host cell, and only few proteins have a prokaryotic origin, coming either from the membranes of the endosymbiont itself or from intracellular bacterial parasites (Tyra et al., 2007; Fischer, 2011). Surprisingly in contrast, the chloroplast OE largely originated from, and still resembles the outer membrane of the Gram-negative cyanobacterial-like endosymbiont (Block et al., 2007). In the chloroplast IE, numerous metabolite and ion transporter proteins have been identified and characterized thoroughly with respect to their physiological role and molecular mechanisms (Weber and Linka, 2011; Finazzi et al., 2015). These channels and transporters are hydrophobic, mainly

TABLE 1 | Proteins involved in chloroplast Fe acquisition and homeostasis as described throughout the text.

		Localization						
Name	**AGI**	**ee**	**AT_C**	**cTP**	**α-TM**	**Putative function**	**Evidence**	**Reference**
At-FRO7	At5g49740	ENV (GFP)	n.p.	y	10	Fe (III) reduction	*fro7* ko activity in yeast	Jeong et al., 2008
At-PIC1	At2g15290	IE (GFP, IMB)	IE	y	4	Fe uptake	*pic1* ko; *PIC1* ox yeast comp.	Duy et al., 2007b; Duy et al., 2011
Nt-PIC1	n.a.	ENV (GFP)	n.a.	y	4	Fe uptake	*Nt-PIC1* RNAi + ox yeast comp.	Gong et al., 2015
At-NiCo	At2g16800	ENV (GFP)	IE	y	6	Complex with PIC1 metal binding/transport	Interaction y2h metal binding domain	Duy et al., 2011; Eitinger et al., 2005
Os-ZN (=NiCo)	n.a.	THY?[1] (GFP, ind. IMB)	n.a.	y	6–7	ROS protection	*Os-zn* pm	Li et al., 2010
At-YSL4	At5g41000	TON (GFP)	n.p.	n	14	Fe efflux	*ysl4* + *ysl4ysl6* ko	Divol et al., 2013; Conte et al., 2013
At-YSL6	At3g27020	ENV (IMF, IMB) TON (GFP)	n.p.	n	14	Fe efflux	*ysl4* + *ysl4ysl6* ko *YSL6* ox	Divol et al., 2013; Conte et al., 2013
At-MAR1/IREG3	At5g26820	ENV?[2] (YFP)	n.p.	y	11	NA or Fe/NA uptake	*mar1* ko *MAR1* ox	Conte et al., 2009
At-NAP14	At5g14100	STR (GFP)	IE	y	0	Metal homeostasis and/or metal transport	*nap14* ko	Shimoni-Shor et al., 2010
At-Mlf1	At5g42130	–	IE	y	3–6	Fe transport	*mlf1* ko	Tarantino et al., 2011
Zm-FDR3	n.a.	C[3] (ind. IMF)	n.a.	?	0	Possible transport of Fe-protein	*Zm-FDR3* ox in tobacco yeast comp.	Han et al., 2009
At-FER1	At5g01600	STR*	STR	y	0	Protection against oxidative stress Fe storage	*fer1/3/4* triple ko *fer2* ko	Ravet et al., 2009a; Ravet et al., 2009b;
At-FER2	At3g11050	STR*	n.p.	y	0			
At-FER3	At3g56090	STR*	STR	y	0			
At-FER4	At2g40300	STR*/M (IMB)	STR	y	0	Metal homeostasis	*fer4* klao/frataxin mutant	Murgia and Vigani, 2015
At-NEET	At5g51720	C/M (GFP, IMB) STR (YFP, IMG)	STR	y	0	Fe–S/Fe cluster transfer	*neet* kd *in vitro* Fe-S/Fe transfer	Nechushtai et al., 2012; Su et al., 2013

Names including plant species, Arabidopsis gene identifiers (AGI), the localization defined by experimental evidence (ee) and in proteomic studies (AT-CHLORO database, AT_C; Ferro et al., 2010), predicted chloroplast targeting peptides (cTP), and α-helical membrane-spanning domains (α-TM) according to the Aramemnon database (Schwacke et al., 2003), as well as putative functions and evidence described in the respective references are listed. [1]*: Please note that GFP-signals for Os-ZN have a ring-like appearance and immunoblots are only "indirect" (ind.), i.e., with antibodies directed against the GFP-tag. Thus, a localization of Os-ZN in chloroplast envelopes similar to its ortholog At-NiCo might be possible.* [2]*: For At-MAR1/IREG3, YFP signals stain the entire chloroplast and cannot discriminate between thylakoid and envelope membranes.* [3]*: The authors in Han et al., 2009 suggest presence of Zm-FDR3 in the thylakoid lumen, although "indirect" immunofluorescence against a MYC-tagged FDR3 protein only indicates general appearance in chloroplasts. *: for overview on chloroplast STR localisation of ferritin proteins in various plant species, see Briat et al., 2010a. At, Arabidopsis thaliana; C, chloroplast; comp., complementation; ENV, envelope; GFP, in vivo GFP targeting; IE, chloroplast inner envelope membrane; IMB, immunoblot; IMF, immunofluorescence; IMG, immunogold; kd, knock-down mutant; ko, knockout mutant; M, mitochondrium; n, no; n.a., not applicable; n.p., not present; Nt, Nicotiana tabacum; Os, Oryza sativa; ox, over-expressing line; pm, point mutation; ROS, reactive oxygene species; STR, chloroplast stroma; THY, thylakoid membranes; TON, tonoplast; y2h, yeast two hybrid; y, yes; Zm, Zea mays.*

α-helical membrane proteins facilitating the exchange of ions, and metabolic products with the cytoplasm. The characteristic channels of the outer membrane in Gram-negative bacteria and chloroplast OE, however, span the membrane in the form of β-strands that are organized to form a barrel-like pore structure (Duy et al., 2007a; Zeth and Thein, 2010).

Fe transport across the two envelope membranes of chloroplasts (import and export) is still not well understood (for overview see Nouet et al., 2011; Finazzi et al., 2015). However, research in the last decade has provided increasing evidence, which suggests that several families of proteins may play a role in Fe transport in chloroplasts. To date, proteins that shuttle Fe across the OE have not been identified, nevertheless, they might be similar to the ligand gated, TonB-dependent, β-barrel receptor channels in the outer membrane of Gram negative bacteria, i.e., FecA for Fe(III) citrate in *E. coli* or TBDTs (TonB-dependent transporters) in cyanobacteria (for overview see Chakraborty et al., 2007; Duy et al., 2007a; Kranzler et al., 2013). Direct and unequivocal experimental evidence for integration into the chloroplast IE membrane so far is only provided for the permease PIC1 (see below). However, several other Fe transporters, which have been localized to the chloroplast envelope, most likely are targeted to the IE membrane as well, while none of them has been ascribed to be integral to thylakoids. The current knowledge about Fe-transport and homeostasis proteins in chloroplasts is described below and summarized in **Figure 1** and **Table 1**.

PIC1 Participates in Chloroplast Fe Uptake and Plant Fe Homeostasis

The protein PIC1 (permease in chloroplasts 1) in *Arabidopsis* was identified in a screen for metal transport proteins as the first molecular component involved in plastid Fe-transport (**Figure 1**; Duy et al., 2007b). This integral membrane protein contains four membrane-spanning α-helices and was localized to the IE membrane of chloroplasts by *in vivo* GFP-targeting and immunoblot analysis (see **Table 1**). Interestingly PIC1 represents an ancient permease, clearly originating from the few proteins that were inherited by the cyanobacterial-like endosymbiont (see above; Duy et al., 2007b; Fischer, 2011). The function of PIC1 in Fe transport was verified by the growth complementation of a Fe uptake-deficient yeast strain where PIC1 could increase yeast cellular Fe-levels. Furthermore, PIC1 overexpression lines (PIC1ox) accumulate about 2.5-times more Fe in chloroplasts than wild-type plants (Duy et al., 2011). Thus, the functional analyses in yeast and the phenotypes of *pic1* loss-of-function mutants and PIC1ox lines clearly show that PIC1 participates in plastid Fe-uptake as well as Fe-homeostasis pathways throughout the plant. Plants lacking PIC1 are characterized by a strong dwarf, albino phenotype resembling Fe-deficiency symptoms in plants. In addition, *pic1* mutant plants display disrupted leaf mesophyll organization and a severely impaired development of chloroplasts, which suggests Fe shortage within the organelle (Duy et al., 2007b). Only recently, the function of PIC1 in plastid Fe-transport has been further supported by studies of *PIC1* knockdown and overexpression lines in tobacco (*Nicotiana tabacum*) plants, which showed similar phenotypes to the *Arabidopsis* PIC1 mutants (Gong et al., 2015). Furthermore, Nt-PIC1 was also able to complement growth of Fe-deficient yeast.

Surprisingly, on the one hand *Arabidopsis pic1* knockout plastids/chloroplasts in meristems and leaves show an accumulation of ferritin protein clusters, which most likely are loaded with Fe (Duy et al., 2007b). In addition to their role as Fe-storage proteins during germination, plastid-intrinsic ferritins function in Fe sequestration when Fe is present in excess, thereby protecting cells against oxidative damage (Ravet et al., 2009a; Briat et al., 2010b). On the other hand, the phenotype of PIC1ox mutants clearly resembles that of ferritin loss-of-function plants under Fe excess, showing severe defects in flower and seed development that result in reduced seed yield and germination rates (Ravet et al., 2009a; Duy et al., 2011). Flowers of PIC1ox mutants contain more Fe, while other transition metals are unaffected, whereas seeds show a significant reduction in the concentration of this metal. Furthermore, PIC1 transcript levels are slightly up-regulated in leaf ferritin knockouts (Ravet et al., 2009a). All these findings detailed above point toward a close reciprocal correlation between PIC1 and ferritin which might be explained as follows: (i) In leaves of *pic1* loss-of-function plants, chloroplast-intrinsic Fe levels are low and therefore thylakoid membrane systems are degraded. However, at least in seed and meristem tissues, a plastid Fe-uptake bypass pathway exists, which allows germination and slow growth of *pic1* lines. When leaves mature, Fe-uptake mediated by PIC1 becomes dominant, and cytosolic Fe-levels increase transiently due to the blockage in chloroplast Fe-uptake of *pic1* plants, which in turn leads to oxidative stress and induction of ferritin expression. The plastid-intrinsic ferritin accumulating in *pic1* knockouts thus is most likely induced by a cytosolic signal transduction pathway and stores Fe coming from degraded thylakoids. (ii) In contrast, plastid levels of free Fe-ions significantly increase in both, PIC1ox lines under Fe sufficient and ferritin knockouts under Fe-excess growth conditions. In this situation, plants have to cope with plastid-intrinsic oxidative stress that leads to similar phenotypes concerning flower and, in particular, seed development. Transcriptional profiling of *pic1* and PIC1ox plants revealed that major changes occurred in genes related to metal transport and homeostasis and future studies might contribute to unravel plastid- and nucleus-driven signaling processes under disturbed Fe homeostasis (Vigani et al., 2013).

The permease PIC1 has also been described as Tic21 (translocon at the IE membrane of chloroplasts of 21 kDa), a putative IE translocon component, which could participate in import of nuclear-encoded plastid proteins from the cytosol (Teng et al., 2006). The *Arabidopsis* protein Tic21, which is identical to PIC1, was identified by forward genetics using a screen for mutants defective in chloroplast protein import (Sun et al., 2001). The proposed function of Tic21/PIC1 as essential translocon component in the chloroplast IE by Teng et al. (2006) is mainly based on the accumulation of unprocessed plastid precursor proteins in *tic21/pic1* knockout lines, defects in IE protein translocation in isolated chloroplasts of a sub-lethal *tic21/pic1* mutant, and co-immunoprecipitation of Tic21/PIC1 with other major protein import translocon components. However, a direct functional analysis of Tic21/PIC1 for protein transport is lacking, and analyses of Duy et al. (2007b) failed to detect residual plastid precursor proteins in identical *pic1/tic21* mutant lines. Moreover, plastid-localized ferritin was not only accumulating in *pic1/tic21* plastids, but also properly processed, indicating that PIC1/Tic21 is not involved in protein translocation (for detailed discussion see Duy et al., 2007b). In 2009, a protein complex of about 1 MDa was identified at the chloroplast IE membrane containing the putative translocon channel Tic20, a large fraction (about 900 kDa) of yet unidentified membrane proteins, and also small amounts of Tic21/PIC1 (Kikuchi et al., 2009). The authors hypothesized that this complex might function as a general TIC protein translocation core complex, where Tic21/PIC1 is loosely associated to the periphery. More recent publications, however, which lead to the identification of the other proteins in this putative TIC protein translocation core, demonstrated that Tic21/PIC1 does not co-purify with this 1 MDa complex (Kikuchi et al., 2013; Nakai, 2015). Therefore, the previously described attachment of PIC1/Tic21 to TIC translocon component proteins such as Tic20 might have been generated by the high membrane protein density in the chloroplast IE, rather than by specific protein interactions.

PIC1/Tic21 displays a close phylogenetic relationship to cyanobacterial proteins annotated as putative permease components functioning in solute and/or ion transport across membranes (Duy et al., 2007b). The ortholog of PIC1/Tic21 in *Synechocystis* sp. PCC 6803, Sll1656, can functionally complement *pic1/tic21* loss-of-function plants (Lv et al., 2009)

and the growth of Fe uptake-deficient yeast mutants (Duy et al., 2007b). In contrast, PIC1/Tic21 shares little sequence similarity to protein translocon channels in the chloroplast IE (e.g., Tic20) or to those in the inner membrane of mitochondria (Tim17 and Tim23; Inaba and Schnell, 2008; Balsera et al., 2009), although all of them share a similar structure containing four predicted α-helical membrane domains. According to Gross and Bhattacharya (2009), however, a possible dual function of PIC1/Tic21 in iron transport and protein import is not mutually exclusive, arguing that the beneficial effect of new functional properties (such as protein import) would be an evolutionary addition to its function as an ancient solute permease of cyanobacterial origin.

In addition to the PIC1 function in Fe accumulation in yeast cells (Duy et al., 2007b; Gong et al., 2015), and in *Arabidopsis* chloroplasts when over-expressed (Duy et al., 2011), the interaction of PIC1 with the putative metal transport protein NiCo (Eitinger et al., 2005) at the plastid IE membrane, points to a role in Fe transport (Duy et al., 2011). Furthermore, transcript levels of At2g16800 - one of the two *Arabidopsis* NiCo genes - were increased in *PIC1*ox mutant flowers (Duy et al., 2011). *In vivo* GFP-targeting of the corresponding protein shows characteristic ring-shaped signals of chloroplast envelope proteins, which is supported by the occurrence of At2g16800 in IE proteomics (see **Table 1**; Eitinger et al., 2005; Ferro et al., 2010; Duy et al., 2011). A point mutation of the *NiCo* ortholog in rice (named Os-ZN, for zebra-necrosis protein) resulted in a protein that - although most likely mislocalized to thylakoids (see above and **Table 1**) - caused a yellow-stripe, necrotic leaf phenotype (Li et al., 2010), which was light-dependent and related to ROS production. Therefore, a role of PIC1-NiCo in an iron translocon complex at the IE of plastids is very likely (see **Figure 1**; Duy et al., 2011). Given that NiCo proteins contain specific metal binding domains (Eitinger et al., 2005), a possible molecular mechanism for PIC1-NiCo interaction in *Arabidopsis* IE membranes, could involve Fe binding by At-NiCo and its subsequent transfer to the permease PIC1. However, more direct functional assays for metal transport still need to be established to study the molecular mechanisms in detail.

Other Proteins Participating in Fe Transport Across the Chloroplast Envelope

Two transporters from the "yellow stripe 1-like" family of *Arabidopsis*, YSL4 and YSL6, have been characterized as potential plastid Fe-efflux transporters (**Figure 1**; Divol et al., 2013). Both genes are up-regulated in response to Fe excess and at least one of them, YSL6, was unequivocally localized to the chloroplast envelope by immunoblot and immuno-fluorescence analysis (**Table 1**; Divol et al., 2013). However, if YSL6 integrates into the OE or IE, still remains an open question, since the protein could not be identified in proteomic analyses of chloroplast proteins. Furthermore, neither YSL4 nor YSL6 contain a classical, N-terminal chloroplast targeting peptide (see **Table 1**), a feature which is more common for OE than for IE proteins. Phenotypical characterization of single and double knockout mutants showed that Fe accumulated in the chloroplasts, and this occurred concomitantly with an increase in ferritin, whereas ubiquitous over-expression of YSL4 and YSL6 caused sensitivity to Fe and a decrease of Fe in chloroplasts (Divol et al., 2013). In addition, the coordinated expression of these YSL genes with ferritin genes in embryos and senescent leaves prompted the authors to propose their physiological role in detoxifying Fe during plastid differentiation in embryogenesis and senescence (Divol et al., 2013). The role of these transporters, however, remains controversial since due to proteomic data and GFP-targeting they have also been associated to transport of metal ions across tonoplast and ER membranes (see **Table 1**; Conte et al., 2013).

The *Arabidopsis* "multiple antibiotic resistance 1/iron regulated 3" (MAR1/IREG3) protein, which most likely transports aminoglycoside antibiotics into plastids and has been localized to chloroplasts via targeting of a YFP-tag (**Table 1**), also plays a role in cellular Fe homeostasis (Conte et al., 2009; Conte and Lloyd, 2010). Because MAR1/IREG3 expression is down-regulated by Fe deficiency and MAR1 overexpressing plants show leaf chlorosis that can be rescued by Fe addition, it has been proposed that MAR1/IREG3 may play a role in Fe chelation, storage, or sequestration (Conte et al., 2009). In addition, MAR1/IREG3 belongs to the IREG/ferroportin transporter family that includes IREG1/FPN1 and IREG2/FPN2, which mediate metal transport across the plasma membrane in the root stele and tonoplast, respectively (Schaaf et al., 2006; Morrissey et al., 2009). Interestingly, aminoglycosides can use polyamine inward transport systems to enter eukaryotic cells (Van Bambeke et al., 2000), and one of the most important natural chelators of Fe, nicotianamine (NA) is a polyamine (for overview, see Curie et al., 2009). These observations have prompted the hypothesis that MAR1/IREG3 may transport NA or an Fe-NA complex into the plastid (**Figure 1**; Conte et al., 2009; Conte and Lloyd, 2010). Further indications of the role of this protein in Fe homeostasis were found in a study of quantitative trait locus (QTL) mapping for *Arabidopsis* seed mineral concentrations, where this gene was found in two QTLs associated with the seed Fe trait (Waters and Grusak, 2008).

A bioinformatics approach using cyanobacterial Fe-transporter genes as queries revealed the existence of a plastid-localized, non-intrinsic ABC transporter protein, NAP14 in *Arabidopsis*. *At-NAP14* (also named ABCI11 according to ABC transporter nomenclature [Verrier et al., 2008]) encodes for a non-membrane intrisic, nucleotide binding domain (NBD) subunit of an ABC transporter complex, similar to the FutC unit of the FutABC Fe transporter in cyanobacteria (Shimoni-Shor et al., 2010). Although *in vivo* GFP targeting by Shimoni-Shor et al. (2010) shows signals in the chloroplast stroma, the At_CHLORO database (Ferro et al., 2010) associates this protein to the IE membrane, indicating an attachment of NAP14 to a membrane-intrinsic protein component (see **Figure 1**, **Table 1**). The iron concentration in shoots of *nap14* loss of function mutants is dramatically increased (18 times higher than in wild-type plants), whereas in roots it is approximately 50% lower. Moreover, this mutant showed damage to chloroplast structures, exhibited severe growth defects, strong chlorosis and a deregulation of the Fe-homeostasis genes (Shimoni-Shor et al., 2010). Based on these findings, either a function in regulating plastid Fe-homeostasis or a direct role as part of

a plastid Fe ABC-transporter complex have been proposed. NAP14 could also be involved in Fe–S cluster biogenesis similar to NAP7/SufC, a stroma localized NBD-NAP protein (Xu and Möller, 2004; Balk and Schaedler, 2014). Furthermore, high-throughput proteomic studies have demonstrated the presence of several ABC transporters of still unknown function in the IE membrane, including some "half-size" proteins such as NAP8/ABCB28 (consisting of 1 membrane-intrinsic permease and 1 soluble NBD domain [Verrier et al., 2008]), as well as NAP13/ABCI10, a relative to NAP14 (Ferro et al., 2010; Gutierrez-Carbonell et al., 2014), whose roles in plastid Fe homeostasis deserve further studies. These proteomic studies also indicated the presence of the half-size ABC transporter STA1/ATM3/ABCB25 in the chloroplast envelope. Initially, this transporter was associated to Fe–S cluster export from the mitochondria (see Bernard et al., 2013) but recently, its role in glutathione polysulfide export from the mitochondria for Fe–S cluster assembly in the cytosol has been described (Schaedler et al., 2014). Although STA1/ATM3/ABCB25 is clearly localized in the inner mitochondrial membrane, its presence in chloroplast envelope proteomes suggests a possible dual localization that may be verified by future studies.

Another reasonable candidate for Fe import into plastids is At-Mfl1 (mitoferritin-like1). This gene was found in an attempt to uncover mitochondrial Fe-transporters in *Arabidopsis* using the template mitoferrin 2 (MFRN2), a mitochondrial Fe importer in non-erythroid cells from *Danio rerio* (zebra fish; Tarantino et al., 2011). At-Mfl1 belongs to the mitochondrial carrier family, is annotated as an IE protein in the AT_CHLORO database, has been detected in the envelope proteome of various plant species [see Ferro et al., 2010, Supplemental Table S10 and references therein], and contains a predicted chloroplast targeting peptide (**Table 1**). The expression of this gene is mainly localized in rosette leaves, it is upregulated with Fe excess and correlates with that of genes coding for proteins involved in chloroplast metabolism including PIC1 (Tarantino et al., 2011). *At-Mlf1* loss-of-function mutantlines present lower seedling and leaf Fe-concentrations than wild-type plants and a reduced expression of *At-ferritin1* (Tarantino et al., 2011), suggesting a putative role for Mfl1 as a chloroplast Fe-transporter (**Figure 1**). The observation that mutants with a loss-of-function are viable and fertile implies that the role of At-Mfl1 is redundant or that it plays a specific function only when Fe is in excess.

In maize, differential display screening allowed the identification of the Fe-deficiency inducible gene Zm-FDR3 (*Zea mays* Fe-deficiency related 3; Yin et al., 2000). Yeast complementation studies indicated that this protein can transport Fe and/or Cu (Han et al., 2009). Because orthologs of this gene were not found in any strategy I plant including *Arabidopsis*, tobacco was used to study Zm-FDR3 function. In transgenic tobacco, overexpressing a MYC-tagged Zm-FDR3, this protein was presumably localized to chloroplasts by immunofluorescence labeling directed against the tag (**Table 1**). Since the protein contains no predicted membrane-spanning domains, it could be most likely in the stroma or the thylakoid lumen. Based on an *in silico* prediction, the latter is proposed by Han et al. (2009) (**Figure 1**). Transgenic tobacco plants, expressing Zm-FDR3 displayed higher seed Fe concentration, lower chlorophyll concentration but higher photosynthetic performance, therefore suggesting a role in plastid Fe-homeostasis. This protein does not have homology to other proteins in higher plants but contains domains similar to members of the bacterial type III secretion system, involved in the secretion of effector proteins into a host cell, which might imply the involvement of Zm-FDR3 in the transport of an Fe-containing protein (Han et al., 2009). The existence of such a mechanism in plants and specifically in thylakoids is untypical but plausible. For instance, the assembled Rieske Fe–S protein of the cytochrome b6/f complex can utilize the TAT protein import pathway for its translocation into the thylakoid membrane (Molik et al., 2001). However, knowledge on Zm-FDR3 structure and exact localization is very limited, since the protein contains no predicted membrane-spanning domains and no chloroplast targeting peptide (see **Table 1**).

PLASTID Fe HOMEOSTASIS

As a sink for most of the cellular Fe, plastids are likely to be involved in sensing and regulation of Fe concentration within the whole plant and changes in the plastid Fe-status may trigger different adaptation responses depending on the plant developmental stage (Vigani et al., 2013). In spite of this important putative role, little is known about processes governing Fe homeostasis and signaling in plastid organelles and in their communication with other cellular compartments. For general and tissue-specific subcellular Fe distribution see (Kim et al., 2006; Roschzttardtz et al., 2013; Mary et al., 2015).

Fe Trafficking and Storage in Plastids

Ferritins are conserved eukaryotic proteins and their homo-oligomeric structure of 24 assembled subunits forms a hollow core that can accommodate up to 4,000 Fe(III) ions (for overview see Briat et al., 2010a,b). Plant ferritins are nuclear encoded and *Arabidopsis* contains four ferritin genes (**Table 1**), three of which encode for chloroplast targeted proteins, and one for a protein targeted to seed plastids (Petit et al., 2001; Ravet et al., 2009b). For an overview on plastid stroma localization of ferritin proteins (**Figure 1**) in various plant species we refer to Briat et al. (2010a). Furthermore ferritins - in particular At-FER4 via immunoblots (see **Table 1**) - have also been described in mitochondria (Tarantino et al., 2010; Murgia and Vigani, 2015). In contrast to seed tissue, in mature leaf chloroplasts ferritin proteins are less abundant, however, they accumulate in chloroplasts under Fe excess conditions (Briat et al., 2010b) and thereby play an important role in buffering Fe in its free ionic form and preventing oxidative stress (Ravet et al., 2009a). Whereas in humans the chaperone involved in Fe delivery to ferritin has been discovered (Shi et al., 2008), in plants the machinery involved in the delivery of Fe to ferritin is largely unknown (Briat et al., 2010a,b). Given the active redox nature of Fe, the involvement of metallo-chaperones or chelating molecules in this process is more than likely,

and might be similar to the CCS chaperone that delivers Cu to the Cu/Zn superoxide dismutase (SOD) in plastids (Cohu et al., 2009). The mechanism underlying the release of Fe from ferritin is even more enigmatic. On one hand, *in vitro* studies with animal ferritins suggest that this release requires Fe chelators or reducing agents. On the other hand, *in vivo* studies have demonstrated Fe release by proteolytic degradation of the protein (see Briat et al., 2010a,b for details). Regarding the observed correlation of PIC1 and ferritin function in chloroplasts (see above), a role for PIC1 in handover of imported Fe to the ferritin protein shell might be possible as well.

Plastid organelles harbor an independent pathway for the assembly of Fe–S clusters, the SUF (sulfur mobilization) system, which derives from their cyanobacterial ancestor (Balk and Pilon, 2011). Several components of this pathway have been elucidated and include a cysteine desulfurase (NFS2), scaffold proteins including the SUFBCD complex and NFU proteins, and the cluster transfer and insertion proteins (SUFA, HCF101, GRXS14 and GRXS16; see Couturier et al., 2013; Balk and Schaedler, 2014 for overview). However, the proteinaceous source of Fe for plastid Fe–S cluster synthesis is still unknown. Another Fe–S cluster protein that has been described to play a role in Fe homeostasis in the chloroplast belongs to the NEET family, which is involved in a large array of biological processes in mammalian cells. Although their mode of function is largely unknown, their 2Fe–2S cluster is labile, thereby allowing a role in Fe–S/Fe transfer within cells (Zuris et al., 2011). The *Arabidopsis* protein, At-NEET, most likely is involved in Fe–S/Fe cluster transfer to an acceptor protein and initially was dually localized to chloroplasts and mitochondria by GFP-targeting and immunoblot analysis (**Table 1**; Nechushtai et al., 2012). This protein has been categorized as a plastid protein in AT_CHLORO, and its sub-organellar localization within the chloroplast stroma (immunogold labeling) has been recently reported (Su et al., 2013). At-NEET knockdown plants obtained by RNA interference (RNAi) accumulate more Fe and present higher ROS levels (Nechushtai et al., 2012). The growth of At-NEET knockdown seedlings is insensitive to high, but sensitive to low Fe-levels, strongly suggesting that this protein is involved in Fe transfer, distribution, and/or management. Furthermore, At-NEET knockdown mutants present a dramatic decrease in catalase abundance, a heme enzyme that detoxifies ROS, suggesting also a role of this protein in the supply of heme Fe (Nechushtai et al., 2012).

Another candidate to maintain Fe homeostasis in the plastid is NA. This polyamine has a role in metal chelating in phloem sap, vacuoles and cytoplasm (Pich et al., 1997; Haydon et al., 2012). The presence of NA in plastids has not been studied so far. However, as pointed out by Divol et al. (2013), the leaves from the tomato mutant *chloronerva*, which does not contain NA, present electron dense inclusions of Fe and phosphor in the chloroplast that are absent in wild-type and do not correspond to ferritin (Becker et al., 1995). Thus, these inclusions might indicate a role of NA in maintaining Fe in a soluble form in plastids.

Cross Talk with Vacuoles and Mitochondria

Both, chloroplasts and mitochondria are rich in Fe-containing proteins and although they are most likely major control points in the Fe homeostasis network, little is known about the mechanisms involved in their communication (see Vigani et al., 2013). In these two compartments, Fe is a major constituent of proteins belonging to their respective electron transfer chains and therefore Fe deficiency has a strong impact on their performances. Under Fe deficiency, the mitochondrial electron transport chain undergoes modifications aiming to bypass the Fe-rich complexes, mainly complex I, by the action of alternative NAD(P)H dehydrogenases (Vigani et al., 2009; Vigani and Zocchi, 2010) and this occurs concomitantly with increases in the glycolytic pathway (López-Millán et al., 2000; Zocchi et al., 2007). At the same time, Fe deficiency has a strong impact on thylakoid PSI, the highest Fe sink of the photosynthetic electron chain, and causes a remodeling of the antenna complexes that alters the stoichiometry of both PSI and PSII (Abadia, 1992; Moseley et al., 2002). While in the green microalgae *Chlamydomonas reinhardtii*, mitochondria are more resistant than chloroplasts to Fe deficiency, therefore suggesting a preference for Fe delivery to mitochondria under Fe starvation (Naumann et al., 2007; Nouet et al., 2011), nothing is known about the preference for Fe allocation between these compartments in vascular plants. Indirect evidences exist to support the existence of a cross talk between mitochondria and chloroplasts since for instance loss of function mutants of MIT, the mitochondrial iron transporter for Fe uptake, show lower chlorophyll content and altered ferritin gene expression (Bashir et al., 2011).

The phenotype of the double knockout mutant *ysl4ysl6* suggests a close communication between vacuoles and plastids in embryo tissue during seed germination. In this mutant, the expression of the transporters NRAMP3 and NRAMP4, which are responsible for vacuolar Fe-export during germination, is dramatically down-regulated (Divol et al., 2013). In addition, the double *nramp3 nramp4* mutant does not contain FER2, the seed stable ferritin isoform, which suggests a decrease of Fe in the plastid (Ravet et al., 2009b), and this has been explained by the prevention of Fe from exiting the vacuole (Divol et al., 2013). However, as previously discussed in the same publication, the expression of these genes may not occur in the same cells and therefore the cross-talk between the vacuolar NRAMP3/4 and the plastid YSL4/6 must involve sensing of Fe in the plastid and signaling to adjust Fe release from the vacuole.

The Role of Plastids in Seed Fe-Filling

Metal loading in seeds depends on both, root soil uptake and remobilization from source tissues, including senescent leaves. Senescent organs are a major source for nitrogen but little is known about their contribution to metal remobilization. As the sink for most of the plant Fe, it is tempting to speculate that plastids may play a role by providing Fe in this process. During leaf senescence, the chloroplast is one of the first organelles to be degraded. This occurs gradually: initially pigment degradation occurs in the chloroplast whereas the degradation of stromal

proteins takes place in the vacuole. These processes reduce the number and size of plastids and allow for further degradation via chlorophagy (Wada and Ishida, 2009; Pottier et al., 2014). RuBisCo from the chloroplast stroma of senescing leaf cells is a major source of remobilized nitrogen for seed filling. In this context the role of Fe-rich plastid proteins in Fe remobilizing should be further explored. Furthermore, chloroplasts could also provide Fe when this metal is limited in the environment because autophagy for nutrient remobilization can be triggered upon nutrient starvation and stress (Pottier et al., 2014). These hypotheses are consistent with the strong expression of YSL6 in senescent leaves and its putative role in controlling Fe release from the plastid during senescence (Divol et al., 2013). On the other hand, transcriptomic analyses have also shown that senescence occurs concomitantly with an upregulation of vacuolar Fe-transporters such as NRAMP3 (Thomine et al., 2003; Pottier et al., 2014), suggesting that in physiological conditions both the vacuole and the plastid can take part in Fe remobilization.

CONCLUDING REMARKS

In summary, research in the last decade has led to the discovery of several Fe-transport proteins in plastids. Detailed phenotypic analyses of the corresponding mutants in the model plant *Arabidopsis*, already allow us to obtain a quite accurate description of the role of these proteins in plant Fe homeostasis and general physiology. Unfortunately, exact and unequivocal localization at the sub-cellular and sub-organelle level is still missing for most of these membrane proteins. Future research thus definitely requires unbiased and independent approaches such as *in vivo* GFP targeting in combination with specific immuno-localization and membrane proteomic analyses as well as *in silico* predictions of targeting signals and *in vitro* import assays into organelles. Furthermore, Fe-transport proteins in the chloroplast OE still await their discovery. Major questions to be answered include the exact transport mechanisms, their respective structure/function relationships and the nature of their substrates such as free Fe–ions or chelates. Established - e.g., yeast, *E. coli* -, but also new heterologous systems for functional transport assays such as Gram-positive *Lactococcus lactis*, might lead to more detailed answers in future research.

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REVIEW
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Copper Delivery to Chloroplast Proteins and its Regulation

*Guadalupe Aguirre and Marinus Pilon**

Biology Department, Colorado State University, Fort Collins, CO, USA

Copper is required for photosynthesis in chloroplasts of plants because it is a cofactor of plastocyanin, an essential electron carrier in the thylakoid lumen. Other chloroplast copper proteins are copper/zinc superoxide dismutase and polyphenol oxidase, but these proteins seem to be dispensable under conditions of low copper supply when transcripts for these proteins undergo microRNA-mediated down regulation. Two ATP-driven copper transporters function in tandem to deliver copper to chloroplast compartments. This review seeks to summarize the mechanisms of copper delivery to chloroplast proteins and its regulation. We also delineate some of the unanswered questions that still remain in this field.

Keywords: plastocyanin, photosynthesis, copper deficiency, copper transporting P-type ATPase, polyphenol oxidase, superoxide dismutase, Cu-miRNA

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****Correspondence:***
Marinus Pilon
pilon@colostate.edu

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INTRODUCTION

It is estimated that at least a third of all known proteins require a metal cofactor (Waldron et al., 2009). Copper (Cu) is essential for most living organisms including higher plants. In living cells Cu is predominantly found in two redox states, Cu^{2+} (cupric) and Cu^{+} (cuprous). The utility of Cu is related to its capacity to switch redox states in the cellular environment (i.e., become oxidized and reduced while bound to a protein) and the majority of Cu proteins (∼90%) found in nature function as oxidoreductases (Waldron et al., 2009). In green tissue of plants a large fraction of the Cu is present within the chloroplasts, corresponding to roughly a third of the Cu in leaves of soil-grown, well-fertilized, *Arabidopsis thaliana* (Shikanai et al., 2003). The fraction of Cu in a leaf that is allocated to plastids probably varies with Cu supply, however, (Burkhead et al., 2009). In this review we aim to describe the mechanism of Cu delivery to chloroplast proteins and its regulation. Emphasis is given on new models and outstanding questions.

CHLOROPLAST COPPER PROTEINS

In chloroplasts, three major Cu proteins have been described (see **Figure 1**). In the stromal compartment, an isoform of copper/zinc superoxide dismutase is active. The enzyme is encoded by the *CSD2* gene in *Arabidopsis thaliana* (Kliebenstein et al., 1998). CSD2 maturation has an absolute requirement for a conserved CCS called CCS (Abdel-Ghany et al., 2005b; Chu et al., 2005; Huang et al., 2012) which functions to deliver and insert Cu into this enzyme. In contrast, the major cytosolic isoform of copper/zinc superoxide dismutase (CSD1 in *Arabidopsis*) can mature without CCS, albeit with low efficiency (Huang et al., 2012). Although copper/zinc superoxide

Abbreviations: CCO, Cytochrome-*c* oxidase; CCS, Copper chaperone for SOD; CSD, Cu/ZnSOD or Cu/Zn-superoxide dismutase; HMA, Heavy Metal ATPase; PAA, P-type ATPase of *Arabodopsis*; PC, *Plastocyanin*; PPO, *polyphenol oxidase*; SPL7, Squamosa Promotor binding-Like 7.

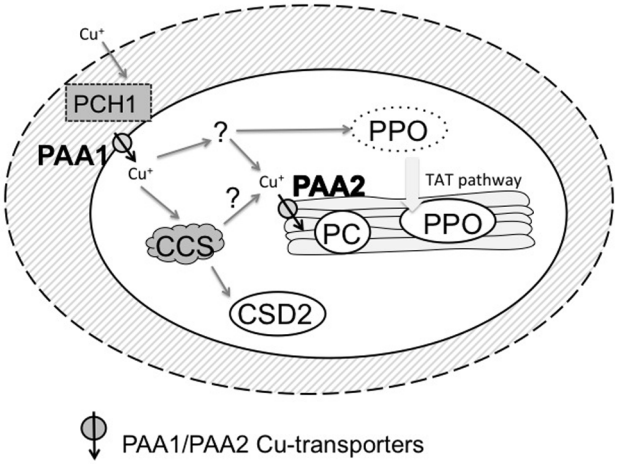

FIGURE 1 | Chloroplast copper proteins and delivery systems. CSD2 (copper/zinc superoxide dismutase) is active in the stroma and receives Cu ions from CCS (the copper chaperone for superoxide dismutase). PC is active in the thylakoid lumen. PPO is found in the thylakoid lumen but may receive its Cu ion in the stroma before entering the thylakoid space via the TAT protein translocation pathway. PAA1 is a copper transporter in the envelope required to supply Cu ions to CSD2, PC and presumably also PPO. PAA1 receives Cu ions from the plastid copper chaperone 1 (PCH1). Cu ions may reach PCH1 in the inter membrane space of the envelope via diffusion through porins in the outer membrane, perhaps bound to low molecular weight chelators such as glutathione. Alternatively, PCH1 might pick up Cu ions in the cytosol and diffuse in via pores in the outer membrane. PAA2 is a homologs copper transporter in the thylakoid membrane required to deliver Cu ions to PC. A protein donating Cu ions to PAA2 has not been identified yet.

dismutases are highly conserved in plants and other eukaryotes, their biological significance in plants has been elusive. A report of strong phenotypes for a loss of function (knock-down) mutant of CSD2 (Rizhsky et al., 2003) was in retrospect based on a insertion mutant that upon further study did not seem to affect CSD2 protein expression at all (Cohu et al., 2009). Phenotypes of CCS loss-of function mutants, which have no detectable CSD2 activity and very low CSD1 activity, are very mild when compared to the wild-type (Dugas and Bartel, 2008; Cohu et al., 2009) or require extreme stress to become noticeable (Sunkar et al., 2006). Redundancy with chloroplastic FeSOD may be one factor affecting Cu/ZnSOD loss of function phenotypes (Cohu et al., 2009). The green alga *Chlamydomonas reinhardtii* does not encode for Cu/ZnSOD but it contains FeSOD in the chloroplast.

Plastocyanin is a small Cu protein in the thylakoid lumen and serves as an electron carrier between the cytochrome-b_6f complex and PSI in plants and is essential for photo-autotrophic growth in plants (Weigel et al., 2003). In contrast, similar to some cyanobacteria, *Chlamydomonas* can use the heme-containing cytochrome-c_6 when Cu becomes limiting, as an alternative to PC (Kropat et al., 2015). In most plant genomes including *Arabidopsis* and poplar two PC isoforms are encoded. The two isoforms have essentially equivalent function regarding electron transport activity but in *Arabidopsis* one protein isoform (PC1) accumulates at a lower level whereas a second isoform (PC2) is much more abundant but also more sensitive to Cu availability at the protein level (Abdel-Ghany, 2009; Pesaresi et al., 2009). In poplar, both PC isoforms seem to be expressed at a comparable level (Ravet et al., 2011). PC was the first plant Cu enzyme that was cloned and its biogenesis is very well studied. The PC precursor has a bipartite N-terminal targeting sequence consisting of first a transit peptide, which functions to bring the protein into the stroma, followed by a signal sequence that serves for interaction with the ATP-dependent SEC machinery for translocation to the thylakoid lumen (Smeekens et al., 1986). Because protein transport over the envelope and thylakoid membranes via the SEC pathway requires unfolded polypeptide substrates, Cu must insert into apo-PC in the thylakoid lumen (for a review on chloroplast protein transport see Jarvis, 2008).

A third abundant Cu enzyme is PPO, also known as tyrosinase, which is present in the thylakoid lumen of many plants including poplar and spinach where it was discovered as the first Cu enzyme in plants (Arnon, 1949). However, PPO is absent from *Arabidopsis* (Schubert et al., 2002) and *Chlamydomonas*. The PPO enzymes contain 2 Cu atoms per monomer. *In vitro*, PPO can catalyze the formation of ortho-diphenols or ortho quinones from mono-phenols or ortho-dihydroxyphenols (See Mayer, 2006). The PPO substrates are not found in the thylakoid lumen, but are stocked in other cellular compartments such as vacuoles, and cell disruption by herbivory would allow PPO to form the dark-brown colored products in disrupted cells that make plant tissue less digestible (Mayer, 2006). Thus, PPO is thought to function in defense to herbivory or pathogen attack (Constabel et al., 2000; Wang and Constabel, 2004). As is the case with PC, the PPO precursors contain a bipartite targeting sequence at their N-terminus, but in the case of PPOs the thylakoid transfer sequence contains the twin arginine sequence motif, which means that PPO should use the TAT system for thylakoid transfer (see Jarvis, 2008). Because the TAT system can translocate folded peptides this implies that PPO might acquire its two Cu atoms in the stroma.

PRINCIPLES OF COPPER HOMEOSTASIS

It helps to understand chloroplast Cu transport in the context of overall Cu homeostasis and the properties of Cu ions. Among biologically active metal ions, copper binds the most tightly to its ligands. Both cupric and cuprous ions bind tightly to S and N-containing ligands (Irving and Williams, 1948; Waldron et al., 2009). These ligands are abundant in the cytosol, either as parts of amino acid functional groups in proteins (particularly cysteine, methionine, and histidine) or in low molecular weight compounds such as glutathione (cysteine). Within the reducing environment of the cytosol, free thiols in proteins and glutathione are especially strong ligands for Cu^+ ions (Waldron et al., 2009). Therefore, the cytosol will have a very high chelating capacity for Cu ions when compared to an extracellular or exoplasmic compartment (e.g., vacuole) and indeed only very low free Cu ion concentrations are found inside cells (Rae et al., 1999). The very high cellular chelation capacity for Cu means that effectively a concentration gradient for free Cu ions should exist over the

plasma membrane with a relatively low free Cu concentration in the cytosol, and this should help to provide a driving force for Cu ion uptake from the extracellular space. Conversely, export of Cu ions from the cytosol or a compartment with similar properties such as the chloroplast stroma should require metabolic energy. The strong tendency of Cu to bind to intracellular sites also makes it possible for Cu ions to replace other metals, causing toxicity. For this reason and in order to ensure correct delivery of Cu^{+}, intracellular Cu-chaperones exist which scavenge Cu^{+} and deliver it to specific targets via protein-protein interactions. Finally, sensors that control Cu homeostasis should have a high affinity for the ion.

CELLULAR COPPER TRANSPORTERS

Before turning to chloroplast-specific transport we want to first give a brief overview of cellular Cu transport and regulation because this affects the chloroplast also. Four classes of transporters have been implicated in Cu transport: COPT, ZIP, YSL, and HMA (see Williams et al., 2000; Burkhead et al., 2009).

Roles of COPT, ZIP, and YSL Transporters

Copper enters eukaryotic cells by means of the CTR Cu transporters. The *Arabidopsis* genome contains six CTR-like sequences, which are called COPT1-6. COPT4 does not seem to be active (Sancenon et al., 2003). COPT 1, 2, and 6 are likely active at the cell surface (Sancenón et al., 2004; Jung et al., 2012), COPT3 may be active in an internal membrane and COPT5 is active in the tonoplast (Garcia-Molina et al., 2011; Klaumann et al., 2011). CTR/COPT transporters likely function as channels or carriers specific for Cu^{+} where the direction of transport is toward the cytoplasm (Eisses and Kaplan, 2005). The driving force for Cu^{+} uptake can be provided by both the strong capacity of the cytosol for Cu^{+} chelation as well as the typical orientation of the membrane potential, which is positively charged on the exoplasmic side due to proton exporting pumps. In the case of plant cells, reductases of the FRO family (Fro4/5) facilitate Cu uptake by reducing extracellular Cu^{2+} to Cu^{+}, providing COPT proteins with a substrate (Bernal et al., 2012).

Whereas the role of COPT transporters is fairly well delineated, the picture is far less clear for the ZIP (Zrt and Irt like proteins; divalent metal transporters) and YSL (Yellow Stripe Like) transporter families. ZIP2 is clearly upregulated by Cu deficiency (Wintz et al., 2003; Bernal et al., 2012). ZIP2 and ZIP4 were reported to complement a yeast *ctr1* Cu uptake mutant (Wintz et al., 2003) but this result was not reproduced for ZIP2 in a more recent study, which instead indicated ZIP2 functions in Zn and Mn transport (Milner et al., 2013). The Yellow Stripe-Like (YSL) metal transporters, which were first identified in corn, are involved in the long-distance transport of metal-bound nicotianamine in *Arabidopsis* and rice (Waters et al., 2006; Chu et al., 2010; Zheng et al., 2012). For YSL family members YSL1, 2 and 3 roles in redistribution of Cu from leaves during senescence have been reported (Waters et al., 2006; Chu et al., 2010). YSL2 is strongly up-regulated in Cu deficiency at the transcript level in an SPL7-dependent process (Bernal et al., 2012). In excess-copper conditions, YSL1 and YSL3 proteins are down-regulated through sumoylation in *Arabidopsis* (Chen et al., 2011).

HMA Transporters

An important class of Cu transporters is formed by a subclass of the metal transporting P1b-type ATPases or HMA proteins as they are called in plants (Williams et al., 2000; Hanikenne and Baurain, 2014). A schematic model for this type of transporter is given in **Figure 2**. Members of this family of transporters exist in two conformational states called E1 and E2. In all mechanistically studied P-type ATPases, hydrolysis of ATP after substrate binding is utilized to convert the E1 form of the enzyme to the E2 state in which a conserved ASP residue is phosphorylated. Subsequent release of the substrate on the *trans* side allows for phosphatase activity, which brings the enzyme back to the E1 state (for review see Rosenzweig and Argüello, 2012). In virtually all known P-type ATPases the ATP is bound on the cytoplasmic side and the substrates are exported from

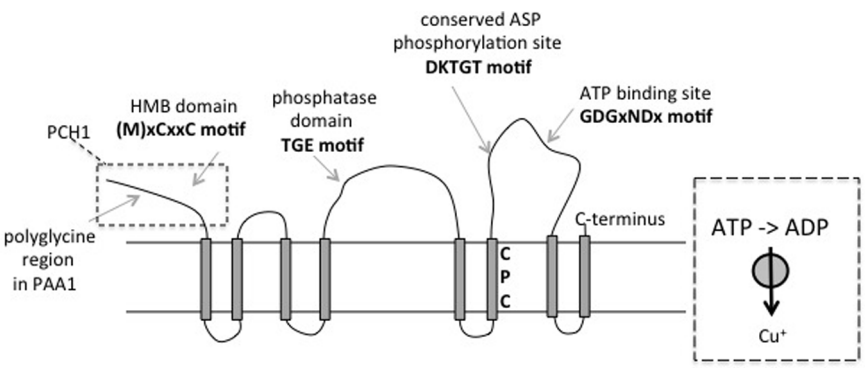

FIGURE 2 | Topology model of Cu+ transporting P-type ATPases such as PAA1 and PAA2. The proteins have eight transmembrane segments. Major domains and sequence motifs are indicated. The region that corresponds to the copper chaperone PCH1, encoded in *Arabidopsis* by a PAA1 alternative splice form, is indicated in a box. The box on the right gives a simplified depiction of the protein in the same topology, which is used in **Figure 3** also.

there (Rosenzweig and Argüello, 2012). The sequence motif CPC in TM region 6 is characteristic for Zn and Cu transporters (Hanikenne and Baurain, 2014). The specificity for Cu^+ substrate is further determined by specific sequences in transmembrane regions 7 and 8 (Mandal et al., 2004). In other classes of P-type ATPases, the energy released by ATP hydrolysis can be used to pump ions such as H^+, Ca^{2+}, and Na^+ against steep electrochemical gradients. In the cases of Zn and Cu it is evident that such large concentration gradients do not exist. However, the high chelating capacity of the cytosol for Zn and Cu ions presents a sizable thermodynamic barrier for export, which necessitates the utilization of metabolic energy for the transport of these ions from this compartment. Specific metallo chaperones are used to sequester the correct ion in the cytosol and deliver it to the cognate metal transporter via ligand exchange (Rosenzweig and Argüello, 2012). One of the best characterized Cu chaperones is ATX (anti-oxidant) from yeast, which has a ferredoxin-like fold and contains the consensus Cu binding motif MxCxxC. All Cu chaperones for P1B-type ATPases are similar and therefore this type of chaperone is called ATX-like (Pufahl et al., 1997). Most metal transporting P-type ATPases have one or more N-terminal heavy metal-binding (HMB) domain(s) which are also known as metal binding domains (MBD; (Hanikenne and Baurain, 2014). For Cu^+ transporters these domains are structurally similar to ATX (Rosenzweig and Argüello, 2012). Work with bacterial Cu^+ transporters has indicated that the HMB domain can accept metal from a ATX-like metallochaperone with a similar ferredoxin fold structure. Because the transporters maintain ATPase and transport activity without the HMB domain, and because this domain was shown to be inhibitory for ATPase activity in some cases, it can be proposed that the function of the HMB domain *in vivo* is perhaps regulatory (Rosenzweig and Argüello, 2012), but this has not been addressed in plants. The Cu chaperones that function with Cu-transporting ATPases bind via electrostatic interactions with a platform formed by a loop associated with transmembrane region 2 of the transporter and deliver sequentially two Cu^+ ions directly to the transmembrane domains 6–8 that harbor the metal binding/transport sites (González-Guerrero and Argüello, 2008; Rosenzweig and Argüello, 2012).

Eight metal transporting P-Type ATPases are encoded in the *Arabidopsis* genome with homologs in most green eukaryotes (Williams et al., 2000; Hanikenne and Baurain, 2014). For four of the *Arabidopsis* HMA proteins a direct role in Cu homeostasis has been shown. HMA7 transports Cu from the cytoplasm to an endomembrane compartment where it can associate with the Cu binding site of the ethylene receptors (Hirayama et al., 1999). HMA5 in the plasma membrane removes Cu from the cell to allow xylem loading in the roots and prevent cellular Cu overload (Andres-Colas et al., 2006). HMA5 and 7 are likely to receive Cu from the ATX and or CCH cytosolic Cu chaperones for which interaction has been shown using yeast-two-hybrid assays (Puig et al., 2007). Two Cu transporting P-Type ATPases, PAA1/HMA6 and PAA2/HMA8 have been localized in plastids and these are described in more detail further below together with HMA1 a third ATP-driven metal transporter in the chloroplast.

COPPER HOMEOSTASIS

Because it affects chloroplast Cu transport pathways, it is necessary to briefly discuss cellular Cu homeostasis. When cellular Cu levels drop, a conserved transcription factor called SPL7 (Squamosa Promoter binding-Like 7) activates the transcription of a set of genes that together mediate a response to low Cu (Yamasaki et al., 2009). SPL7 in plants shares sequence similarity with *Chlamydomonas* CRR1 (the Copper Response Regulator) and activates transcription of genes that have a Cu Response element (CuRe) with the consensus sequence GTAC in it (Kropat et al., 2005; Yamasaki et al., 2009). SPL7 targets include several of the COPT transporters (COPT 1, 2, 6) and the potential cupric reductases FRO4 and 5 (Yamasaki et al., 2009; Bernal et al., 2012). Several highly conserved microRNAs, together called the Cu-microRNAs are also targets of SPL7 (Yamasaki et al., 2009; Bernal et al., 2012). These microRNAs in turn down-regulate the expression of Cu proteins including CSD2 and the PPOs (Yamasaki et al., 2007; Abdel-Ghany and Pilon, 2008; Cohu et al., 2009; Ravet et al., 2011). Since PC is not a target of a Cu-microRNA it can be hypothesized that the Cu-microRNAs serve to maintain a larger pool of Cu available for PC maturation, which is essential for photosynthesis (Burkhead et al., 2009). MiR398 targets CSD1, CSD2, and CCS as well as COX5b, a potential subunit of mitochondrial CCO but not a part of the Cu-binding core of this enzyme (Yamasaki et al., 2007). Mir397 and MiR408 target laccases and MiR408 also targets apoplastic plantacyanin (Abdel-Ghany and Pilon, 2008). MiR1444 targets PPO isoforms (Ravet et al., 2011). In *Chlamydomonas,* no miRNA regulation of Cu proteins exists but CRR1 regulates Cu economy by promoting the expression of cytochrome-c_6 and turnover of PC; further transcriptional regulation by CRR1 aims to further economize Cu use and optimize Fe acquisition in *Chlamydomonas* (Kropat et al., 2005, 2015).

COPPER TRANSPORT TO CHLOROPLAST PROTEINS

In the chloroplast two conserved Cu transporters have been identified (**Figure 1**). PAA1/HMA6 is localized in the chloroplast envelope (Shikanai et al., 2003; Catty et al., 2011). PAA2/HMA8 is localized in the thylakoids (Abdel-Ghany et al., 2005a). GFP fusions (Abdel-Ghany et al., 2005b), proteomic data (Catty et al., 2011; Tomizioli et al., 2014), and direct biochemical localization data, (Blaby-Haas et al., 2014) support the localization of these two proteins in *Arabidopsis*. In addition, PAA2/HMA8 was observed by immune EM in thylakoids in soybean (Bernal et al., 2007). Both PAA1 and PAA2 display all the characteristic motifs and domains expected for a Cu-transporting P-type ATPase (Abdel-Ghany et al., 2005a). A schematic structure is depicted in **Figure 2**. Phenotypes of *Arabidopsis* mutants indicated that both transporters are required for efficient maturation of PC, whereas only PAA1 is required for CSD2 activity. In addition, PAA1 mutants have lower Cu content in the entire chloroplast whereas PAA2 mutants have lowered Cu in the thylakoids (Abdel-Ghany et al., 2005a). Thus the two transporters function in tandem,

as indicated in **Figure 1**. Homologs of PAA1 and PAA2 are present in eukaryotic green organisms including *Chlamydomonas* (Hanikenne and Baurain, 2014).

As discussed above, Cu-transporting P-type ATPases are expected to transport Cu away from the ATP binding site and to accept Cu from a metallo- chaperone. For PAA1 the topology in the inner envelope has been determined, which showed that the protein has its ATP binding site and thus also its substrate binding site on the intermembrane space side of the inner envelope membrane (Blaby-Haas et al., 2014). In *Arabidopsis*, two major splice forms are found for the PAA1 mRNA. One splice form, the full length mRNA, encodes for the full transporter. A second splice form is truncated and has an early stop codon, which leads in translation to a protein that only includes the transit peptide and HMB domain (Blaby-Haas et al., 2014). Since HMB domains are structurally very similar to Cu-chaperones it was proposed that this splice form encodes a chloroplast Cu chaperone called PCH1. PCH1 is conserved, in some plants it is formed from the alternative splice form of PAA1; in other plants gene duplication allowed a PAA1 sequence to evolve to encode the chaperone. Immunoblot analyses showed that the small Cu chaperone indeed is present in plants (Blaby-Haas et al., 2014). Direct biochemical measurements of ATPase activity for purified recombinant PAA1 and PAA2 showed that PCH1 can donate Cu to PAA1 but not to PAA2. Thus PCH1 seems to function in Cu delivery to PAA1 in the envelope intermembrane space of the chloroplast (Blaby-Haas et al., 2014). For PAA2 the topology is not determined directly. PAA2 functions to provide Cu to the lumen and thus we can expect PAA2 to have its ATP binding site in the stroma.

No regulation of transcript or protein levels has been observed in response to Cu availability for PAA1. For PAA2, however, regulation has been observed at the post-translational level. When *Arabidopsis* is grown on media with elevated Cu, much less PAA2 protein accumulates (Tapken et al., 2012). The mRNA of PAA2 is not affected by Cu feeding of plants, but instead Cu feeding mediates the turnover of the PAA2 protein via the conserved stromal CLP protease system (Tapken et al., 2015a). The observation that PAA2 is more stable in *paa1* mutants suggests that PAA2 turnover requires Cu in the chloroplast (Tapken et al., 2012). Interestingly, loss of pc2 resulted in increased PAA2 turnover Tapken et al. (2012). In a *paa1/pc2* double mutant, however, the regulation of PAA2 turnover was maintained similar to the wild type (Tapken et al., 2015b). Thus PC2 protein itself is not involved in PAA2 regulation. The CLP complex does not seem to be regulated by Cu (Tapken et al., 2015a). Rather, it can be proposed that PAA2 in association with Cu is more susceptible to turnover and this state is promoted by elevated Cu availability in the stroma or the absence of a Cu acceptor in the lumen.

It is not clear yet if there is a Cu chaperone for PAA2 in the stroma. *In vitro* experiments showed that CCS could interact with PAA2 and deliver Cu to stimulate ATPase activity of PAA2. The reported effect on the V_{max} of PAA2 for CCS bound to Cu was small in comparison to only Cu^+ ions (Blaby-Haas et al., 2014). In addition, knock-out of CCS does not affect PC activity at all and CCS is also hardly expressed when Cu is below moderate availability due to miR398 regulation. Yet under these conditions PC still matures efficiently (Cohu et al., 2009). Because the CLP protease cleaves PAA2, it also cannot be excluded that the N-terminal HMB domain of PAA2 remains as a proteolytic fragment and this could be a chaperone for PAA2 in the stroma. But the free PAA2 HMB domain bound to Cu^+ did not stimulate PAA2 ATPase activity at all and did not display Cu transfer activity (Blaby-Haas et al., 2014). Perhaps PAA2 receives Cu from a low molecular weight chelator such as glutathione in the stroma. Indeed ATPase activity is observed for PAA2 with only Cu^+ present and in a phosphorylation assay PAA2 displayed a much higher affinity for free Cu compared to PAA1, which requires a chaperone (Blaby-Haas et al., 2014; Sautron et al., 2015).

EFFECTS OF COPPER-MICRORNAS ON CU DISTRIBUTION IN CHLOROPLASTS

A potential miR408 target site is present in PAA2/HMA8 genes. However in *Arabidopsis* this site is not used (Bernal et al., 2012; Tapken et al., 2012). It seems likely that more indirect microRNA regulation via miR398 and miR1444 has important consequences for chloroplast Cu delivery to PC. To maintain growth and development of the photosynthetic apparatus during lower Cu availability, Cu delivery to PC should be prioritized. Induction of miR398 causes a drastic reduction in CCS and CSD2 protein expression whereas miR1444 will prevent PPO expression. This should allow remaining Cu in the stroma to be transported via PAA2 to PC (Burkhead et al., 2009). Could there be significance to the use of the TAT pathway by PPO? In agreement with its function in defense PPO is strongly induced by wounding. If PPO can pick up Cu in the stroma it would give plants a way to further prioritize Cu use in the emergency situation of biological attack. In such a case further maturation of PC is envisioned to be a lower priority.

OUTSTANDING QUESTIONS

The chloroplast Cu delivery system is now one of the best-understood trace metal delivery systems. However, several questions remain regarding the function of PAA1 and PAA2 as a system.

Firstly, alternative transport routes must exist for Cu in chloroplasts. All the tested *paa1* and *paa2* loss of function mutants are suppressed by elevated levels of Cu in the growth medium and suppression is more evident for *paa1* mutants (Abdel-Ghany et al., 2005a). Because both sequence analyses and immunoblot analyses have shown that all *paa1* alleles except for *paa1-3* and all *paa2* alleles are truly null mutants, the implication is that low affinity alternative Cu transport pathways must exist (Tapken et al., 2012; Blaby-Haas et al., 2014; Boutigny et al., 2014).

Another still puzzling observation is that *paa1/paa2* double mutants are seedling lethal even when given extra Cu in the media (Abdel-Ghany et al., 2005a). This observation is puzzling because synthetic lethality for two independent loci can be taken as support for functions of the encoded gene products in parallel pathways. Both *paa1* and *paa2* mutants are not lethal on soil,

both are suppressed by Cu feeding and according to our model the two proteins function in sequence and not in parallel. Could we be wrong and could PAA1 and PAA2 function in parallel in the same membrane? This would require mis-targeting in the chloroplast of a fraction of the "normally" envelope-localized PAA1 to thylakoids or *vice versa* of PAA2 to the envelope. Such "mis-targeting" might explain both the suppression by Cu and the lethal phenotype of the double mutant. Given that PAA1 requires interaction with PCH1 and that PAA2 cannot accept Cu from PCH1 (Blaby-Haas et al., 2014) it seems unlikely that mis-targeted PAA1 or PAA2 could be functional. Similarly, modeling approaches have suggested that PAA2 but not PAA1 is suitable for Cu delivery to PC by direct docking between the transporter and apo-plastocyanin (Sautron et al., 2015). Rather, we favor the idea that loss of two high affinity transporters restricts Cu transport to the thylakoids so much that low affinity transport activities can no longer compensate (Abdel-Ghany et al., 2005a).

How did PAA1 and PAA2 evolve? The chloroplast has evolved via endosymbiosis from a cyanobacterial ancestor in which Cu also needed to be supplied to PC and to CCO in the same membrane. In the cyanobacterium *Synechocystis PCC6803* two Cu-transporters, CtaA and PacS, and a Cu chaperone, ScAtx, have been described (Phung et al., 1994; Tottey et al., 2001, 2002). CtaA was thought to be located in the bacterial envelope inner membrane, ScAtx is cytosolic and PacS is in the thylakoid membrane (see **Figure 3**). It was proposed that CtaA functions as a Cu importer based on physiological measurements and Cu contents of mutant cells; and that PacS functions to transport Cu into the thylakoid lumen, which is important for PC maturation and the prevention of toxic effects of Cu in the bacterial cytosol (Tottey et al., 2001, 2002). Sequence comparisons show that both PAA1 and PAA2 are more similar to CtaA and thus PacS and ScAtx1 as well as COX seem to be lost from the chloroplast system after endosymbiosis (Abdel-Ghany et al., 2005a; Hanikenne and Baurain, 2014). The V_{max} kinetic parameter measured for both PAA1 and PAA2 is comparable to that of CtaA and about 10-fold lower than the V_{max} reported for PacS, which further supports the notion that PAA1 and PAA2 are more similar to CtaA (Blaby-Haas et al., 2014). Thus, it can be proposed that both PAA1 and PAA2 are derived from a CtaA-like cyanobacterial sequence. Which steps would have been required for the evolution of the chloroplast Cu-transporting P-type ATPases?

The scenario where CtaA functions as a Cu importer in the cyanobacterium (which is *not* as it is depicted by us in **Figure 3**) seems at first glance straightforward but there are, in our opinion, two issues with this model (Tottey et al., 2001, 2002). One is that CtaA in the cellular importer orientation should have a topology that is non-canonical from an evolutionary perspective in that it would have its ATP binding site not on the cytoplasmic side of the bacterial inner membrane but in the periplasm. Alternatively, CtaA could have its ATP binding site in the cytosol and import Cu toward its ATP binding site but in this case it would be the only Cu-ATPase that transports toward the cytoplasm and it would require a unique mechanism (this we consider a very unlikely scenario). Therefore we favor the idea that CtaA has a toplogy as indicated in **Figure 3**. The other issue associated with CtaA functioning as an importer would be that PAA2, if indeed derived from a CtaA-like sequence, would have flipped its topology in the membrane to now have its ATP binding site in the stroma of the chloroplast (equivalent to the bacterial cytosol). It is hard to envision a mechanism for this. Is there another, perhaps more parsimonious scenario for the evolution of PAA1 and PAA2 from a CtaA-like sequence? We propose the model in **Figure 3**. In this scenario CtaA is in an orientation in the cyanobacterium where it serves a biosynthetic function that allows it to donate Cu to exoplasmic (non-cytosolic) Cu enzymes such as PC and CCO. In the case of COX maturation, perhaps copper chaperones are utilized equivalent to the well-described factors that mediate COX maturation in mitochondria in the intermembrane space (Carr and Winge, 2003). CtaA in the topology proposed by us might be located in the inner membrane of the envelope. In addition, or alternatively, CtaA is in a hypothetical internal vesicle that would allow it to also facilitate Cu delivery to PC and COX in the thylakoids via vesicular transport. We believe that the model in **Figure 3** for CtaA function can be reconciled with all published data regarding phenotypes of cyanobacterial *ctaA* mutants and interactions with ScAtx (Tottey et al., 2001, 2002). For the evolution of PAA2 and PAA1 in chloroplasts we propose that they derive from a cyanobacterial CtaA protein perhaps present in an internal vesicle.

The model as depicted in **Figure 3** also has apparent problems, but we think that these issues can all be resolved. First, the model in **Figure 3** invokes that Cu enters the cyanobacterial cell via an alternative, not yet described, route. Low affinity yet somehow substantial Cu ion uptake by bacteria apparently does occur and is perhaps favored by the membrane potential and the high Cu chelating capacity of the cytosol relative to extracellular spaces. Indeed, many bacteria have extensive ATP-dependent Cu extrusion systems required to avoid Cu toxicity even if they lack specific Cu import pathways (Outten et al., 2001). Another possible weakness of the model as presented in **Figure 3** is that evidence for the types of vesicles that allow Cu trafficking is lacking in present day cyanobacteria. However, these types of vesicles would be by definition transient and we only need to invoke that an ancestor of the chloroplast utilized such vesicles. The final possible issue with the model now depicted in **Figure 3** is that PAA1 would have a flipped topology relative to CtaA. However, we postulate that the presence of the poly glycine domain of PAA1 serves as an envelope retention signal that at the same time forces insertion of the transporter in the correct new orientation. A poly glycine containing stretch was postulated to serve as an envelope retention signal for chloroplast TOC75 protein (Inoue and Keegstra, 2003). The poly glycine-containing stretch is a hallmark of PAA1 proteins and not found in PAA2 homologs (Abdel-Ghany et al., 2005a; Blaby-Haas et al., 2014; Hanikenne and Baurain, 2014).

Another question is whether there also is a pathway for the export of Cu from plastids? To regulate ion H^+, Ca^{2+}, and Na^+ ion concentrations, cellular membrane systems are typically equipped with transport pathways for both directions. Indeed, in plants COPTs provide transport to the cytosol while HMA5 and 7 provide export. Is a similar scenario in place in the chloroplast? Is there a Cu exporter for thylakoids and the envelope membranes? In *Chlamydomonas* one additional function of PC next to

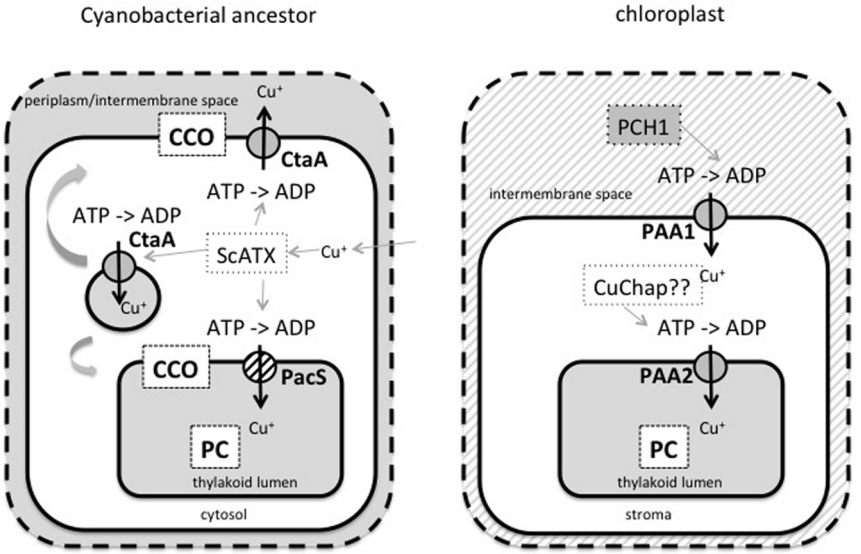

FIGURE 3 | Model for the evolution of chloroplast Cu transport from a hypothetical cyanobacterial ancestor of the chloroplast. Cyanobacteria, such as *Synechocystis*, contain two major Cu proteins, PC in the thylakoids and CCO in both the thylakoids and inner membrane of the envelope. In these cyanobacteria two Cu-transporting P-type ATPases called CtaA and PacS are present together with a copper chaperone ScATX that can interact with both transporters. ScATX serves to sequester Cu ions that entered the cytosol by low affinity pathways and to deliver the Cu ions to CtaA and PacS. PacS is in the thylakoid membranes and loss of function affects both Cu tolerance and PC maturation. CtaA is presumably present in the inner membrane and maybe in addition in an internal vesicle. We propose that CtaA has the indicated topology, which allows the protein to deliver Cu to the exoplasmic side of membranes to allow maturation of both the CCO complex and PC. If CtaA is active in an internal vesicle then Cu ions can reach CCO and PC via a vesicular transport mechanism involving membrane fusion. After endosymbiosis gave rise to the chloroplast, both PacS and ScATX were lost. Nucleus encoded PAA1 and PAA2 then evolved from CtaA with the addition of a transit peptide for chloroplast targeting and for PAA1 also a poly-glycine stretch to ensure envelope retention and correct (novel) topology. In addition, alternative splicing or gene duplication allowed the evolution of the novel Cu chaperone PCH1 from PAA1/CtaA. In analogy to the use of the N-terminus of PAA1 as a copper chaperone, PAA2 may use its N-terminal region as a Cu chaperone in the stroma (CuChap, hypothetical at present) that might be derived from the full transporter via proteolytic processing. Alternatively PAA2 may receive Cu ions that bound to low molecular weight chelators in the stroma or from CCS protein (not shown).

electron transport might be that it serves as a Cu store upon which the cell can rely in times of impending deficiency (Kropat et al., 2015). In this state, CRR1 mediates expression of the Fe containing functional PC alternative cytochrome-c_6 while PC expression is turned off and PC turnover induced, perhaps via a Deg-type protease (Kropat et al., 2015). The Cu coming out of PC can then be utilized for other functions such as CCO in mitochondria and ferric reductase activities at the cell surface (Kropat et al., 2015). This scenario would rely on Cu export from the thylakoids and chloroplasts. Similarly, during senescence in higher plants Cu from plastids might be re-purposed for use in seeds or storage in stem parts. For *Arabidopsis* it was estimated that less than 30% of Cu going to seeds comes from senescing rosettes, which might imply that Cu re-routing from plastids is a minor pathway (Waters et al., 2006). What could be the Cu exporter? The P-type ATPase HMA1 is located in chloroplast envelope membranes (Seigneurin-Berny et al., 2006). It was originally proposed that HMA1 protein functions in Cu homeostasis based on phenotypic characterization of yeast expressing recombinant HMA1 (Seigneurin-Berny et al., 2006). Furthermore, a lower Cu content and reduced stimulation of ATPase activity by Cu ions for chloroplasts isolated from plants that lack HMA1 was observed, which led to the suggestion that HMA1 might function as an alternative for PAA1 in mediating Cu import into plastids (Seigneurin-Berny et al., 2006). However, a careful analysis of *paa1/hma1* double mutants suggests that HMA1 is unlikely to transport Cu into plastids since the double mutant is virtually identical to the single *paa1* mutant for the maturation of both PC and CSD2 (Boutigny et al., 2014). Overexpression of HMA1 in different backgrounds caused higher Cu levels to be observed in chloroplasts of the *paa1* mutant background but not in the wild-type background (Boutigny et al., 2014). We conclude that HMA1 affects Cu homeostasis somehow but the substrate (and direction of transport) for HMA1 remains unclear (see for a more extensive review and data on this topic see: Boutigny et al., 2014).

Another possible Cu export pathway might be formed by members of the YSL family, which was first described for its involvement in Fe acquisition. Recently two YSL members (YSL4 and 6) were described in the chloroplast envelope where they have a function in preventing Fe overload (Divol et al., 2013). Perhaps these proteins might also mediate some Cu export. Alternatively, Cu might be recycled from chloroplasts present in vacuoles following autophagy. Autophagy of plastids in lytic vacuoles might be an important mechanism to recycle precious micronutrients such as Cu and this field deserves more attention in the future (for a review see Blaby-Haas and Merchant, 2014).

Finally, a question is how Cu reaches the chloroplast surface, a question that is important to answer in view of the strong capacity of Cu to bind to proteins and thus to be

sequestered in the cytosol (Burkhead et al., 2009). Perhaps cellular architecture should be considered here. Lysosomes, vacuoles and related compartments may play a role in providing Cu to the photosynthetic machinery. Under Zn deficiency, *Clamydomonas* sequesters Cu in a lysosome-related compartment, perhaps to prevent displacement of Zn with Cu in important Zn enzymes. This process is CRR1-dependent (Hong-Hermesdorf et al., 2014). It is not clear, however, if plants have a similar mechanism in place. The COPT5 transporter in the tonoplast membrane of plants allows Cu to re-enter the cytosolic Cu pool (Garcia-Molina et al., 2011; Klaumann et al., 2011). COPT5 is especially important during Cu starvation, a condition where it can be envisioned that it is beneficial to recycle precious Cu from organelles that are no longer needed, or damaged. In plant cells, the vacuole takes up a large volume and many organelles including chloroplasts are seen under the microscope to be pressed to the side of the cell by the vacuole. Under low Cu availability, close contact between the tonoplast and chloroplast envelopes would be an especially advantageous arrangement for Cu delivery to plastids. A very short distance between COPT5 and the chloroplast envelope surface would mean that Cu ions have to diffuse only a very small distance via outer membrane porins to reach PCH1 and PAA1 in the envelope and can virtually avoid the cytoplasm. It will be interesting to address these various questions in further research.

AUTHOR CONTRIBUTIONS

GA drafted the first manuscript. MP edited and finalized the MS.

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frontiers in Plant Science

MINI REVIEW
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Calcium Flux across Plant Mitochondrial Membranes: Possible Molecular Players

Luca Carraretto[1], Vanessa Checchetto[1,2], Sara De Bortoli[1], Elide Formentin[1,3], Alex Costa[4,5], Ildikó Szabó[1,6] and Enrico Teardo[1,6]**

[1] Department of Biology, University of Padova, Padova, Italy, [2] Department of Biomedical Sciences, University of Padova, Padova, Italy, [3] Department of Life Science and Biotechnology, University of Ferrara, Ferrara, Italy, [4] Department of Biosciences, University of Milan, Milan, Italy, [5] CNR, Institute of Biophysics, Consiglio Nazionale delle Ricerche, Milan, Italy, [6] CNR, Institute of Neurosciences, Padova, Italy*

Plants, being sessile organisms, have evolved the ability to integrate external stimuli into metabolic and developmental signals. A wide variety of signals, including abiotic, biotic, and developmental stimuli, were observed to evoke specific spatio-temporal Ca^{2+} transients which are further transduced by Ca^{2+} sensor proteins into a transcriptional and metabolic response. Most of the research on Ca^{2+} signaling in plants has been focused on the transport mechanisms for Ca^{2+} across the plasma- and the vacuolar membranes as well as on the components involved in decoding of cytoplasmic Ca^{2+} signals, but how intracellular organelles such as mitochondria are involved in the process of Ca^{2+} signaling is just emerging. The combination of the molecular players and the elicitors of Ca^{2+} signaling in mitochondria together with newly generated detection systems for measuring organellar Ca^{2+} concentrations in plants has started to provide fruitful grounds for further discoveries. In the present review we give an updated overview of the currently identified/hypothesized pathways, such as voltage-dependent anion channels, homologs of the mammalian mitochondrial uniporter (MCU), LETM1, a plant glutamate receptor family member, adenine nucleotide/phosphate carriers and the permeability transition pore (PTP), that may contribute to the transport of Ca^{2+} across the outer and inner mitochondrial membranes in plants. We briefly discuss the relevance of the mitochondrial Ca^{2+} homeostasis for ensuring optimal bioenergetic performance of this organelle.

Keywords: higher plants, mitochondria, calcium channels and transporters, calcium homeostasis, physiological processes

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Richard Sayre,
New Mexico Consortium at Los Alamos National Labs, USA

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Michael G. Palmgren,
University of Copenhagen, Denmark
Chun Pong Lee,
The University of Western Australia, Australia

****Correspondence:***
Enrico Teardo
enrico.teardo@unipd.it;
Ildiko Szabo
ildi@civ.bio.unipd.it

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INTRODUCTION

Mitochondria and Calcium Homeostasis

Molecular identification and pharmacological characterization of mitochondria-located ion channels allowed a deep understanding of the crucial importance of these proteins for organelle function and even for determining cell fate in animals (Leanza et al., 2014; Szabo and Zoratti, 2014). In plant mitochondria the current knowledge is unfortunately more limited than in the animal system and only few electrophysiological studies deal with plant mitochondrial ion channels. These include the voltage-dependent anion channel (VDAC), e.g., (Abrecht et al., 2000;

Berrier et al., 2015) of the outer mitochondrial membrane (OMM) and those of the inner membrane (IMM), i.e., a large conductance Ca^{2+}-insensitive potassium channel (Matkovic et al., 2011), a mitochondrial chloride channel (Matkovic et al., 2011) presumably corresponding to PIMAC (plant inner membrane anion channel) studied by classical bioenergetics (for review see Laus et al., 2008), a large-conductance Ca^{2+}-activated BK-type potassium channel (Koszela-Piotrowska et al., 2009) and an ATP-dependent potassium channel K (ATP; De Marchi et al., 2010; Jarmuszkiewicz et al., 2010; Matkovic et al., 2011). In almost all studies either purified inner mitochondrial vesicles or purified proteins have been exploited upon incorporation into artificial membrane via the black lipid bilayer technique, because application of the patch clamp electrophysiological technique is experimentally very demanding (De Marchi et al., 2010). In addition to electrophysiological investigation, in-depth bioenergetic studies proved the existence and the relevance of potassium-permeable pathways (e.g., Pastore et al., 1999; Trono et al., 2014, 2015), of anion transport (Laus et al., 2008), of the proton-gradient dissipating uncoupling proteins (Vercesi et al., 2006) and of the permeability transition pore (Vianello et al., 2012; Zancani et al., 2015) in the context of plant mitochondrial physiology. Plant mitochondria have been shown to contain an uptake system for Ca^{2+} as well (Hanson et al., 1965; Dieter and Marme, 1980; Martins and Vercesi, 1985; Carnieri et al., 1987; Silva et al., 1992; Zottini and Zannoni, 1993): however, the molecular identification of the pathways mediating Ca^{2+} flux is still far from complete. **Figure 1** summarizes the currently known/hypothesized pathways in plant mitochondria.

Understanding the routes and regulation of mitochondrial Ca^{2+} entry and exit would bring to an important advancement in the field and would help to determine the impact of Ca^{2+} homeostasis on bioenergetic efficiency and on the function of this organelle in plants. It has to be underlined that amongst the different signal transduction mechanisms Ca^{2+} plays a prominent role as a secondary messenger. A highly negative membrane potential in mitochondria (−150 to −180 mV) represents a huge driving force for the uptake of Ca^{2+} and other cations (Szabo and Zoratti, 2014) therefore calcium influx and efflux have to be under tight control in order to avoid calcium overload. In animal cells, transient accumulation of Ca^{2+} in intracellular organelles shapes cytosolic Ca^{2+} signals (Rizzuto et al., 2012) and a similar concept has been suggested for plants (Stael et al., 2012; Nomura and Shiina, 2014). The emerging view is that the influx of Ca^{2+} into the mitochondrial matrix can be induced by a variety of abiotic stresses such as heat, oxidative and salt stress, anoxia or extracellular ATP and extracellular Ca^{2+} (Logan and Knight, 2003; Xiong et al., 2006; Loro et al., 2012; Schwarzlander et al., 2012; Zhao et al., 2013; Rikhvanov et al., 2014; He et al., 2015; Pu et al., 2015), but the extent of uptake is variable among different tissues and species (Martins and Vercesi, 1985) as is the case also for the animals, (e.g., Fieni et al., 2012). The so far available data suggest that Ca^{2+} fluxes into and out of mitochondria might also shape the cytosolic "Ca^{2+} signature" (Stael et al., 2012; Nomura and Shiina, 2014), however, direct *in vivo* experimental proof has to be obtained in favor of this idea. In fact, in a recent study, where the calcium uptake into mitochondria has been impaired by deletion of the MCU regulator MICU (see below), cytoplasmic calcium transients appeared unaltered (Wagner et al., 2015).

Clear-cut demonstration of stimuli-induced Ca^{2+} uptake into mitochondria in intact plant tissues has been obtained thanks to the mitochondria-targeted genetically encoded Ca^{2+} sensors, aequorin (Logan and Knight, 2003) and Cameleon (Loro et al., 2012). Mitochondrial Ca^{2+} accumulation correlated to the intensity of Ca^{2+} increase in the cytoplasm. However, the molecular players of Ca^{2+} uptake and release remain to be largely elucidated. In addition, mostly indirect evidence indicate that in plant mitochondria, similarly to animal ones (Rizzuto et al., 2012), matrix Ca^{2+} levels regulate tricarboxylic acid (TCA) cycle enzymes (He et al., 2015) and oxidative phosphorylation (Wagner et al., 2015).

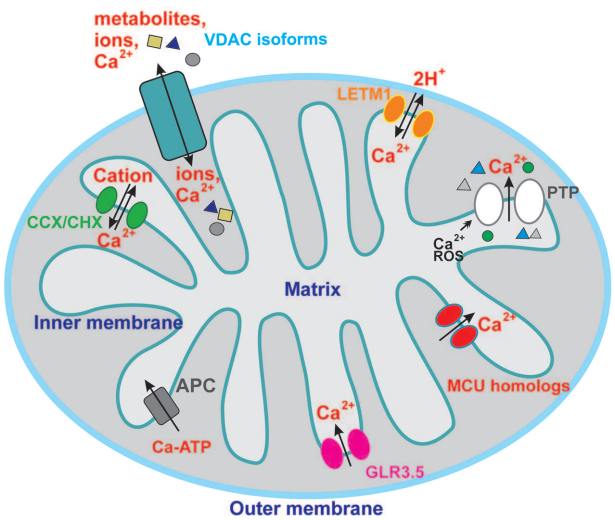

FIGURE 1 | Overview of the putative calcium-permeable ion channels and transporters in plant mitochondria. For sake of clarity, only channels and transporters putatively involved in calcium transport are shown, so the channels mediating flux of other ions are not depicted here. See text for further details.

Ca^{2+}-Flux Mediating Pathways in Animal Mitochondrial Membranes

Several players participating in calcium homeostasis have been identified over the last decades. We briefly mention these pathways, although it has to be kept in mind, that while knowledge from the animal field might be a source of inspiration, plant mitochondria do not necessarily take advantage of the same systems.

Voltage-dependent anion channel (VDAC) isoforms are universally recognized as the main pathways for flux of metabolites and ions across the OMM (see, e.g., Shoshan-Barmatz et al., 2010; Checchetto et al., 2014; Mertins et al., 2014; Madamba et al., 2015). VDACs, although defined as anion channels, can conduct a substantial flow of Ca^{2+}, as demonstrated both *in vitro* and *in vivo* for the mammalian

protein (Gincel et al., 2001; Rapizzi et al., 2002; Bathori et al., 2006; Israelson et al., 2007; Rizzuto et al., 2009; Shoshan-Barmatz et al., 2010). As to Ca^{2+} uptake across the IMM, the 40 kDa protein (MCU) has been proposed to be the channel-forming component of the Ca^{2+} uniporter (Baughman, 2011; De Stefani et al., 2011). The uptake of Ca^{2+} via the uniporter has been linked to energy production (De Stefani et al., 2015). Lack of MCU in knock-out mice causes only modest defects in skeletal muscle strength and minor metabolic changes (Pan et al., 2013; Pendin et al., 2014) but MCU was shown to be important for bioenergetic performance in other models (Huang et al., 2013; Wu et al., 2015). In addition to MCU, several additional proteins such as MICU1 (Perocchi et al., 2010), EMRE (Sancak et al., 2013), MCUR1 (Mallilankaraman et al., 2012), and MCUb (a dominant negative MCU isoform; Raffaello et al., 2013) as well as MICU2 (Patron et al., 2014) were reported to be essential components and/or regulators of the mammalian MCU complex (MCUC; De Stefani et al., 2015; Foskett and Philipson, 2015). MICU1/2 proteins are EF-hand proteins with the ability to regulate mitochondrial Ca^{2+} uptake and MCU channel activity upon Ca^{2+} binding (Patron et al., 2014). EMRE was proposed to mediate the physical interaction between MCU and MICU1/MICU2 dimer and has recently been shown to fine-tune calcium-regulation of the channel activity on the matrix side (Vais et al., 2016). MCUR1 also affects mitochondrial Ca^{2+} uptake, however the underlying mechanism is still highly debated (Paupe et al., 2015; Vais et al., 2015).

Mitochondrial Ca^{2+} uptake might take place via additional or alternative components as well. In animals, LETM1 has been proposed to possess a $Ca^{2+}/2H^{+}$ electroneutral antiporter activity and to take up Ca^{2+} (Jiang et al., 2013; Doonan et al., 2014; Tsai et al., 2014). The yeast homolog Mdm38 however affects potassium homeostasis (Nowikovsky et al., 2004) and impacts mitochondrial translation, independently of its ion transporter function (Bauerschmitt et al., 2010). The nature of the ions transported by LETM1 is still highly debated (Nowikovsky et al., 2012; Nowikovsky and Bernardi, 2014). In addition, the solute carrier 25A23 (SLC25A23) has been shown to interact with MCU and was proposed to play an important role in mitochondrial Ca^{2+} influx (Hoffman et al., 2014).

It also has to be mentioned that calcium uptake is linked to cell death pathways via induction of the PTP (Bernardi et al., 2015). The PTP, first characterized in mammalian cells, is a channel responsible for the permeability increase of the inner mitochondrial membrane under specific conditions (Zoratti and Szabo, 1995; Bernardi et al., 2015). PTP can be activated by different stimuli such as high matrix Ca^{2+} concentration (in 100s μM range) and oxidative stress, leading to swelling of mitochondria and dissipation of energy. In animals, when PTP opens only transiently, it possibly mediates Ca^{2+} release from mitochondria (Bernardi and von Stockum, 2012) together with the recently identified $3Na^{+}/Ca^{2+}$ antiporter (NCX; Palty et al., 2010). In addition, a still unidentified Na^{+}-insensitive Ca^{2+} release system, possibly a H^{+}/Ca^{2+} antiporter plays a role (Nowikovsky et al., 2012). Over the last decades VDAC, the adenine nucleotide carrier, the benzodiazepine receptor and cyclophilin D (CypD) were proposed in different combinations as the main components of the mammalian PTP (Zoratti and Szabo, 1995; Bernardi et al., 2015). The discovery that oxidative stress and application of elevated $[Ca^{2+}]$ results in channel formation by the dimeric form of the F-ATP synthase, opened a new perspective to the field (Giorgio et al., 2013). A recent work proposed instead, that mitochondrial spastic paraplegia 7 (SPG7), a nuclear-encoded mitochondrial metalloprotease (m-AAA) which interacts with CypD and VDAC1 and with a paraplegin-like protein AFG3L2, is essential for the PTP complex formation (Shanmughapriya et al., 2015). However, PTP could still be opened in the absence of SPG7, although at higher matrix calcium concentrations, suggesting that SPG7, similarly to CypD, acts a regulator rather than a crucial pore-forming moiety of the PTP (Bernardi and Forte, 2015). Interestingly, apart from their proteolytic roles, the *m*-AAA proteases mediate ATP-dependent membrane dislocation of the heme-binding reactive oxygen scavenger protein Ccp1 (Tatsuta et al., 2007), possibly linking PTP activation to oxidative stress.

Ca^{2+} FLUX-MEDIATING PATHWAYS IN PLANT MITOCHONDRIAL MEMBRANES

VDAC of the Outer Mitochondrial Membrane

In higher plants, similarly to animals, functionally distinct isoforms of VDAC exist. In particular, in *Arabidopsis* various isoforms displaying distinct subcellular localization (in the plasma membrane, mitochondria, chloroplasts, and plastids) and function have been identified (Smack and Colombini, 1985; Pottosin, 1993; Clausen et al., 2004; Tateda et al., 2011; Homble et al., 2012; Robert et al., 2012; Takahashi and Tateda, 2013; Michaud et al., 2014). Their roles, as assessed mostly by using T-DNA insertion knockout mutants of *Arabidopsis*, include regulation of development (Tateda et al., 2011; Robert et al., 2012; Pan et al., 2014), regulation of the hypersensitive response/programmed cell death (Lacomme and Roby, 1999; Tateda et al., 2009, 2011), of the response to abiotic stress (Li et al., 2013; Zhang et al., 2015) and import of tRNA into mitochondria (Salinas et al., 2006). Whether any of these functions requires Ca^{2+} flux across the OMM (or across other membranes) mediated by VDACs is unclear, however AtVDAC1 has been shown to interact in two-hybrid yeast system with CBL1, a Ca^{2+}-sensor (Li et al., 2013). Despite detailed electrophysiological characterization of several isoforms (Blumenthal et al., 1993; Pottosin, 1993; Mlayeh et al., 2010; Godbole et al., 2011; Berrier et al., 2015), experimental evidence is still missing to understand whether and how VDAC proteins influence Ca^{2+} flux across plant endomembranes.

Homologs of the Mitochondrial Calcium Uniporter (MCU) in the Inner Mitochondrial Membrane

In the *Arabidopsis thaliana* genome six genes are present which can be identified as putative MCU channel proteins with predicted mitochondrial targeting, since they display

sequence similarity with the mammalian MCU counterparts and contain the conserved DVME (Asp-Val-Met-Glu) selectivity filter sequence (Stael et al., 2012). Varying number of homologs can be identified in the genome of other higher plants as well (see Aramemnon http://aramemnon.uni-koeln.de/). Whether all these isoforms are indeed targeted to mitochondria, whether they form ion channels able to provide a permeation pathway for Ca^{2+} and whether the various isoforms operate in different tissues and/or at different developmental stages still awaits clarification. Recently obtained experimental evidence indicates that at least one of the isoforms is indeed targeted to mitochondria in *Arabidopsis* (Carraretto et al., 2016). The discovery showing that lack of the only existing isoform of the regulator, AtMICU, in *Arabidopsis* alters mitochondrial Ca^{2+} uptake points to a functional conservation of the core-components of the MCU complex in plants (Wagner et al., 2015). Interestingly, even though Ca^{2+} uptake into mitochondria and basal Ca^{2+} are significantly higher in AtMICU-less plants than in WT plants, respiration and mitochondrial morphology are only slightly affected and plant development is normal (Wagner et al., 2015). As to EMRE, its close homologs do not seem to be present in higher plants, while one of the two homologs of MCUR1 in *Arabidopsis* has been described as a plant specific subunit of complex IV (Millar et al., 2004; Klodmann et al., 2011). In summary, the plant homologs of MCU and MICU1 are certainly excellent candidates to be key players in mitochondrial Ca^{2+} homeostasis, but experimental proof for the ability of MCU proteins to form Ca^{2+}-permeable channels is still lacking.

Glutamate Receptor 3.5, LETM1/Mdm38 and Adenine Nucleotide/Phosphate Carriers (APCs) of the Inner Membrane

Similarly to the animal mitochondria, alternative calcium flux-mediating pathways seem to exist also in higher plants. Our knowledge in this respect is restricted mostly to *Arabidopsis*, since T-DNA insertion mutants of this model plant are available and widely used. In *Arabidopsis* deletion of both isoforms of LETM is lethal, probably due to the requirement of LETM proteins for mitochondrial protein translation and accumulation (Zhang et al., 2012). A recent study provided evidence that members of the APC family of *Arabidopsis* mediate a time dependent uptake of [^{45}Ca] (in the form of Ca-ATP) *in vitro* (Lorenz et al., 2015). This system is homolog of the above mentioned SLC25A23. The transport rate of AtAPC2 was however low and was completely blocked by 25-fold excess of Mg^{2+}, suggesting that *in vivo* Ca^{2+} flux might take place via this transporter only under specific conditions in plant mitochondria.

Another study located an alternative spliced isoform of a member of the glutamate receptor family, AtGLR3.5 to mitochondria. Although there is no direct evidence thus far demonstrating that the subfamily 3 member AtGLR3.5 functions as Ca^{2+}-permeable ion channel, the close homolog AtGLR3.4 and the AtGLR1.4 and AtGLR1.1 pores behave as Ca^{2+}-permeable non-selective cation channels when expressed in heterologous systems (Tapken and Hollmann, 2008; Vincill et al., 2012; Tapken et al., 2013). In addition, inner membrane vesicles isolated from spinach chloroplasts and containing members of the GLR subfamily 3 harbor a glutamate/glycine-induced Ca^{2+}-permeable activity which is sensitive to known animal ionotropic glutamate receptor antagonists (Teardo et al., 2010). Studies using *Atglr3.3* mutant plants showed that Ca^{2+} uptake induced by glutamate in *Arabidopsis* into hypocotyls and root cells is correlated with the presence of AtGLR3.3 (Qi et al., 2006). Therefore, similarly to the other members of the subfamily 3, AtGLR3.5 is expected to work as Ca^{2+}-permeable channel. In accordance, Ca^{2+} dynamics measurements performed using the Cameleon probe targeted to mitochondria in WT and mutant plants lacking AtGLR3.5 revealed that AtGLR3.5 might mediate Ca^{2+} uptake into mitochondria, at least in response to specific stimuli (e.g., wounding (Teardo et al., 2015).

In agreement with the mitochondrial localization and predicted activity of AtGLR3.5, plants lacking this protein harbor mitochondria with profoundly altered ultrastructure: a dramatic loss of cristae and swelling with the matrix becoming translucent can be observed. The exact mechanism leading to these changes is not known, even though *AtGLR3.5* topology indicates that the glutamate (agonist)-binding domains are located in the cytosol, possibly sensing the cytosolic glutamate/aminoacid concentration (Teardo et al., 2015). Whether, a reduced Ca^{2+} uptake in the *Atglr3.5* KO plants leads to morphological changes via reduction of the oxidative phosphorylation remains to be established. It is interesting to note that complex I inhibition in mammalian mitochondria leads to similar morphological changes to that observed for *Atglr3.5* KO plants (Ramonet et al., 2013).

Permeability Transition Pore

Plant mitochondria can also undergo Ca^{2+}-induced permeability transition, (Arpagaus et al., 2002; Petrussa et al., 2004; Vianello et al., 2012), an event linked to nitric oxide-induced cell death (Saviani et al., 2002) and to programmed cell death (for updated review see Zancani et al., 2015). Whether the "life-sustaining" F-ATP synthase forms the PTP in plants as well, still has to be clarified, but the thylakoid membrane, which also contains this ATP-producing machinery, harbors a high-conductance channel resembling PTP (Hinnah and Wagner, 1998). Ccp1 (see above) shares high sequence homology with ascorbate peroxidase (APX), with one of the isoforms dually targeted to mitochondria and chloroplasts (Chew et al., 2003). This further supports previous findings in the literature that ROS regulates PTP also in plants. A BLAST search in the *Arabidopsis* database reveals that it contains several ATP-dependent metalloproteases (FtsH proteases) that show high aminoacid sequence similarity to SPG7 (see above; e-values ranging from $2e^{-169}$ to $8e^{-58}$, with aminoacid identities in the range of 36–49%) and to AFG3L2 (e-value of 0 and 50% identity is found for AtFtsH3 and AtFtsH10). Among those with the highest score, AtFtsH3 and AtFtsH10 are present in mitochondria (Piechota et al., 2010).

Several plant putative Ca^{2+}/cation exchangers, for example CCX1, CCX3, and CCX4 (Schwacke et al., 2003), score for mitochondrial localization according to Aramemnon database although in most species rather poorly. Experimental evidence

in favor of their localization in mitochondria versus secretory pathways or of a dual localization is missing. Furthermore, for AtCCX3 it has been established that it functions as an endomembrane-localized H^+-dependent K^+ transporter (Morris et al., 2008). A putative cation/proton exchanger, AtCHX25 has also a predicted mitochondrial localization, but whether it mediates K^+/H^+ exchange like some members of the AtCHX family or Ca^{2+}/H^+ exchange has still to be established (Sze et al., 2004; Evans et al., 2012; Chanroj et al., 2013).

Under biologically relevant conditions (e.g., during oxidative stress), the above-described uptake and efflux pathways for calcium might cooperate. For example, Ca^{2+} influx-triggered Ca^{2+} release has been linked to pulsing of the mitochondrial membrane potential, a phenomena proposed to yield a transient uncoupling leading to reduced ROS production (Schwarzlander et al., 2012).

In summary, a combination of genetics, fluorescent probe imaging, electrophysiology, bioenergetics and physiology will hopefully provide answers to the numerous open questions related to mitochondrial calcium homeostasis in plants.

AUTHOR CONTRIBUTIONS

All authors contributed to the works published by our groups which are described in the minireview. Furthermore, all authors actively participated in writing the manuscript.

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REVIEW
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Modulation of Potassium Channel Activity in the Balance of ROS and ATP Production by Durum Wheat Mitochondria—An Amazing Defense Tool Against Hyperosmotic Stress

*Daniela Trono [1], Maura N. Laus [2], Mario Soccio [2], Michela Alfarano [2] and Donato Pastore [2]**

[1] *Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di Ricerca per la Cerealicoltura, Foggia, Italy,* [2] *Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, Università di Foggia, Foggia, Italy*

In plants, the existence of a mitochondrial potassium channel was firstly demonstrated about 15 years ago in durum wheat as an ATP-dependent potassium channel ($PmitoK_{ATP}$). Since then, both properties of the original $PmitoK_{ATP}$ and occurrence of different mitochondrial potassium channels in a number of plant species (monocotyledonous and dicotyledonous) and tissues/organs (etiolated and green) have been shown. Here, an overview of the current knowledge is reported; in particular, the issue of $PmitoK_{ATP}$ physiological modulation is addressed. Similarities and differences with other potassium channels, as well as possible cross-regulation with other mitochondrial proteins (Plant Uncoupling Protein, Alternative Oxidase, Plant Inner Membrane Anion Channel) are also described. $PmitoK_{ATP}$ is inhibited by ATP and activated by superoxide anion, as well as by free fatty acids (FFAs) and acyl-CoAs. Interestingly, channel activation increases electrophoretic potassium uptake across the inner membrane toward the matrix, so collapsing membrane potential ($\Delta\Psi$), the main component of the protonmotive force (Δp) in plant mitochondria; moreover, cooperation between $PmitoK_{ATP}$ and the K^+/H^+ antiporter allows a potassium cycle able to dissipate also ΔpH. Interestingly, $\Delta\Psi$ collapse matches with an active control of mitochondrial reactive oxygen species (ROS) production. Fully open channel is able to lower superoxide anion up to 35-fold compared to a condition of ATP-inhibited channel. On the other hand, $\Delta\Psi$ collapse by $PmitoK_{ATP}$ was unexpectedly found to not affect ATP synthesis *via* oxidative phosphorylation. This may probably occur by means of a controlled collapse due to ATP inhibition of $PmitoK_{ATP}$; this brake to the channel activity may allow a loss of the bulk phase Δp, but may preserve a non-classically detectable localized driving force for ATP synthesis. This ability may become crucial under environmental/oxidative stress. In particular, under moderate hyperosmotic stress (mannitol or NaCl), $PmitoK_{ATP}$ was found to be activated by ROS, so inhibiting further large-scale ROS production according to a feedback mechanism; moreover, a stress-activated phospholipase A_2 may generate FFAs, further activating the channel. In conclusion, a main property of $PmitoK_{ATP}$ is the ability to keep in balance the control of harmful ROS with the mitochondrial/cellular bioenergetics, thus preserving ATP for energetic needs of cell defense under stress.

Keywords: plant mitochondria, potassium channel, oxidative phosphorylation, reactive oxygen species, hyperosmotic stress, durum wheat

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Ildikò Szabò, University of Padova, Italy

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Marco Zancani, University of Udine, Italy

****Correspondence:***
Donato Pastore
donato.pastore@unifg.it

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THE MITOCHONDRIAL POTASSIUM CHANNELS FROM DURUM WHEAT AND OTHER PLANT SOURCES

To date, there is evidence of the existence in plant mitochondria of at least four different types of K^+ channels (**Table 1**): the ATP-sensitive K^+ channels ($PmitoK_{ATP}$ and other similar); the K^+ channel insensitive to ATP; the large conductance K^+ channel activated by Ca^{2+} ($mitoBK_{Ca}$); the large conductance K^+ channel insensitive to Ca^{2+} and sensitive to iberiotoxin (mitoBK).

ATP-sensitive K^+ Channels

The existence of a K^+ channel has been demonstrated for the first time in mitochondria from etiolated seedlings of durum wheat. This was achieved by means of measurements of decrease of electrochemical membrane potential ($\Delta\Psi$) due to externally added K^+ to energized mitochondria, as well as by swelling experiments in which K^+ influx into mitochondria was checked by monitoring absorbance decrease of mitochondrial suspension in isosmotic KCl solution (Pastore et al., 1999). The channel was found to be an ATP-sensitive K^+ channel and was named Plant $mitoK_{ATP}$ channel ($PmitoK_{ATP}$) in analogy with the animal counterpart, the $mitoK_{ATP}$. In durum wheat mitochondria (DWM) the $PmitoK_{ATP}$-mediated $\Delta\Psi$ decrease is specifically induced by K^+ (Cs^+ and Rb^+), whereas it is less evident in the presence of Na^+ or Li^+; the rate of K^+ uptake by DWM shows a hyperbolic dependence on the K^+ concentration with a Km of about 2 mM, which is significantly lower compared to the value of 32 mM measured for the $mitoK_{ATP}$ purified from rat liver mitochondria (Paucek et al., 1992). Moreover, the K^+ transport through the $PmitoK_{ATP}$ depends on $\Delta\Psi$; notably, the channel is activated by hyperpolarization with a fast increase of activity between 140 and 175 mV. Similarly to the animal counterpart, the $PmitoK_{ATP}$ is inhibited by ATP and, to a lesser extent, by ADP; it is also activated by diazoxide and by thiol-group reagents, such as mersalyl and *N*-ethylmaleimide (NEM). Contrarily to $mitoK_{ATP}$, the $PmitoK_{ATP}$ does not require Mg^{2+} for the ATP inhibition, it is activated rather than inhibited by palmitoyl-CoA and it is not inhibited by glyburide. The $PmitoK_{ATP}$ also differs from plant inward rectifying channels of non-mitochondrial membranes as it is not inhibited by Al^{3+}, Ba^{2+}, and TEA^+. Activation by CoA and inhibition by NADH and Zn^{2+} are typical features of the $PmitoK_{ATP}$ (Pastore et al., 1999).

In DWM, the $PmitoK_{ATP}$ is highly active and may cooperate with the K^+/H^+ antiporter. The operation of a K^+/H^+ exchanger in mammalian mitochondria has long been known (for review, see Bernardi, 1999; Xu et al., 2015), with the molecular identity in yeast and humans proposed by Zotova et al. (2010). The existence of a very active K^+/H^+ antiporter has been reported also in plant mitochondria (Diolez and Moreau, 1985) and some potential candidate genes have been reported by Sze et al. (2004). In DWM, the occurrence of a negligible ΔpH and of a high $\Delta\Psi$ is in line with the existence of a powerful K^+/H^+ antiporter (Trono et al., 2011). The cooperation between $PmitoK_{ATP}$ and K^+/H^+ antiporter allows the operation of a K^+ cycle that causes the re-entry of H^+ into the matrix, thus collapsing the proton motive force (Δp) (Pastore et al., 1999; Trono et al., 2004, 2011) by dissipating, in particular, the $\Delta\Psi$, which represents the main part of Δp in plant mitochondria (Douce, 1985; **Figure 1**). Interestingly, it has been demonstrated that the rate-limiting step of the K^+ cycle is represented by the electrophoretic K^+ influx *via* $PmitoK_{ATP}$ (Pastore et al., 1999). In this respect, $PmitoK_{ATP}$ strongly differs from the mammalian counterpart. Indeed, in mammalian mitochondria the K^+ cycle cannot uncouple completely, because the maximal rate of the cycle, that corresponds to the V_{max} of the K^+/H^+ antiporter, is only about 20% of the maximal rate of proton ejection by the respiratory chain (Garlid and Paucek, 2003). Indeed, in heart mitochondria, the increased K^+ influx associated to K^+ channel opening is small and it was found to depolarize by only 1–2 mV (Kowaltowski et al., 2001). In rat liver mitochondria some $\Delta\Psi$ decrease was observed which depended on KCl concentration (up to about 20 mV at 100 mM KCl), but it was compensated by an increase in ΔpH so that the Δp remained almost constant (Czyz et al., 1995).

The patch clamp technique, for the first time successfully applied to plant mitochondria, confirmed the existence in DWM of a cation channel inhibited by ATP, probably referable to the original $PmitoK_{ATP}$ (De Marchi et al., 2010). This channel (i) is inhibited by ATP but not by Mg^{2+}, Ba^{2+}, Ca^{2+}, and TEA^+, (ii) does not discriminate between K^+ and Na^+, (iii) has a conductance of 150 pS, (iv) has a strong voltage dependence and rectification.

In addition to the $PmitoK_{ATP}$ from DWM, the existence of ATP-sensitive K^+ channels has been afterward demonstrated by different research groups in mitochondria from different plant species and tissues/organs: pea stem (Petrussa et al., 2001), soybean suspension cell cultures (Casolo et al., 2005), embryonal masses of two coniferous species (Petrussa et al., 2008a), *Arum* spadix and tuber (Petrussa et al., 2008b), potato tuber (Matkovic et al., 2011) and potato cell cultures (Fratianni et al., 2001).

As for the K^+ selective and ATP-inhibited K^+ channel identified in pea stem mitochondria, evidence has been reported about its sensitivity to cyclosporine A (CsA; Petrussa et al., 2001), which has led to the assumption that it is involved in programmed cell death (PCD). In line with this hypothesis, the authors showed that, in these mitochondria, K^+ channel activation with CsA causes the rupture of the outer mitochondrial membrane and an increase in permeability transition of the inner membrane with consequent release of pyridine nucleotides and cytochrome *c* from mitochondria (Chiandussi et al., 2002; Petrussa et al., 2004). The involvement of the K^+ channel in mitochondrial swelling

Abbreviations: ADP/O, ratio between phosphorylated ADP and reduced oxygen; AOX, Alternative Oxidase; ATP D.S., ATP detecting system; CsA, cyclosporine A; DWM, durum wheat mitochondria; FFA, free fatty acid; IMAC, Inner Membrane Anion Channel; NEM, *N*-ethylmaleimide; NO, nitric oxide; OXPHOS, oxidative phosphorylation; PIMAC, Plant Inner Membrane Anion Channel; PLA_2, phospholipase A_2; $PmitoK_{ATP}$, Plant mitochondrial ATP-sensitive K^+ channel; PCD, programmed cell death; PUCP, Plant Uncoupling Protein; RC, respiratory control; RNS, reactive nitrogen species; ROS, reactive oxygen species; UCP, Uncoupling Protein; Δp, proton motive force; $\Delta\Psi$, electrochemical membrane potential.

TABLE 1 | Plant mitochondrial K^+ channels and their main modulators.

K^+ channel	Plant source	Activators	Inhibitors	Reference
ATP-sensitive				
$PmitoK_{ATP}$	Durum wheat seedling	Diazoxide, GTP, mersalyl, NEM, $\Delta\Psi$, superoxide anion, FFAs, acyl-CoAs	ATP, ADP, NADH, Zn^{2+}	Pastore et al. (1999, 2007, 2013); Trono et al. (2004, 2011); Laus et al. (2011)
ATP-sensitive cation-channel	Durum wheat seedling		ATP	De Marchi et al. (2010)
K^+_{ATP} channel	Pea stem	CsA, NO, FFAs	ATP, H_2O_2	Petrussa et al. (2001, 2004); Chiandussi et al. (2002)
	Soybean cell culture	CsA, NO, H_2O_2	ATP	Casolo et al. (2005)
	Picea abies and *Abies cephalonica* cell culture; *Arum* spadix and tuber	CsA	ATP	Petrussa et al. (2008a,b)
ATP-regulated K^+ channel	Potato tuber	Diazoxide	ATP, 5-hydroxydecanoate, glyburide	Matkovic et al. (2011)
ATP-insensitive				
ATP-insensitive K^+ transport	Potato tuber, tomato fruit, maize coleoptile		Quinine	Ruy et al. (2004)
Ca^{2+}-activated				
$mitoBK_{Ca}$	Potato tuber	Ca^{2+}, NS1619	ATP, iberiotoxin	Koszela-Piotrowska et al. (2009)
Ca^{2+}-insensitive				
mitoBK	Potato tuber		Iberiotoxin, charybdotoxin	Matkovic et al. (2011)

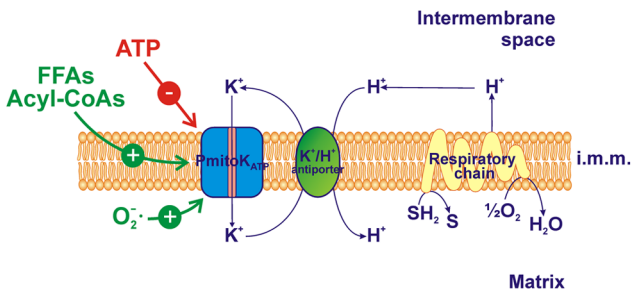

FIGURE 1 | $PmitoK_{ATP}$ functioning and modulation in durum wheat mitochondria (DWM). $PmitoK_{ATP}$ catalyzes the electrophoretic K^+ uptake across the inner membrane toward the matrix, so lowering membrane potential; the cooperation between $PmitoK_{ATP}$ and the K^+/H^+ antiporter allows a K^+ cycle able to dissipate also ΔpH, the second component of the proton motive force generated by the respiratory chain. ATP inhibits the channel while free fatty acids (FFAs), acyl-CoAs and superoxide anion activate it. The reducing equivalent flux through the respiratory chain to molecular oxygen and the coupled proton ejection into the intermembrane space are also indicated. The topology of ATP, FFAs, acyl-CoAs and ROS interaction with $PmitoK_{ATP}$ is not considered. SH_2, reduced substrates; S, oxidized substrates; i.m.m., inner mitochondrial membrane.

and cytochrome *c* release has been confirmed during H_2O_2-induced or NO-induced PCD of soybean suspension cell cultures (Casolo et al., 2005). A role of K^+ channel in thermogenic tissues has also been postulated. Notably, *Arum* spadix mitochondria possess a highly active K^+ channel, which mediates K^+ influx only when the over-reduction of the electron transport chain is lowered by Alternative Oxidase (AOX); under this condition, K^+ accumulation inside mitochondria mediated by the K^+ channel may contribute to prevent mitochondrial shrinkage consequent to the non-coupled respiration (Petrussa et al., 2008b).

An ATP-regulated K^+ channel with a conductance of 164 pS has also been detected in potato tuber mitochondria by Matkovic et al. (2011). Similarly to $mitoK_{ATP}$, this channel causes low K^+ influx, which leads to a modest decrease in $\Delta\Psi$ (up to a few mV); moreover, it is activated by diazoxide and blocked by ATP, 5-hydroxydecanoate and glyburide.

ATP-insensitive K^+ Channels

A highly active ATP-insensitive K^+ channel has been described in potato, tomato and maize mitochondria (Ruy et al., 2004). This channel has a greater selectivity for K^+ compared to $PmitoK_{ATP}$, it is insensitive to NADH, 5-hydroxydecanoate and glyburide, typical modulators of ATP-sensitive K^+ channels, and it is sensitive to quinine, a broad-spectrum inhibitor of K^+ channels. Similarly to the $PmitoK_{ATP}$, the ATP-insensitive K^+ channel allows the import of large quantities of K^+ inside mitochondria; this determines a significant increase in the respiration rate in state 4, that could potentially lead to a reduction in the ability of phosphorylation of these mitochondria.

Large-conductance Ca^{2+}-activated K^+ Channel

Six years ago, for the first time, the existence of a large-conductance (502–615 pS) and Ca^{2+}-activated K^+ channel in mitochondria from potato tuber was shown by Koszela-Piotrowska et al. (2009). The authors demonstrated that, in the presence of KCl and under conditions in which the ATP-sensitive K^+ channels are inhibited by ATP, a decrease of $\Delta\Psi$ occurs, which is stimulated by both Ca^{2+} and NS1619, this latter being an opener of BK_{Ca} channels, and inhibited by iberiotoxin, an inhibitor of both plasma membrane K^+ channels and BK_{Ca}.

Large-conductance Ca^{2+}-insensitive K^+ Channel

Mitochondria from potato tubers also possess a large-conductance (312 pS) and Ca^{2+}-insensitive K^+ channel (mitoBK;

Matkovic et al., 2011). Similarly to the mitoBK$_{Ca}$ channel, the mitoBK channel is inhibited by iberiotoxin. Charybdotoxin, another K^+ channel blocker, also inhibits the potato tuber mitoBK channel. The authors reported evidence that, under phosphorylating conditions, the coupling parameters of potato tuber mitochondria remain unchanged in the presence of high K^+ level, thus indicating that the K^+ channel present in these mitochondria functions as energy-dissipating system that is not able to divert energy from oxidative phosphorylation (OXPHOS).

It is noteworthy that the occurrence of some K^+ pathways has been demonstrated by swelling experiments in mitochondria from other plant species and tissues/organs, including (i) etiolated seedlings of bread wheat, spelt, rye, barley and lentil, (ii) green leaves of triticale, maize, spinach and durum wheat, (iii) pea roots, (iv) potato and topinambur tubers (Pastore et al., 1999; Laus et al., 2011). However, to date, no information is available about ATP and/or Ca^{2+} modulation of these K^+ pathways.

Molecular Identities of Mitochondrial K^+ Channels

In spite of the important role of mitochondrial K^+ channels in cell physiology, knowledge of their molecular identities is difficult to obtain; so it proceeds only slowly and remains still limited.

Information about the molecular structure of PmitoK$_{ATP}$ and other plant mitochondrial K^+ channels is still very scarce and molecular identity remains unknown. At our best knowledge, only a report is available that regards the partial purification from potato of a mitoK$_{ATP}$, which was suggested to contain Kir and SUR type subunits (Paucek et al., 2002). The identification by means of a proteomic approach of a β regulatory subunit of a Kv channel has also been reported in rice mitochondria (Tanaka et al., 2004), although the identity of the channel associated with it remains unknown. With regard to possible candidate genes, a strong mitochondrial targeting is predicted for the *Shaker*-type Kv genes AKT1 or AKT1-like in several plant species, including barley, grape, rice and bread wheat, but not *Arabidopsis thaliana* (De Marchi et al., 2010). In particular, in wheat root an AKT1-like channel with a strong prediction of mitochondrial localization has been cloned (Buschmann et al., 2000). In addition, only one Kir-like channel has been identified in the *A. thaliana* genome, which is the single-pore K^+ channel AtKCO3. However, this channel does not show any sequence homology with Kir6.1 and Kir6.2 and its mitochondrial targeting is predicted with low probability and only by some localization prediction tools (De Marchi et al., 2010).

With regard to the PmitoK$_{ATP}$, some hypotheses about the possible molecular identity of this channel have been proposed based on its electrophysiological properties. In particular, the voltage dependence of the DWM channel prompted the authors to propose that it might correspond to the single-pore KCO3 channel (De Marchi et al., 2010), although this proposal is in contrast with the high conductance and the high permeability to Na^+ of the observed channel activity. The possibility that the DWM channel may be a *Shaker*-like channel with altered characteristics, such as the lack of β-subunit or, alternatively, the presence of another regulatory protein, may be also considered. In particular, one possibility may be that an ABC-transporter protein is associated with AtKCO3 or AKT1-like subunits to give rise to the ATP-dependent DWM channel activity. Moreover, in the light of both the high conductance and the poor selectivity for K^+ over Na^+, the possibility that the observed DWM channel may be a non-selective cation channel (NSCC) cannot be excluded (De Marchi et al., 2010).

As for other plant mitochondrial K^+ channels, immunological studies have suggested the molecular identity of the ATP-regulated K^+ channel (Matkovic et al., 2011) and of the mitoBK$_{Ca}$ (Koszela-Piotrowska et al., 2009) detected in potato tuber mitochondria by electrophysiological studies (see above). Indeed, immunoreactivity with antibodies raised against the human pore-forming subunits of the Kir6.0-family, as well as against the α- and β-subunits of the mammalian plasma membrane BK$_{Ca}$ channel, has confirmed the presence in these mitochondria of an ATP-regulated K^+ channel and of a large-conductance Ca^{2+}-activated iberiotoxin-sensitive K^+ channel, structurally similar to the mammalian mitoK$_{ATP}$ and mitoBK$_{Ca}$ channels, respectively (Koszela-Piotrowska et al., 2009; Matkovic et al., 2011).

Substantial efforts are still needed to achieve the molecular identification of plant mitochondrial K^+ channels.

PHYSIOLOGICAL MODULATORS OF THE PmitoK$_{ATP}$

Inhibition by ATP

As said above, PmitoK$_{ATP}$ activity is inhibited by ATP, with the inhibition occurring at the outer side of the inner membrane (Laus et al., 2008; **Figure 1**). ATP inhibition of PmitoK$_{ATP}$ does not require the presence of Mg^{2+} ions (Pastore et al., 1999; De Marchi et al., 2010), so differing from the mammalian mitoK$_{ATP}$ (Garlid, 1996). Moreover, PmitoK$_{ATP}$ differs from the mammalian counterpart for another important aspect of ATP inhibition. The mitoK$_{ATP}$ activity is strongly inhibited by very low ATP concentrations, with half inhibition of 22–40 μM (Garlid, 1996; Garlid and Paucek, 2003); this suggests that the degree of mitoK$_{ATP}$ opening *in vivo* should be not easily modulated by ATP (Garlid and Paucek, 2003), whose concentration in mammalian cells falls in the millimolar range. Conversely, PmitoK$_{ATP}$ affinity for ATP is significantly lower (from 10- to 15-fold) than that of the mammalian counterpart. Indeed, a non-competitive inhibition by ATP on the PmitoK$_{ATP}$ was observed with a Ki of about 0.3 mM (Pastore et al., 1999); consistently, a $K_{0.5}$ of 0.5 mM was measured by patch clamp technique (De Marchi et al., 2010). These results suggest a possible PmitoK$_{ATP}$ regulation by ATP *in vivo*. With respect to this point, the nucleotide triphosphate concentration measured in plant cell by means of NMR analysis is 0.9–1.2 mM (Gout et al., 1992). Moreover, about 70% of nucleotide triphosphate concentration is formed by ATP, as measured in perchloric acid extract (Roby et al., 1987). Considering these data, a cytosolic ATP concentration of 0.6–0.8 mM may be assumed. Moreover, it should be also considered that the apparent Km for K^+ uptake by PmitoK$_{ATP}$ is 2.2 mM (Pastore et al., 1999) and that plant mitochondria show a high cytosolic K^+ concentration

homeostatically maintained at high levels (80–100 mM; Leigh and Wyn Jones, 1984). In the light of these data, and on the basis of a rough calculation according to the Michaelis–Menten equation, a PmitoK$_{ATP}$ functioning equal to 20–45% of the maximal activity could be reached at physiological ATP concentrations; moreover, a change of channel functioning may be expected as a response to changing ATP concentrations. Consistently, in different mitochondrial preparations, in the presence of 0.7, 0.5 and 0.3 mM ATP, an activation of PmitoK$_{ATP}$ was measured with respect to 0.9 mM ATP condition, equal to 25.3 ± 4.3%, 37.2 ± 3.2%, 44.7 ± 7.4% (SE, $n = 4$), respectively; in the absence of ATP, the activation was equal to 184.7 ± 13.2% (Soccio et al., 2013).

It is noteworthy that, among the mitochondrial dissipative systems, ATP inhibition is not a peculiar characteristic of (P)mitoK$_{ATP}$. Indeed, ATP, GTP, and GDP, have been widely demonstrated to act as inhibitors of Uncoupling Proteins (UCPs) from animal and plant sources (Hourton-Cabassa et al., 2004). As far as DWM is concerned, ATP is the main inhibitor of Plant Uncoupling Protein (PUCP), while GDP and GTP are less effective (Pastore et al., 2000). In particular, 0.5 mM ATP was found to determine an inhibition of about 70% of the rate of the linoleate-induced $\Delta\Psi$ decrease in succinate-respiring mitochondria; consistently, in mitochondria depolarized by linoleate, 30 μM ATP was able to cause about 50% $\Delta\Psi$ recovery (Pastore et al., 2000). Moreover, in DWM, ATP at physiological concentrations was found to strongly inhibit (45–85%) also the Plant Inner Membrane Anion Channel (PIMAC), probably acting at the outer side of the inner membrane (Laus et al., 2008). So, ATP counteracts the transport of both K^+ and Cl^- through the inner membrane of DWM. A strong ATP inhibition (50–80%) was also observed on the PIMAC of topinambur tuber mitochondria (Laus et al., 2008).

Activation by ROS

In the plant cell, mitochondria and chloroplasts represent the major site of reactive oxygen species (ROS) generation. In particular, the Complex I and especially the Complex III of the mitochondrial electron transport chain are involved in the non-enzymatic one-electron reduction of molecular oxygen to generate superoxide anion; at the level of the Complex III superoxide anion may be released not only into the matrix but also on the outer side of the inner mitochondrial membrane (Blokhina and Fagerstedt, 2010). A key role in the superoxide anion production is played by the redox state of the ubiquinone pool. In fact, ubiquinone over-reduction increases the lifetime of the ubisemiquinone radical, which, in turn, promotes single electron transfer to molecular oxygen, so generating superoxide anion. Superoxide anion produced in the mitochondrial matrix may generate H_2O_2 in an enzymatic reaction catalyzed by a matrix-localized Mn-superoxide dismutase. Plant mitochondria are known to generate also reactive nitrogen species (RNS). In particular, nitrite reduction to nitric oxide (NO) occurs at Complex III and Complex IV of the electron transport chain (Igamberdiev et al., 2014).

The activation of a mitochondrial K^+ channel by superoxide anion has been reported for the first time in DWM (Pastore et al., 1999; **Figure 1**). In particular, an about 100% stimulation of the PmitoK$_{ATP}$ activity was observed as a consequence of mitochondria incubation with the superoxide anion-producing system, consisting of xanthine *plus* xanthine oxidase. Moreover, a partial prevention of activation was observed when the reaction medium was added with superoxide dismutase, able to remove superoxide anion. Two years later, activation by superoxide anion has also been reported for the mammalian mitoK$_{ATP}$ by Zhang et al. (2001a). The authors found that reconstituted myocardial mitoK$_{ATP}$ was markedly activated by superoxide anion and that the activation was completely prevented by pretreatment with the sulfhydryl alkylating compound NEM. This result suggests that the activation by superoxide anion of the mitoK$_{ATP}$ may be dependent on its direct action on the sulfhydryl groups of the channel protein. Successively, in isolated rat heart mitochondria the mitoK$_{ATP}$ activity was found to be strongly enhanced by stimulation of endogenous mitochondrial ROS generation or mitochondrial treatment with H_2O_2; this occurred in a manner probably dependent on redox sensors located in the sulfonylurea receptor of the channel (Facundo et al., 2007). On the other side, evidence has been reported that in rat heart mitochondria the mitoK$_{ATP}$ is not activated by superoxide anion but it is activated indirectly by NO and H_2O_2, through the activation of protein kinase Cε (Costa and Garlid, 2008). A direct activation of the mitoK$_{ATP}$ by NO was instead observed in cardiac submitochondrial particles (Ljubkovic et al., 2007).

With regard to other plant mitochondria, a regulation of the K^+ channels by ROS and RNS has also been reported in pea steam mitochondria (Chiandussi et al., 2002). Notably, in these mitochondria the K^+_{ATP} channel resulted to be inhibited by H_2O_2 and activated by NO. As already observed for the animal counterpart, also in pea steam mitochondria the NO-induced activation of the K^+ channel may be dependent on a nitrothiosylation reaction occurring between NO^+ and specific sulfhydryl groups present in the protein forming the channel (Chiandussi et al., 2002).

It should be outlined that in plants a regulation by ROS and RNS was also observed on K^+ channels located in plasma membranes. Indeed, in *A. thaliana*, hydroxyl radical was found to induce a large outward-rectifying K^+ current attributed to GORK, a guard cell-type constitutive outward-rectifying root plasma membrane K^+ channel (Demidchik et al., 2010), whereas heterologously expressed SKOR was found to be modulated by H_2O_2 (Garcia-Mata et al., 2010). Also, Xia et al. (2014) recently demonstrated that NO lowers AKT1 channel-mediated K^+ uptake in *A. thaliana* root cells by modulating vitamin B6 biosynthesis, whereas a NO-induced up-regulation of the AKT1 gene was reported in mangrove plant, which contributes to K^+/Na^+ balance (Chen et al., 2013).

As far as the effect of ROS and RNS on other plant mitochondrial dissipative systems, an activation of PUCP by both H_2O_2 and superoxide anion has been reported in DWM (Pastore et al., 2000) and topinambur (Paventi et al., 2006). Indeed, the addition of H_2O_2 and superoxide anion to succinate-respiring DWM, partially depolarized by low linoleate concentrations (4–8 μM), was found to determine a further and complete

$\Delta\Psi$ collapse; moreover, when H_2O_2 or superoxide anion was added before 8 μM linoleate, the linoleate-induced $\Delta\Psi$ decrease occurred to a higher extent and at a higher rate (about 40%). To date, no information about PUCP modulation by NO is available; nevertheless, sensitivity of PUCP activity to changes of *S*-nitrosoglutathione reductase levels has been recently reported in *A. thaliana* transgenic cell lines, thus suggesting that PUCP activity might be modulated by nitrosothiols/NO content (Frungillo et al., 2013). Moreover, in suspension cells of *Petunia hybrida* (Wagner, 1995) and tobacco (Vanlerberghe and McIntosh, 1996), AOX gene expression was found to be induced by H_2O_2. Conversely, in DWM, AOX activity was demonstrated to be insensitive to H_2O_2 and superoxide anion, while activation was found to be dependent on photorespiratory and malate metabolism (Pastore et al., 2001, 2003). Similarly, the lack of effect of NO on the AOX activity (Millar and Day, 1996) and its ability to induce AOX gene expression (Huang et al., 2002) were reported. However, although ROS may act as activators of some transport pathways, it should be underlined that an excess of harmful ROS also causes inhibition of some other transports in DWM (Pastore et al., 2002).

Activation by Free Fatty Acids and Acyl-CoAs

As demonstrated by means of swelling experiments in KCl solutions, linoleate and other free fatty acids (FFAs), including the non-physiological 1-undecanesulphonate and 5-phenylvalerate, added to isolated DWM at a concentration of 10 μM, activated K^+ uptake *via* PmitoK$_{ATP}$ by 2–4-fold (Laus et al., 2011; **Figure 1**). The FFA-induced activation of PmitoK$_{ATP}$ is not associated to the depletion of endogenous Mg^{2+}, so differing from that of the mammalian K^+ channels (Schönfeld et al., 2003 and references therein). Also in pea stem mitochondria FFAs appear to stimulate the K^+_{ATP} channel activity in dissipating $\Delta\Psi$ (**Table 1**, Petrussa et al., 2004). Regulation by FFAs in plants is observed on plasma membrane K^+ channels, too; in particular, in guard cells, FFAs have been reported to activate the inwardly rectifying K^+ channel and to inhibit the outward K^+ channel (Lee et al., 1994).

Interestingly, in DWM, K^+ transport, evaluated by swelling experiments, was also found to be stimulated by acyl-CoA esters (**Figure 1**). The acyl-CoA-mediated stimulation of K^+ uptake resulted much higher (5–12-fold) than that induced by the corresponding FFAs (Laus et al., 2011). With regard to acyl-CoAs, PmitoK$_{ATP}$ behavior differs from that of the rat liver mitoK$_{ATP}$, whose activity is strongly inhibited by palmitoyl-CoA and oleoyl-CoA (Garlid, 1996; Paucek et al., 1996), and more closely resembles that of mammalian plasma membrane K$_{ATP}$ channels. Indeed, acyl-CoAs represent one of the main classes of activators of plasma membrane ATP-regulated K^+ channels in pancreatic beta cells (Bränström et al., 2007 and references therein; Webster et al., 2008 and references therein) and cardiac muscle cells (Liu et al., 2001; Schulze et al., 2003).

The FFA/acyl-CoA-induced stimulation of K^+ transport observed in DWM by means of swelling experiments was also confirmed by $\Delta\Psi$ measurements. Indeed, the addition to succinate-respiring DWM of 5-phenylvalerate or palmitoyl-CoA, both unable to activate the PUCP, was found to stimulate the depolarization induced by K^+ uptake *via* PmitoK$_{ATP}$. This stimulation was partially recovered/prevented by the addition of ATP, thus suggesting that FFAs/acyl-CoAs are activators of PmitoK$_{ATP}$ able to modulate its sensitivity to ATP (Laus et al., 2011). It is noteworthy that this stimulation is enhanced when FFAs and acyl-CoAs are present together. Indeed, when palmitate at concentrations ranging between 20 and 50 nmol mg^{-1} of protein was added to DWM suspended in a KCl medium containing palmitoyl-CoA at a physiological concentration (2.5 μM) (Larson and Graham, 2001), a synergistic action was observed able to determine a very strong activation (up to 11-fold) of PmitoK$_{ATP}$. Moreover, activation of K^+ transport by FFAs/acyl-CoAs resulted a property common to other plant mitochondria isolated from different mono/dicotyledonous species, including bread wheat, barley, triticale, maize, lentil, pea and topinambur, as well as from different organs, such as root, tuber, leaf and shoot (Laus et al., 2011).

Interestingly, PmitoK$_{ATP}$ activation was also observed after addition to DWM of small amount of a bee venom phospholipase A_2 (PLA_2), an enzyme able to specifically hydrolyze membrane phospholipids at the *sn*-2 position to yield FFAs and lysophospholipids. This suggests that activation of PmitoK$_{ATP}$ by FFAs/acyl-CoAs may occur *in vivo* as a result of endogenous generation of FFAs from membrane phospholipids and their metabolism (Laus et al., 2011). The recent discovery of a PLA_2 activity in DWM and mitochondria from other plant species (barley, spelt, maize and tomato) and tissues/organs (tuber and green and etiolated seedlings; Trono et al., 2013) is in line with the proposed physiological regulation of PmitoK$_{ATP}$ by FFAs/acyl-CoA derivatives.

With regard to other dissipative systems, FFAs are known to be strictly required for UCP activation in both plant and animal mitochondria (for reviews, see Vercesi et al., 2006; Echtay, 2007). Moreover, Sluse et al. (1998) reported that FFAs may act as AOX inhibitors. In addition to dissipative systems, long-chain FFAs have also been reported to activate the Inner Membrane Anion Channel (IMAC), the K^+ uniport and the K^+/H^+ antiport in rat liver mitochondria (Schönfeld et al., 2003, 2004). Recently, stimulation by polyunsaturated FFAs of mitoBK$_{Ca}$ has also been reported in human astrocytoma U87 MG cell lines (Olszewska et al., 2014). With regard to DWM, it has been shown that these mitochondria possess a so active PUCP that very low FFA concentrations (8–12 μM) are enough to quickly and completely collapse $\Delta\Psi$ (Pastore et al., 2000, 2007). Besides the dissipative systems, also DWM-PIMAC was found to be inhibited by unsaturated and, to a lesser extent, saturated FFAs (Laus et al., 2008), an opposite behavior compared to the animal counterpart. In practice, in DWM, FFAs activate K^+, but inhibit Cl^- uptake; this behavior may limit excess swelling and inner membrane rupture under physiological conditions that induce PLA_2 activation and high FFA generation. Moreover, no inhibition of DWM-PIMAC was observed in the presence of acyl-CoA esters (Laus et al., 2008), although palmitoyl-CoA is known to inhibit IMAC (Halle-Smith et al., 1988).

EFFECT OF PmitoK$_{ATP}$ MODULATION ON MITOCHONDRIAL ROS PRODUCTION AND ATP SYNTHESIS *VIA* OXIDATIVE PHOSPHORYLATION

Activation of PmitoK$_{ATP}$ and Control of ROS Production

A dramatic stimulation of mitochondrial generation of superoxide anion and other harmful ROS is induced by high $\Delta\Psi$ values and by an over-reduction state of the respiratory chain components. So, according to the "mild uncoupling" theory proposed by Skulachev (1994, 1998), a lowering of $\Delta\Psi$ may markedly decrease superoxide anion generation, so protecting mitochondria from high ROS production. Consistently, evidence has been reported that, in DWM, the activation of PmitoK$_{ATP}$ may lower superoxide anion generation by controlling $\Delta\Psi$ levels. In particular, in DWM that oxidized succinate in 0.5 mM KCl medium, the rate of superoxide anion generation was equal to 42 $\pm$ 8.8 nmol min^{-1} mg^{-1} of protein; this rate was lowered to 22 $\pm$ 5.6 nmol min^{-1} mg^{-1} of protein when mitochondria were suspended in a medium at high (100 mM) KCl concentration (Pastore et al., 1999). Moreover, in DWM that oxidized ascorbate *plus* *N*, *N*, *N*′, *N*′-tetramethyl-*p*-phenylenediamine, the activation of PmitoK$_{ATP}$ by KCl *plus* mersalyl almost completely abolished the superoxide anion generation. This is not surprising, since, as reported above, PmitoK$_{ATP}$ may act together with the active K^+/H^+ antiporter, thus allowing the operation of a K^+ cycle able to induce a $\Delta\Psi$ collapse that, in turn, may lower the mitochondrial ROS generation.

The capability to control mitochondrial ROS generation is not a peculiar property of the PmitoK$_{ATP}$, but it is common to other plant mitochondrial K^+ channels. Notably, in pea stem mitochondria, the activation of the K^+_{ATP} channel by CsA and its inhibition by ATP were found to increase and to decrease, respectively, the depolarization induced by the channel functioning and, consequently, to decrease and to increase the mitochondrial H_2O_2 production. However, the prevention of ROS production was observed only in pea stem mitochondria respiring succinate and not in those oxidizing malate *plus* glutamate, thus indicating that, in these mitochondria, the operation of the channel is able to control only the ROS generation at the level of the Complex III (Casolo et al., 2003). Also in potato tuber mitochondria, the activation of the ATP-insensitive K^+ transport was found to induce a decrease in the mitochondrial H_2O_2 generation (Ruy et al., 2004).

With respect to ROS control, PmitoK$_{ATP}$ may differ from the mammalian counterpart due to the fact that in mammalian mitochondria the K^+ cycle is very low and causes a negligible depolarization (see above). So, in rat heart mitochondria an increase in ROS generation was even observed as a consequence of mitoK$_{ATP}$ activation (Garlid et al., 2013 and references therein). On the other hand, in the same mitochondria an opposite behavior was reported by Facundo et al. (2005), who showed an inhibition of ROS production due to mitoK$_{ATP}$ activation; similarly, inhibition of ROS production was also reported for mitochondria of other mammalian tissues, *e.g.,* brain (Ferranti et al., 2003), liver (Alberici et al., 2009) and spleen (Alberici et al., 2013). These controversial findings in mammalian mitochondria may be dependent on different experimental conditions and effects of K^+ channel on $\Delta\Psi$.

Many other energy-dissipating systems in plant and animal mitochondria have been proposed to efficiently control mitochondrial ROS generation (Kowaltowski et al., 2009; Blokhina and Fagerstedt, 2010). As for purified DWM, PUCP and AOX activation was demonstrated to dampen mitochondrial ROS production. In particular, in succinate-respiring mitochondria a 45% decrease of superoxide anion generation rate was observed in the presence of externally added linoleate, able to activate PUCP (Pastore et al., 2000); this effect was prevented by the addition of bovine serum albumin, able to remove FFAs. Similarly, AOX activation by externally added pyruvate in DWM respiring malate *plus* glutamate was found to reduce by half the rate of superoxide anion generation; the inhibition of AOX by propylgallate restored the rate of superoxide anion generation to the levels measured in the absence of pyruvate (Pastore et al., 2001).

On the whole, *in vitro* modulation of PmitoK$_{ATP}$ due to externally added ATP, FFAs/acyl-CoAs and ROS regulates the mitochondrial $\Delta\Psi$ and ROS generation. Notably, as shown in **Figure 2**, ATP addition to DWM inhibits PmitoK$_{ATP}$ and, consequently, the K^+ cycle; this generates a high $\Delta\Psi$ and a high ROS production (**Figure 2A**). On the contrary, the addition of FFAs and/or their acyl-CoA derivatives, as well as of ROS, which all activate PmitoK$_{ATP}$, determines an increase in the rate of the K^+ cycle, thus leading to a decrease in $\Delta\Psi$ and ROS production (**Figure 2B**).

Unexpected Lack of Inhibition on ATP Synthesis in DWM Depolarized by PmitoK$_{ATP}$

In isolated DWM, PmitoK$_{ATP}$ activity is able to collapse $\Delta\Psi$, but unexpectedly the loss of Δp is unable to inhibit ATP synthesis *via* OXPHOS (Trono et al., 2011). Indeed, succinate-respiring DWM, added with 25 mM KCl, showed a very low $\Delta\Psi$, but both the ATP synthesis and the coupling were found to be not affected. These results were obtained by using three different approaches, that is (i) the continuous monitoring of the ATP synthesis and efflux outside DWM by using an enzymatic ATP detecting system (ATP D.S.), (ii) the enzymatic measurement of the ATP synthesized at the end of a phosphorylation cycle, (iii) the oxygraphic determination of the respiratory control (RC) ratio and of the ratio between phosphorylated ADP and reduced oxygen (ADP/O). The first approach allowed calculation of a rate of ATP appearance outside DWM of 193 $\pm$ 11.8 nmol min^{-1} mg^{-1} of protein and 221 $\pm$ 15.1 nmol min^{-1} mg^{-1} of protein, respectively, in the absence and in the presence of KCl addition, thus suggesting that no significant difference exists between control and KCl-depolarized DWM. This observation was confirmed by the result obtained with the second approach, in which DWM synthesized ATP in the absence of the ATP D.S. and this latter was added at the end of the reaction to assay the amount of ATP produced. Both control and KCl-depolarized DWM were able to completely convert 50 μM ADP into ATP, thus confirming

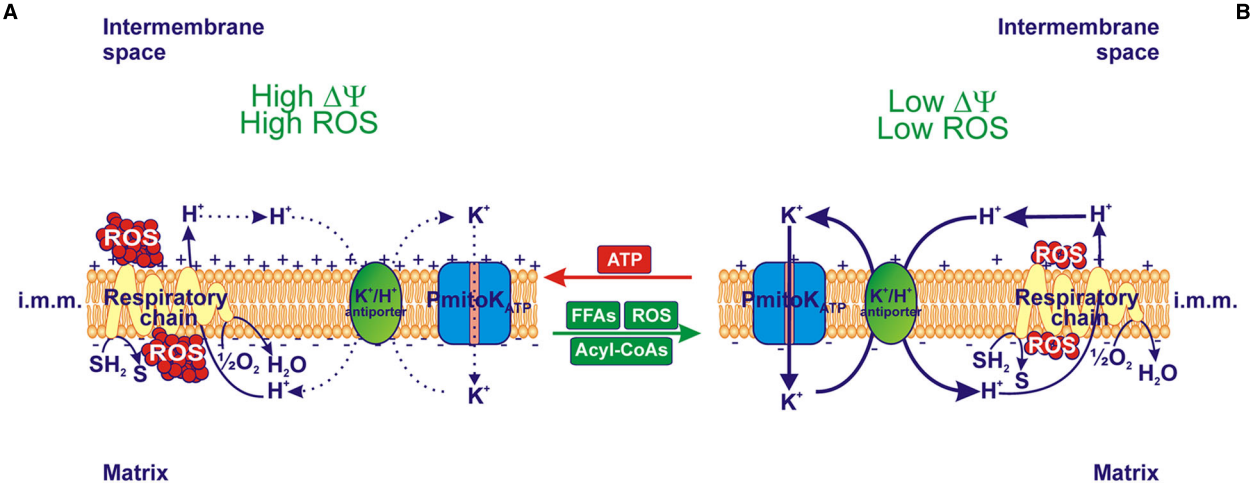

FIGURE 2 | Effect of *in vitro* modulation of PmitoK$_{ATP}$ on $\Delta\Psi$ and reactive oxygen species (ROS) production. (A) When ATP inhibits PmitoK$_{ATP}$ and, as a consequence, the K^+ cycle due to the PmitoK$_{ATP}$-K^+/H^+ antiporter combined function, both high electrical membrane potential ($\Delta\Psi$) and high ROS production are observed. **(B)** On the contrary, in the presence of FFAs and their acyl-CoA derivatives, as well as of ROS, which activate PmitoK$_{ATP}$, K^+ cycle is activated and both low $\Delta\Psi$ and low ROS production are observed. In isolated DWM the degree of PmitoK$_{ATP}$ functioning is dependent on the balance among modulators; a fully opened channel may completely collapse $\Delta\Psi$ and ROS production. The reducing equivalent flux through the respiratory chain to molecular oxygen and the coupled proton ejection into the intermembrane space are also indicated; + and − signs refer to $\Delta\Psi$. The continuous or dotted arrows refer to a more or less active pathway, respectively. SH_2, reduced substrates; S, oxidized substrates; i.m.m., inner mitochondrial membrane.

similar phosphorylative capacity. Finally, the third oxygraphic approach allowed to assess that KCl-depolarized DWM retain RC and ADP/O ratios similar to those observed in the absence of KCl addition. Moreover, the rate of ATP synthesis, calculated by multiplying the ADP/O ratio by the corresponding state 3 oxygen uptake rate (Krömer and Heldt, 1991; Flagella et al., 2006), was 190 ± 6.5 nmol min^{-1} mg^{-1} of protein and 190 ± 11.0 nmol min^{-1} mg^{-1} of protein, respectively, in the absence and in the presence of KCl addition, thus further confirming that the activation of PmitoK$_{ATP}$ in KCl-treated DWM does not affect the ATP synthesis *via* OXPHOS. In the same experiments, as internal controls, classical modes of uncoupling induced by the addition of FCCP, KCl *plus* valinomycin or linoleate, were found to cause fast and complete $\Delta\Psi$ dissipation, as well as a complete impairment of ATP synthesis (Trono et al., 2011).

In order to verify whether a possible increase of ΔpH may compensate the lack of $\Delta\Psi$ to sustain ATP synthesis, measurements of the internal pH, carried out by loading DWM with the fluorescent probe bis-carboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester, were performed to calculate ΔpH. Results showed that no ΔpH increase occurs caused by KCl addition to mitochondria and that the ATP synthesis *via* OXPHOS takes place in the absence of a classically measurable Δp (Trono et al., 2011). Interestingly, these measurements also confirmed that in DWM ΔpH is negligible as in other plant mitochondria and that $\Delta\Psi$ largely represents the main component of Δp (Douce, 1985). A major prediction of the chemiosmotic model is that the phosphorylation potential and the rate of ATP synthesis should depend on the magnitude of the "delocalized" bulk water phase Δp. So, it is feasible that the ATP synthesis *via* OXPHOS in KCl-depolarized DWM should not occur according to the classical chemiosmotic model. Indeed, evidence has been gathered that some energy-transducing membranes trouble this chemiosmotic model in favor of a "localized" theory. For instance, in *Halobacterium halobium* light induced an increase in the ATP synthesis without a concomitant increase in ΔpH and $\Delta\Psi$ (Michel and Oesterhelt, 1980), whereas in thylakoid vesicles photophosphorylation was found to occur in the absence of both $\Delta\Psi$ and ΔpH (Ort et al., 1976). Also, extreme alkaliphilic bacteria from the *Bacillus* species synthesized ATP even in the presence of a very low Δp due to the adverse pH gradient, alkaline outside (Krulwich, 1995 and references therein), whereas in bovine heart submitochondrial particles a decrease in the ATP synthesis was observed as a consequence of the inhibition of the respiration rate, despite Δp remained constant (Sorgato et al., 1980). Moreover Castrejón et al. (1997) reported that, at low phosphate concentration, the addition of KCl to yeast mitochondria caused a Δp collapse, while the rate of ATP synthesis by OXPHOS was maintained at high level (about 60% of that measured in the absence of KCl). In all these papers, the authors converge on the idea that these unusual behaviors are not readily accommodated within a simple chemiosmotic scheme, but an alternative mechanism for the transfer of protons from the respiratory chain to ATP synthase may be invoked.

Consistently, the existence of a "localized" rather than a "delocalized" energy transfer between the complexes of the respiratory chain and the ATP synthase that does not involve the bulk water phase was postulated by Tedeschi (2005a,b). Consistently, Heberle et al. (1994) reported that protons ejected by an integral membrane protein can move laterally along the membrane surface so to reach the usual entry site for protons in the ATP synthase; this long-range proton transfer was found to occur at a rate higher compared to the exchange with the bulk

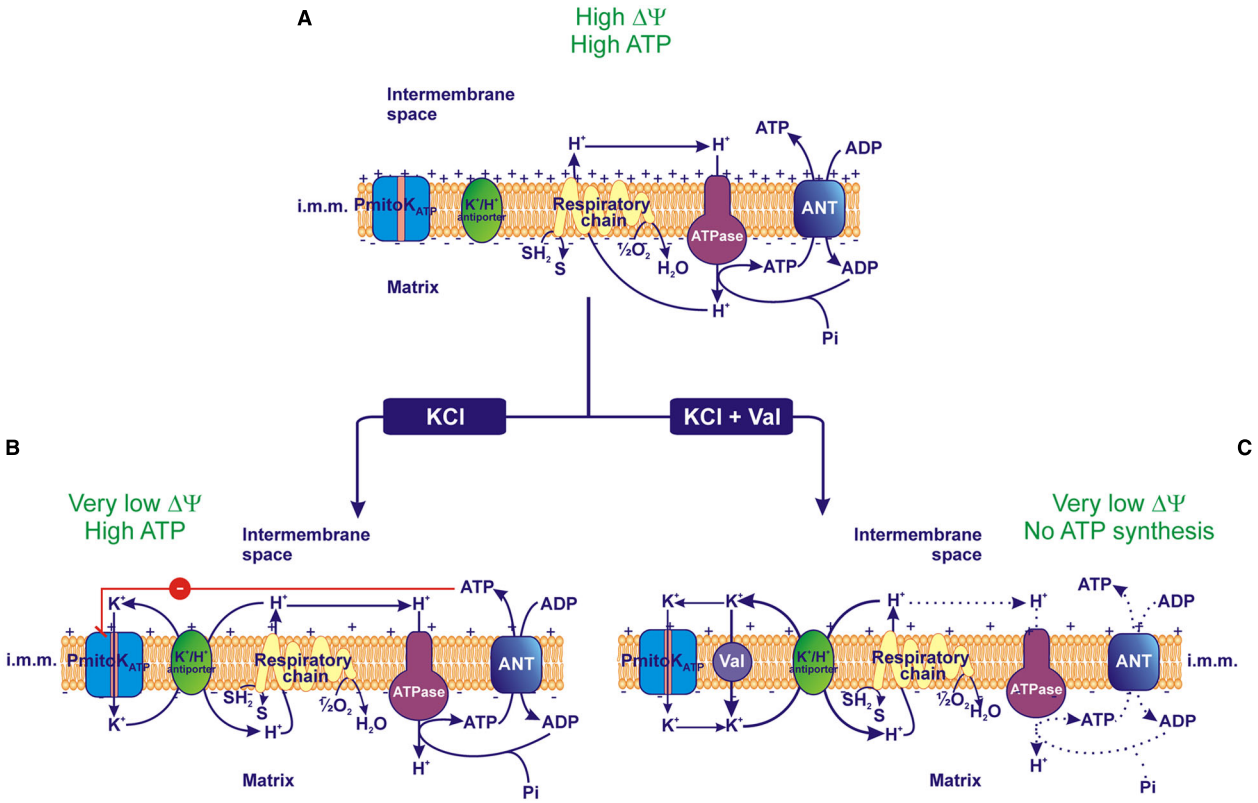

FIGURE 3 | Effect of PmitoK$_{ATP}$ activity on the electrical membrane potential (ΔΨ) and ATP synthesis. (A) In the absence of KCl, PmitoK$_{ATP}$ is inactive and the proton re-entry into the matrix *via* the ATP synthase (ATPase) drives the ATP synthesis according to the classical chemiosmotic scheme. **(B)** In the presence of KCl, PmitoK$_{ATP}$ is active and the concurrent K^+ cycle (see **Figure 1**) competes with ATPase for protons. Since PmitoK$_{ATP}$ represents the rate-limiting step of the cycle, its inhibition by ATP may carefully regulate the rate of the cycle, so that the bulk phase measurable ΔΨ is strongly lowered, but a latent ΔΨ remains feeding the ATPase pathway to regularly accomplish ATP synthesis. **(C)** On the contrary, in the presence of the K^+ ionophore valinomycin (val), PmitoK$_{ATP}$ and its modulation by ATP are bypassed, so the K^+ cycle monopolizes protons and uncouples mitochondria collapsing both ΔΨ and ATP synthesis. The reducing equivalent flux through the respiratory chain to molecular oxygen, the coupled proton ejection into the intermembrane space, the ADP/ATP antiport *via* Adenine Nucleotide Translocator (ANT) and the ATP synthesis *via* ATPase are indicated; + and − signs refer to ΔΨ. The continuous or dotted arrows refer to a more or less active pathway, respectively. SH_2, reduced substrates; S, oxidized substrates; i.m.m., inner mitochondrial membrane.

water phase. Alternatively, studies dealing with the interaction between the cytochrome *caa*$_3$ respiratory chain complex and the F_1Fo-ATP synthase in the extremely alkaliphilic *Bacillus pseudofirmus* OF4 suggested that proton transfer may occur as a direct proton transfer within the membrane during protein-protein interaction probably due to a physical association between the two complexes (Liu et al., 2007).

In this context, the behavior of PmitoK$_{ATP}$-depolarized DWM represents the first evidence of isolated plant mitochondria that lack measurable ΔΨ and ΔpH, but, at the same time, are fully coupled and regularly accomplish ATP synthesis *via* OXPHOS. A possible explanation of this finding may reside in the PmitoK$_{ATP}$ inhibition by ATP; a mechanistic model of this coupling in the absence of measurable bulk phase ΔΨ and ΔpH is represented in **Figure 3**. In DWM suspended in a KCl-free medium the PmitoK$_{ATP}$ is inactive and mitochondria accomplish ATP synthesis according to classical chemiosmosis (**Figure 3A**). On the other hand, in the presence of KCl and ATP, a PmitoK$_{ATP}$ activity exists, but it is inhibited, thus reducing the whole rate of the K^+ cycle; so, a "controlled collapse" is achieved that avoids complete uncoupling. In particular, this ATP-braked activity of PmitoK$_{ATP}$ may strongly reduce the classically detectable bulk phase Δp, but in such a manner to only partially subtract protons to ATP synthase and to retain a latent proton movement, able to sustain ATP synthesis and transport (Pastore et al., 2013; Trono et al., 2014; **Figure 3B**). Consistently, in the presence of valinomycin, the ATP brake of PmitoK$_{ATP}$ activity is overcome, so exacerbating K^+ cycle; under this condition, complete uncoupling occurs, preventing ATP synthesis (**Figure 3C**). In practice, while the classical uncouplers are unable to distinguish among different proton pools, somehow the PmitoK$_{ATP}$/ATP system appears to be able to distinguish the bulk phase Δp from a non-classically detectable driving force for ATP synthesis.

In the light of the above results, it was proposed that, *in vivo*, PmitoK$_{ATP}$ functioning should not affect RC and ADP/O ratios, as well as the rate of ATP synthesis, while an evident effect on Δp is expected because mitochondria truly interface with a high cytosolic KCl concentration (Trono et al., 2011). At this regard, it is interesting to note that the ΔΨ values determined *in vitro*

on isolated KCl-suspended DWM correspond to 60–100 mV in different experiments, that mach well with those measured *in vivo*; therefore, it is feasible that results in DWM depict a physiological condition. Indeed, in plant cells, Igamberdiev and Kleczkowski (2003) applied a new method to evaluate mitochondrial $\Delta\Psi$ from the subcellular ATP/ADP ratios by means of rapid subcellular fractionation of barley leaf protoplasts and calculated values of 70–95 mV under different physiological conditions. In animals, Zhang et al. (2001b) used a conventional fluorescence microscopy combined with three dimensional deconvolution by exhaustive photon reassignment and measured a mitochondrial $\Delta\Psi$ of about 105 mV in fibroblasts and 81 mV in neuroblastoma cells. In addition, in perfused hearts (Wan et al., 1993) and single hepatocytes (Ubl et al., 1996) $\Delta\Psi$ values of 100–140 mV were measured under different metabolic conditions. So, these findings clearly show that, in living cells, mitochondria have a low or very low $\Delta\Psi$, and that ATP synthesis may, however, occur at suboptimal $\Delta\Psi$.

PmitoK$_{ATP}$ AS DWM DEFENSE SYSTEM UNDER HYPEROSMOTIC STRESS

A possible physiological role of the PmitoK$_{ATP}$ under environmental/oxidative stresses derives from its property to act as energy-dissipating system able to control $\Delta\Psi$, ATP synthesis and, above all, ROS production. It is well known that cellular ROS production can be increased as a result of plant exposure to various environmental stresses (Scandalios, 1993; Foyer et al., 1994; Møller, 2001); mitochondria, in particular, are known to increase ROS generation under drought and salt stress (Alscher et al., 1997). Consistently, in DWM purified from hyperosmotically (mannitol or NaCl) stressed seedlings, an increase in the rate of superoxide anion production of about 40% and 120% with respect to the control was found under moderate and severe stress conditions, respectively (Trono et al., 2004). In particular, stress was considered moderate when a starting oxidative stress was observed, without a concomitant damage on substrate oxidation, ATP synthesis and mitochondria intactness. On the other hand, it was considered as severe a stress that induced a drop of substrate oxidation (Trono et al., 2004; Soccio et al., 2010), ATP synthesis (Flagella et al., 2006) and, consequently, a remarkable ATP content decrease (Trono et al., 2011; Soccio et al., 2013), as well as some loss of outer membrane integrity (Trono et al., 2004; Soccio et al., 2010). All these findings were obtained on mitochondria isolated from stressed seedlings in which PmitoK$_{ATP}$ was maintained essentially inactive by carrying out measurements in KCl-free or low-KCl media. On the other hand, when the channel was activated under stress, it was found to deeply affect ROS and ATP production (**Figure 4**).

In the absence of an external stress (**Figure 4A**), mitochondria may synthesize ATP *via* OXPHOS at high rate and ROS are generated at a basal level; consequently, in the balance between ATP and activators, that include ROS and FFAs/acyl-CoAs, ATP inhibition of the PmitoK$_{ATP}$ activity is expected to prevail, thus allowing only basal activity of the channel. The picture changes under stress conditions. Under moderate hyperosmotic stress (**Figure 4B**), the increase of ROS production by mitochondria may activate PmitoK$_{ATP}$, which, in turn, according to a feedback mechanism, may dampen excess ROS production as described above. Moreover, evidence has been reported that the mitochondrial PLA$_2$ activity, recently detected in DWM, is activated by hyperosmotic stress (but not *via* ROS) and it has been proposed to determine the increase *in vivo* of both FFAs and their acyl-CoA derivatives, which further enhance the channel activation (Trono et al., 2013). Consistently, PmitoK$_{ATP}$ activity was found to increase by 57% and 300% under NaCl and mannitol moderate stress, respectively (Trono et al., 2004). Consistently, PmitoK$_{ATP}$ may significantly lower ROS production: in *in vitro* measurements, a mersalyl-activated channel was found to be able to reduce up to 35-fold the rate of superoxide anion generation (Trono et al., 2011).

The most intriguing aspect of PmitoK$_{ATP}$ functioning under moderate stress condition regards its regulation of ATP synthesis. Channel opening for the control of ROS production necessarily implies a $\Delta\Psi$ decrease up to a complete collapse (**Figure 2**); as a consequence, a strong inhibition of ATP synthesis *via* OXPHOS should be observed. Conversely, as stated above, under these experimental conditions, the ATP synthesis is unexpectedly not impaired (Trono et al., 2011). The inhibition by ATP may represent a brake able to finely regulate the K^+ cycle, so that $\Delta\Psi$ is strongly decreased, but ATP synthesis is anyhow preserved, as explained by the "controlled collapse" reported in **Figure 3**. This is a central point in cell defense; in this respect, the need of energy for stress protection and maintenance of tissue functional state under water limiting conditions has been underlined very recently (Simova-Stoilova et al., 2015).

When the level of stress becomes severe (**Figure 4C**), ROS production further increases (+120%) (Trono et al., 2004). At the same time, a notable increase (up to about 25-fold) of FFAs is observed (Laus et al., 2011), that derives from a doubled activity of the mitochondrial PLA$_2$ (Trono et al., 2013). Under these conditions, the balance among PmitoK$_{ATP}$ modulators favors a stronger channel activation by ROS and FFAs/acyl-CoAs over an inhibition by ATP. Consistently, compared to the control, a 300% and 400% increase in channel activity was measured under NaCl and mannitol severe stress, respectively (Trono et al., 2004). This increased PmitoK$_{ATP}$ activity may counteract large-scale ROS production as reported above, but a concomitant $\Delta\Psi$ collapse is observed. Under these conditions, the fully open channel strongly decreases ATP synthesis (up to about a fifth with respect to a condition of inactive channel), but not completely impairs it (Trono et al., 2011), so that a 50–60% of ATP content was preserved (Soccio et al., 2013). As for mitochondrial integrity, interestingly, concurrent inhibition of PIMAC by FFAs prevents large amplitude swelling of mitochondria due to PmitoK$_{ATP}$ activation, thus avoiding outer membrane rupture (Laus et al., 2008).

On the whole, PmitoK$_{ATP}$ appears to be unique among dissipative systems. DWM also possess a very active PUCP and AOX that participate in the control of ROS production, with a cross regulation between PmitoK$_{ATP}$ and PUCP, as well as a clear role of AOX in green tissues (Pastore et al., 2001, 2007; Trono et al., 2006). However, although both uncoupling

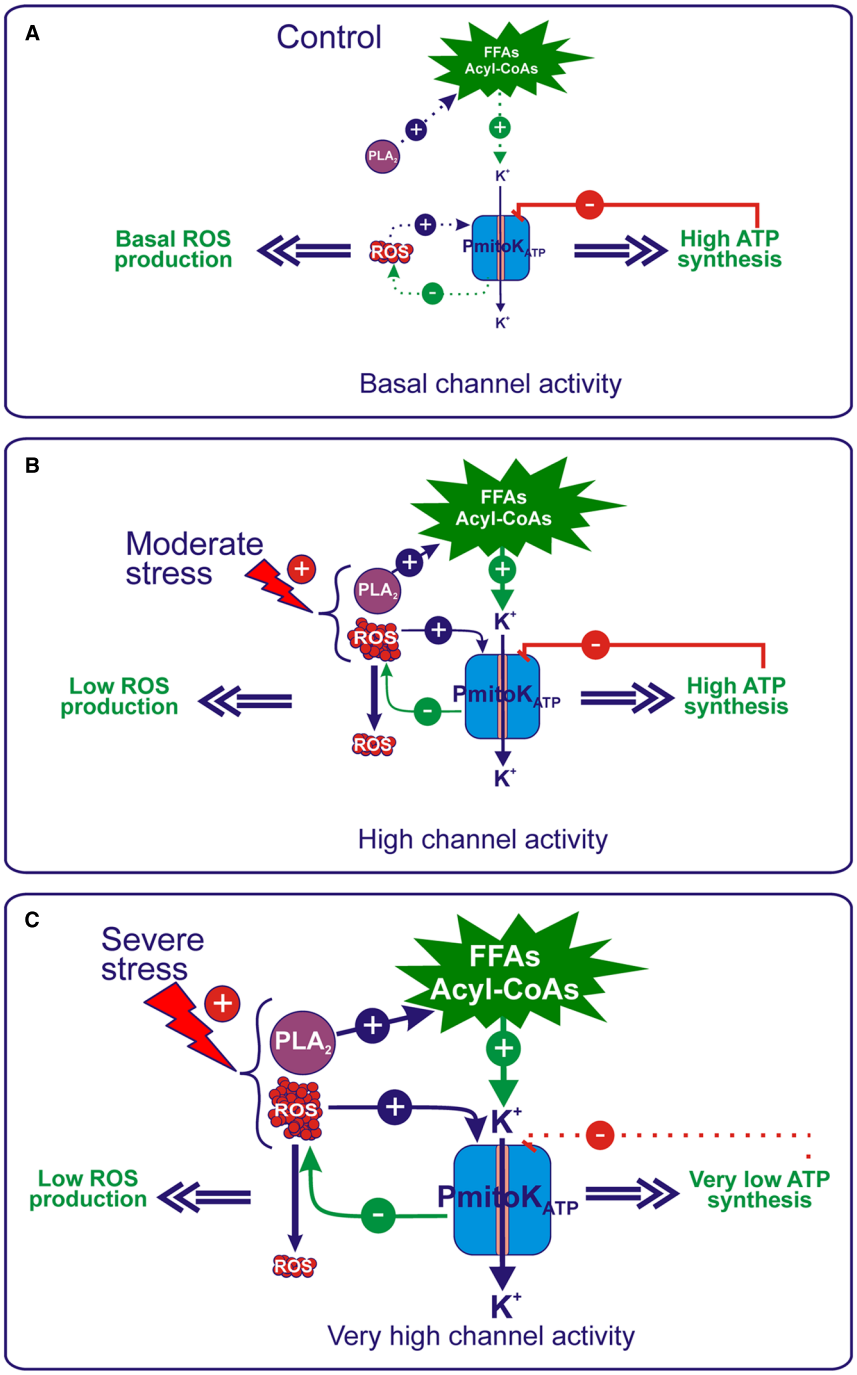

FIGURE 4 | Regulation of ROS production and ATP synthesis by PmitoK$_{ATP}$ under control and hyperosmotic stress conditions. (A) Under control conditions, a basal production of activators occurs, whereas ATP is produced at high level; as a consequence, in the balance of modulators, ATP inhibition of the PmitoK$_{ATP}$ activity prevails, thus allowing only a basal channel activity. **(B)** Under moderate stress conditions, an increase in the mitochondrial generation of ROS occurs that activates PmitoK$_{ATP}$, which, in turn, according to a feedback mechanism, may dampen excess ROS production. Moreover, an activation of the mitochondrial PLA_2 also occurs, that may increase the *in vivo* production of FFAs/acyl-CoAs, which further enhance channel activation. On the other hand, channel inhibition by ATP may represent a brake able to finely regulate the K^+ cycle, so that $\Delta\Psi$ is strongly decreased to control ROS, but the ATP synthesis is not impaired. In this manner, PmitoK$_{ATP}$ meets the cell needs, *i.e.,* to dampen harmful ROS production to curtail oxidative stress and, at the same time, to preserve energy to counteract stress. **(C)** Severe stress conditions determine a further increase in the ROS generation, as well as in the PLA_2 activity, which, in turn, may increase the *in vivo* production of FFAs/acyl-CoAs. Under these conditions, the balance between channel modulators favors stronger channel activation by activators over inhibition by ATP. This further increased PmitoK$_{ATP}$ activity determines a $\Delta\Psi$ collapse able to counteract large-scale ROS production, but, in this case, it leads to a strong decrease in the ATP synthesis, although not complete impairment is observed. For details, see the text. The continuous or dotted arrows refer to a more or less active pathway, respectively.

by PUCP and non-coupling by AOX may dampen superoxide anion production, this control is paid in terms of loss of ATP synthesis (Pastore et al., 2000, 2001, 2007; Trono et al., 2004). On the contrary, PmitoK$_{ATP}$ may balance ROS control and mitochondrial bioenergetics in a crucial moment by preserving ATP synthesis to defend cell; so, this complex basic mechanism may adapt cellular bioenergetics to changing environmental conditions and may oppose environmental/oxidative stress. To date, it is unknown whether this defense mechanism that involves PmitoK$_{ATP}$ in DWM may be operating in other plant species. Further investigations about this aspect should be worthwhile.

AUTHOR CONTRIBUTIONS

DT, MNL, and MS reviewed relevant literature and wrote the manuscript; MA supported analysis of literature and co-wrote the manuscript; DP supervised the review and co-wrote the manuscript.

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The Permeability Transition in Plant Mitochondria: The Missing Link

Marco Zancani, Valentino Casolo, Elisa Petrussa, Carlo Peresson, Sonia Patui, Alberto Bertolini, Valentina De Col, Enrico Braidot, Francesco Boscutti and Angelo Vianello*

Plant Biology Unit, Department of Agricultural and Environmental Sciences, University of Udine, Udine, Italy

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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Edited by:
Ildikò Szabò,
University of Padova, Italy

Reviewed by:
Hao Peng,
Washington State University, USA
Paolo Pinton,
University of Ferrara, Italy

****Correspondence:***
Marco Zancani
marco.zancani@uniud.it

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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.

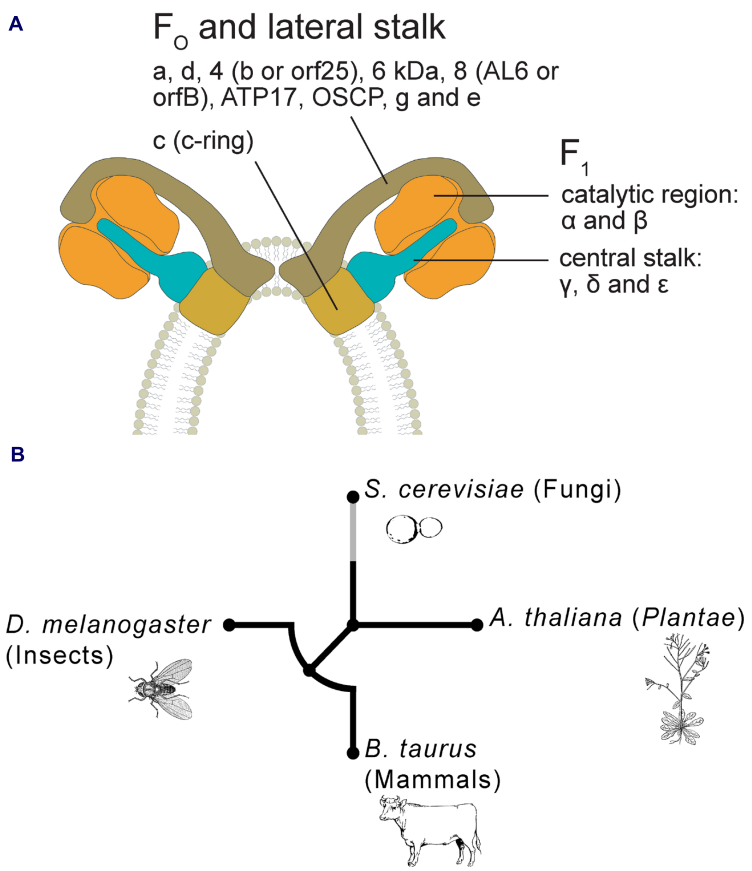

FIGURE 1 | (A) Hypothetical model of PTP in plants, based on F-ATPase dimer formation, as proposed by Bernardi (2013b), Bonora et al. (2013), and Alavian et al. (2014). Plant F-ATPase subunits are organized on the basis of their putative correspondence to the mammalian ones. **(B)** Circular phylogenetic 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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