Ion Channels in Plant Bioenergetic Organelles, Chloroplasts and Mitochondria: From Molecular Identification to Function

Luca Carraretto1, Enrico Teardo1,2, Vanessa Checchetto1, Giovanni Finazzi3,*, Nobuyuki Uozumi4,*, and Ildiko Szabo1,2,*

1Department of Biology, University of Padova, Padova 35121, Italy
2CNR Institute of Neuroscience, University of Padova, Padova 35121, Italy
3UMR 5168 Laboratoire de Physiologie Cellulaire Végétale (LPCV) CNRS/ UJF / INRA / CEA, Institut de Recherches en Technologies et Sciences pour le Vivant (IRTSV), CEA Grenoble, 38054 Grenoble, France
4Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Aobayama 6-6-07, Sendai 980-8579, Japan
*Correspondence: Giovanni Finazzi (giovanni.finazzi@cea.fr), Nobuyuki Uozumi (uozumi@biophy.che.tohoku.ac.jp), Ildiko Szabo (ildi@civ.bio.unipd.it)
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ABSTRACT

Recent technical advances in electrophysiological measurements, organelle-targeted fluorescence imaging, and organelle proteomics have pushed the research of ion transport a step forward in the case of the plant bioenergetic organelles, chloroplasts and mitochondria, leading to the molecular identification and functional characterization of several ion transport systems in recent years. Here we focus on channels that mediate relatively high-rate ion and water flux and summarize the current knowledge in this field, focusing on targeting mechanisms, proteomics, electrophysiology, and physiological function. In addition, since chloroplasts evolved from a cyanobacterial ancestor, we give an overview of the information available about cyanobacterial ion channels and discuss the evolutionary origin of chloroplast channels. The recent molecular identification of some of these ion channels allowed their physiological functions to be studied using genetically modified Arabidopsis plants and cyanobacteria. The view is emerging that alteration of chloroplast and mitochondrial ion homeostasis leads to organelle dysfunction, which in turn significantly affects the energy metabolism of the whole organism. Clear-cut identification of genes encoding for channels in these organelles, however, remains a major challenge in this rapidly developing field. Multiple strategies including bioinformatics, cell biology, electrophysiology, use of organelle-targeted ion-sensitive probes, genetics, and identification of signals eliciting specific ion fluxes across organelle membranes should provide a better understanding of the physiological role of organelar channels and their contribution to signaling pathways in plants in the future.

Key words: ion channels, chloroplasts, mitochondria, cyanobacteria, endosymbiosis, plant physiology


INTRODUCTION

Although over 80% of transport processes occur inside the cells, the mechanisms of ion flux across intracellular membranes are difficult to investigate and remain poorly understood, except for a few cases (e.g., plant vacuoles; Hedrich, 2012; Martinova et al., 2012; Xu et al., 2015). Plastids are the hallmark organelles of all photosynthetic eukaryotes and are the site of major anabolic pathways. Hence, a plethora of metabolite and ion transport systems are found in plastids. Excellent reviews deal with metabolite/ion transporters (Weber et al., 2005; Linka and Weber, 2010; Facchinelli and Weber, 2011; Rolland et al., 2012; Pfeil et al., 2014; Finazzi et al., 2015; Oh and Hwang, 2015), and therefore we focus here on ion channels of plastids. As to mitochondria, similarly to the mammalian organelle, the existence of ion-conducting pathways has been demonstrated previously by classical bioenergetic studies (for reviews, see e.g., Laloï, 1999; Millar et al., 2011; Vianello et al., 2012;
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Pastore et al., 2013; Trono et al., 2014) but the identity of the proteins responsible for these activities is still largely unknown. Both organelles in plant cells serve as major intracellular hubs that supply energy to the cell through the activity of membrane-localized respiratory and photosynthetic chains. To fulfill this role, they require extensive exchange of ions and solutes. In particular, ion transport systems are closely related to the formation of electrochemical potential. Taking into consideration that the mitochondria and chloroplasts are of endosymbiotic origin (Duy et al., 2007; Schleiff and Becker, 2011; Qiu et al., 2013), we address the question of the evolutionary conservation of ion channels in relation to the possibility of exploiting this information for identification of channels in bioenergetics organelles, mainly chloroplasts. Knowledge about the ion channels in bacteria, such as cyanobacteria, is of importance also in relation to the structure–function of ion channels in chloroplasts and mitochondria.

Various classes of membrane transport system are classified into channels and transporters, the former showing high solute permeability per second. Direct measurement of the ion currents by electrophysiological recording enables their function to be evaluated. Presumably a large number of the genes encoding ion channels expressed in ancestor bacteria might be transmitted into the genome in the nucleus of plant cells until they have stabilized in the host cells as mitochondria and chloroplasts. Indeed, one of the major challenges in the field is to understand which of the nucleus-encoded channel-forming proteins are targeted to these organelles, since this is a prerequisite to understand and prove their physiological roles at the level of whole organisms using genetic tools. The sorting process and membrane trafficking of the ion channels into the organelles from the cytosol also involves coordinated control.

CHLOROPLAST ION CHANNELS

Electrophysiology

Chloroplast channel activities of both the outer and inner envelope membranes and thylakoid membranes have been investigated during the last decades either by the technically challenging direct patch clamping of chloroplast envelope membranes and of isolated swollen thylakoid membranes (Schonknecht et al., 1988; Pottosin, 1992, 1993; Pottosin and Schonknecht, 1995, 1996; van den Wijngaard and Vredenberg, 1997; Hinnah and Wagner, 1998; Pottosin et al., 2005) or, in most cases, by incorporating membrane vesicles or purified native/recombinant proteins into planar lipid bilayers (see Table 1 for references). Table 1 summarizes the main characteristics of the ion channels recorded from the three membranes. The chloroplast stroma contains approximately 150 mM K⁺, 50 mM Cl⁻, and 5 mM Mg²⁺ as the main ions (Neuhaus and Wagner, 2000). Several potassium- and divalent cation-selective as well as anionic ion channels have been recorded from all three chloroplast membranes with different conductances and characteristics. Unfortunately, pharmacological characterization of these channels is rather limited and this fact, together with the huge technical difficulty of patch clamping chloroplasts from the completely sequenced Arabidopsis model plants, either the wild-type (WT) or lacking a putative chloroplast channel, renders it difficult to hypothesize about the proteins responsible for most activities. The question also arises whether all observed activities listed in Table 1 correspond to distinct channel proteins. This is most probably not the case; different studies were performed under different experimental conditions, making a direct comparison rather difficult. Furthermore, although in several cases evidence has been obtained for the localization of a given putative channel protein in chloroplasts, direct assessment of its activity is either missing or is studied only with recombinant proteins in non-native membranes (see Table 1). Overall, it appears that while electrophysiology is essential to characterize chloroplast channels, this technique alone is not sufficient to link unequivocally channel activity and the previously observed physiological variations, such as, e.g., calcium uptake into chloroplasts (Stael et al., 2012), increase of Mg²⁺ concentration in stroma during photosynthesis (Hind et al., 1974; Bulychev and Vredenberg, 1976), and thylakoid membrane permeability changes.

Bioinformatics and Proteomics: Toward Molecular Identification

Besides electrophysiological characterization, complementary methods may help to understand what proteins are responsible for the observed activities. All ion channels and transporters of chloroplasts and mitochondria are encoded by the nuclear genome, and the protein products are imported into the organelles during biogenesis after their synthesis in the cytoplasm. Today, several bioinformatic algorithms exist for prediction of chloroplast or mitochondria localization of nucleus-encoded proteins, including the most widely used prediction tools for subcellular localization, namely TargetP (Emanuelsson et al., 2000), MultiLoc2 (Blum et al., 2009), WoLF PSORT (Horton et al., 2007), and ChloroP (Emanuelsson et al., 1999). Table 2 reports all authentic (with proven channel function) or putative ion channels of Arabidopsis (Ward et al., 2009) predicted to be located in chloroplasts.

Most of these algorithms rely on the analysis of the amino acid composition of the N-terminal sequence to detect a putative organelle targeting sequence. However, alternative splicing might lead to products with different N-terminal sequences, thus to targeting of the same protein to different compartments (for reviews, see e.g., Kamieli and Pines, 2005; Yogev and Pines, 2011). In the case of the mammalian system, several mitochondrial channels display dual/multiple localization (e.g., Szabo and Zoratti, 2014; von Charpuius et al., 2015; Balderas et al., 2015). For example, the big-conductance calcium-dependent potassium channel (BKCa) has been demonstrated to be targeted to the mitochondria or the plasma membrane based on alternative splicing (Singh et al., 2013). In plants, relatively few examples of dually localized ion channel proteins are known so far (Elter et al., 2007; Teardo et al., 2011; Robert et al., 2012; Michaud et al., 2014). We have recently demonstrated that an alternative splicing–dependent mechanism accounts for dual organellar localization of the putative channel GLR3.5 of the glutamate receptor family protein, namely to mitochondria and to chloroplasts (Teardo et al., 2015), suggesting that the same mechanisms leading to dual/multiple location are present in animals and plants. Besides the possibility of alternative splicing, other possible difficulties must
<table>
<thead>
<tr>
<th>Name</th>
<th>Localization</th>
<th>Maximal conductance</th>
<th>Selectivity/voltage dependence</th>
<th>Pharmacology</th>
<th>Method of detection</th>
<th>Proposed function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OEP23</strong></td>
<td>OE</td>
<td>466 pS in 250 mM KCl</td>
<td>Cationic, maximal open probability at 0 mV</td>
<td>Reduction of open probability by 10 mM spermine</td>
<td>Recombinant Oep23 of pea in BLM</td>
<td>Solute exchange</td>
<td>(Goetze et al., 2015)</td>
</tr>
<tr>
<td><strong>OEP37</strong></td>
<td>OE</td>
<td>500 pS in 250 mM KCl</td>
<td>Cationic, maximal open probability at 0 mV</td>
<td>Blocked by 2 mM CuCl₂, sensitive to Tic32 prepeptide (in µM range)</td>
<td>Recombinant Oep37 of pea in BLM</td>
<td>Protein import</td>
<td>(Goetze et al., 2006)</td>
</tr>
<tr>
<td><strong>TOC75</strong></td>
<td>OE</td>
<td>1.3 nS in 1 M KCl</td>
<td>Cationic, maximal open probability at 0 mV</td>
<td>Voltage-dependent block by TrOE33</td>
<td>Recombinant TOC75 in BLM</td>
<td>Protein import</td>
<td>(Eckart et al., 2002)</td>
</tr>
<tr>
<td><strong>VDAC homolog</strong></td>
<td>OE or contact sites between OE and IE</td>
<td>520 and 1016 pS in 100 mM KCl</td>
<td>Cationic, closure at &gt;±50 mV (520 pS channel), at &gt;±30 mV (1016 pS channel)</td>
<td>Blocked by Konig polyanion</td>
<td>Patch clamp of chloroplasts from green algae <em>Nitellopsis obtusa</em></td>
<td>Metabolite transport across OE</td>
<td>(Pottosin, 1992, 1993)</td>
</tr>
<tr>
<td><strong>Large-conductance cation channel</strong></td>
<td>Envelope membranes (spinach)</td>
<td>1.14 nS in 250/20 mM K-glutamate</td>
<td>Closed at voltages &gt;50 mV and &lt;−20 mV; slightly cationic</td>
<td>None</td>
<td>Proteoliposomes in BLM</td>
<td>General diffusion pore of OE (porin-like)</td>
<td>(Heiber et al., 1995)</td>
</tr>
<tr>
<td><strong>Calcium-permeable channel</strong></td>
<td>IE</td>
<td>35 pS in 100 mM CaCl₂</td>
<td>Cationic</td>
<td>Blocked by 100 µM DNOX</td>
<td>Purified inner membrane vesicles from spinach chloroplast in BLM</td>
<td>Glutamate receptor</td>
<td>(Teardo et al., 2010)</td>
</tr>
<tr>
<td><strong>Tic110</strong></td>
<td>IE</td>
<td>From 60 to 110 pS in 250 mM KCl</td>
<td>Cationic</td>
<td>Change of selectivity in presence of 20 mM CaCl₂</td>
<td>Recombinant Tic110 from pea in BLM</td>
<td>Protein import channel</td>
<td>(Balsera et al., 2009a)</td>
</tr>
<tr>
<td><strong>FACC cation channel</strong></td>
<td>Envelope membrane</td>
<td>From 40 to 200 pS in 250 mM KCl</td>
<td>Cationic; permeable to K⁺, Na⁺, Ca²⁺, Mg²⁺, low open probability at 0 mV</td>
<td>Blocked by 100 µM Gd³⁺, regulated by pH</td>
<td>Patch clamping of pea chloroplasts</td>
<td>Regulation of stroma alkalinization</td>
<td>(Pottosin et al., 2005)</td>
</tr>
<tr>
<td><strong>ATP-sensitive potassium channel</strong></td>
<td>Envelope membranes</td>
<td>100 pS in 250/20 mM KCl</td>
<td>Cationic; higher open probability at negative voltages</td>
<td>Blocked by 1 mM ATP (added to cis side), partially blocked by 200 µM CsCl</td>
<td>Proteoliposomes in BLM</td>
<td>Electrical balance of H⁺ flux across IE</td>
<td>(Heiber et al., 1995)</td>
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<tr>
<td><strong>Anionic channels</strong></td>
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<tr>
<td><strong>Anion channel</strong></td>
<td>OE or contact sites between OE and IE</td>
<td>160 pS in 100 mM KCl</td>
<td>Anionic, activation at negative voltages</td>
<td>None</td>
<td>Patch clamp of chloroplasts from green algae <em>Nitellopsis obtusa</em></td>
<td></td>
<td>(Pottosin, 1992)</td>
</tr>
<tr>
<td><strong>VDAC-like channel</strong></td>
<td>IE</td>
<td>525 pS in 150 mM KCl</td>
<td>Anionic; open at positive potentials only</td>
<td>3.8 mM succinic anhydride increased open probability</td>
<td>BLM using isolated IE vesicles from spinach</td>
<td>Protein import</td>
<td>(Fuks and Homble, 1995)</td>
</tr>
</tbody>
</table>

Table 1. Channel Activities Observed in Chloroplast Membranes. (Continued on next page)
<table>
<thead>
<tr>
<th>Name</th>
<th>Localization</th>
<th>Maximal conductance</th>
<th>Selectivity/voltage dependence</th>
<th>Pharmacology</th>
<th>Method of detection</th>
<th>Proposed function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anion channel</td>
<td>Envelope membrane (spinach)</td>
<td>60 pS in 100 mM Tris-Cl</td>
<td>Anionic</td>
<td>None</td>
<td>Path clamp of proteoliposomes</td>
<td>Regulation of stromal pH</td>
<td>(Heiber et al., 1995)</td>
</tr>
<tr>
<td>Large-conductance anion channel</td>
<td>Envelope membrane (spinach)</td>
<td>540 pS in 250/20 mM K-glutamate</td>
<td>Closed at voltages &gt;±50 mV, slightly selective for glutamate</td>
<td>None</td>
<td>Proteoliposomes in BLM</td>
<td>Involved in protein import</td>
<td>(Heiber et al., 1995)</td>
</tr>
<tr>
<td>Channels in the Thylakoid Membrane</td>
<td></td>
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</tr>
<tr>
<td>Cationic/unselective channels</td>
<td></td>
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</tr>
<tr>
<td>Unselective large-conductance channel</td>
<td>Thylakoid membrane</td>
<td>620 pS in 20/100 mM KCl (occasionally 1 nS)</td>
<td>Unselective</td>
<td>None</td>
<td>Patch clamp of swollen thylakoid from pea chloroplasts</td>
<td>Correspondent of PTP- role in protein import</td>
<td>(Hinnah and Wagner, 1998)</td>
</tr>
<tr>
<td>Cation channel</td>
<td>Thylakoid membrane</td>
<td>40 pS in 20/100 mM KCl (occasionally 90 pS)</td>
<td>Cationic, permeable also to Mg2+ and Ca2+</td>
<td>None</td>
<td>Patch clamp of swollen thylakoid from pea chloroplasts</td>
<td>Counterbalance of proton entry into the lumen</td>
<td>(Hinnah and Wagner, 1998)</td>
</tr>
<tr>
<td>Cation channel</td>
<td>Thylakoid membrane</td>
<td>60 pS in 100 mM KCl, 21 pS in 50 mM Ca2+, 24 pS in 50 mM Mg2+</td>
<td>Cationic, permeable to K+, Mg2+, and Ca2+; more active at positive potential</td>
<td>None</td>
<td>Patch clamp of swollen thylakoid membrane from spinach</td>
<td>Counterbalance of H+ entry into the lumen</td>
<td>(Pottosin and Schonknecht, 1996)</td>
</tr>
<tr>
<td>Divalent cation channel</td>
<td>Thylakoid membrane</td>
<td>20 pS for Ca2+, 35 pS for Mg2+</td>
<td>Cationic, permeable to divalent cations</td>
<td>None</td>
<td>Thylakoid/liposome vesicle by patch clamp</td>
<td></td>
<td>(Enz et al., 1993)</td>
</tr>
<tr>
<td>Potassium channel</td>
<td>Thylakoid membrane</td>
<td>From 93 to 122 pS in 1000/300 mM KCl and 5 mM MgCl2 (cis side)</td>
<td>Cationic</td>
<td>Inhibited by 50 mM TEACl</td>
<td>Isolated thylakoid membrane vesicles in BLM</td>
<td>Counterbalance of H+ entry into the lumen</td>
<td>(Tester and Blatt, 1989)</td>
</tr>
<tr>
<td>Potassium channel</td>
<td>Thylakoid membrane</td>
<td>100 pS in 100 mM KCl</td>
<td>Cationic</td>
<td>None</td>
<td>Thylakoid/liposome vesicle by patch clamp</td>
<td></td>
<td>(Enz et al., 1993)</td>
</tr>
<tr>
<td>TPK3 potassium channel</td>
<td>Thylakoid membrane</td>
<td>35 pS in 250 mM K-gluconate</td>
<td>Cationic, no voltage dependence</td>
<td>Activated by 100 μM calcium and acidic pH, blocked by 5 mM barium</td>
<td>Recombinant Arabidopsis protein in BLM</td>
<td>Counterbalance of H+ influx into lumen [1]</td>
<td>(Carraretto et al., 2013)</td>
</tr>
<tr>
<td>Anionic channels</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Anion channel</td>
<td>Thylakoid membrane</td>
<td>220 pS in 100 mM KCl</td>
<td>Anionic</td>
<td>None</td>
<td>Thylakoid/liposome vesicle by patch clamp</td>
<td></td>
<td>(Enz et al., 1993)</td>
</tr>
</tbody>
</table>

Table 1. Continued
be considered when trying to predict protein targeting to the organelles based on prediction tools. In multipass transmembrane proteins, like channels, the targeting sequence might be internal. Furthermore and most importantly, protein import pathways different from the classical one via the translocon of the outer and inner membranes of the chloroplasts (TOC/TIC) complex (Strittmatter et al., 2010; Rolland et al., 2012; Paila et al., 2015) operate. Indeed, a largely uncharacterized system mediates the targeting of proteins that lack cleavable targeting signals to the chloroplast (Paila et al., 2015) and an unexpected vesicular transport between the secretory pathway and chloroplast has been discovered in *Euglena* (Slavikova et al., 2005) as well as in higher plants (Villarejo et al., 2005). This latter pathway targets the proteins first to the Sec translocon at the ER and subsequently exploits vesicle trafficking through the endomembrane system for the delivery of the proteins to the chloroplast (Nanjo et al., 2006) (Paila et al., 2015). It is, however, still unclear how frequently and whether these non-canonical pathways, which do not necessarily require an N-terminal targeting sequence, are used for channel proteins. Beside the vesicular system via the endomembrane system, vesicular transport has been proposed to take place also within the chloroplasts, from the envelope membrane to the thylakoid via the COPII-like system (Khan et al., 2013; Karim and Aronsson, 2014). Interestingly, diacidic motifs at the C terminus, which is typical of cargo proteins that are transported by the cytosolic vesicle transport system (Khan et al., 2013) have been identified in TPK3, a thylakoid-located member of the two-pore potassium channel family, which plays an essential role in ion homeostasis in the chloroplast (see below). Finally, a new mRNA-based mechanism of protein targeting to the organelles is emerging. This process could play an essential role in protein targeting to the ER, mitochondria, and chloroplasts (Weis et al., 2013). In this scenario, cis-acting elements in the mRNA determine their subcellular localization, and mRNA become translated only once they have reached the correct location. Thus, given the complexity of the targeting mechanism to organelles, we certainly foresee a considerable increase in the number of organelle-targeted ion channels during the next decade. In addition, proteins annotated with unknown function might turn out to mediate ion transport.

It is important to stress that, once hypothesized by bioinformatic analysis, the subcellular localization of a given protein has to be proven, ideally by biochemical evidence (using specific antibodies), or alternatively, by studying targeting in vivo, using proteins fused with fluorescent proteins. However, this latter method, which can also be exploited for the determination of the cleavage site or the targeting peptide (Candat et al., 2013), does not lack possible pitfalls: GFP, which is most often used for this purpose, is rather large and might affect targeting (especially if fused to the N-terminal part) and assembly of subunits (especially when fused to the C termini), which is necessary for the channel function in the case of multimeric forms. Furthermore, the generally strong overexpression due to strong promoters (e.g., CaMV 35S promoter) might eventually lead to mistargeting. Finally, expression of chloroplast-located proteins in cells lacking mature chloroplasts or in heterologous systems (e.g., *Arabidopsis* proteins in tobacco cells) may yield misleading results. Therefore, definitive and reliable protein localization studies require complementary strategies, also including...
<table>
<thead>
<tr>
<th>Ion channels</th>
<th>Name</th>
<th>Predicted consensus value</th>
<th>Observed localization</th>
<th>Homology to cyanobacterial channel</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Cationic channels</td>
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<td></td>
</tr>
<tr>
<td>AT5G46370</td>
<td>AtTPK2</td>
<td>CP: 6.9</td>
<td>Vacuole</td>
<td>sr1257</td>
<td>(Voelker et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SP: 4.9</td>
<td></td>
<td>44 (4e-05), 35% identity</td>
<td></td>
</tr>
<tr>
<td>AT4G18160</td>
<td>AtTPK3 (two-pore potassium channel)</td>
<td>CP: 5.1</td>
<td>Thylakoid membrane/vacuole</td>
<td>sr1257</td>
<td>(Voelker et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SP: 5.3</td>
<td></td>
<td>48 (3e-06), 30% identity/slr0498</td>
<td>(Voelker et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>41 (3e-04), 33% identity</td>
<td>(Carraretto et al., 2013)</td>
</tr>
<tr>
<td>AT4G32650</td>
<td>AtKC1</td>
<td>CP: 12.3</td>
<td>Endoplasmic reticulum, when heteromericizes with some shaker channels, becomes targeted to the plasma membrane</td>
<td>slr1575</td>
<td>(Reintanz et al., 2002; Duby et al., 2008; Jeanguenin et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M: 10.5</td>
<td></td>
<td>55 (5e-08), 29% identity</td>
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<tr>
<td>AT5G10490</td>
<td>AtMSL2 mechanosensitive channel</td>
<td>CP: 12.1</td>
<td>Chloroplast</td>
<td>gir3870</td>
<td>(Haswell and Meyerowitz, 2006)</td>
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<tr>
<td></td>
<td></td>
<td>M: 7.6</td>
<td></td>
<td>63 (2e-10), 24% identity</td>
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<tr>
<td>AT1G58200</td>
<td>AtMSL3</td>
<td>CP: 3.2</td>
<td>Chloroplast</td>
<td>gir3870</td>
<td>(Haswell and Meyerowitz, 2006)</td>
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<td></td>
<td></td>
<td>M: 6.4</td>
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<td>55 (3e-08), 21% identity</td>
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<tr>
<td>AT5G12080</td>
<td>AtMSL10</td>
<td>CP: 4.8</td>
<td>ER and PM</td>
<td>Gir3870</td>
<td>(Veley et al., 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45 (5e-05)</td>
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<tr>
<td>AT1G05200</td>
<td>AtGLR3.4</td>
<td>CP: 4.4</td>
<td>Chloroplast and plasma membrane</td>
<td>all2911</td>
<td>(Teardo et al., 2011)</td>
</tr>
<tr>
<td></td>
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<td>M: 4.4</td>
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<td>64 (9e-11), 23% identity</td>
<td>(Meyerhoff et al., 2005)</td>
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<tr>
<td>AT4G01010</td>
<td>AtCNGC13</td>
<td>CP: 2.7</td>
<td>Thylakoid membrane</td>
<td>None</td>
<td>(Yin et al., 2015)</td>
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<td>M: 7.8</td>
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<td>AT3G17690</td>
<td>AtCNGC19</td>
<td>CP: 17.0</td>
<td>n.d.</td>
<td>None</td>
<td>(Kugler et al., 2009)</td>
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<td>AT3G17700</td>
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<td>CP: 16.6</td>
<td>n.d.</td>
<td>None</td>
<td>(Kugler et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anionic channels</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT4G27970</td>
<td>AtSLAH2 (putative nitrate anion channel)</td>
<td>CP: 11.4</td>
<td>n.d.</td>
<td>None</td>
<td>(Duby et al., 2008; Maiерhofer et al., 2014)</td>
</tr>
<tr>
<td>AT4G10380</td>
<td>AtNiP5.1 putative boric acid channel</td>
<td>CP: 6.2</td>
<td>Plasma membrane</td>
<td>gir0003</td>
<td>(Takano et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SP: 6.4</td>
<td></td>
<td>138 (2e-33), 39% identity</td>
<td></td>
</tr>
<tr>
<td>AT4G35440</td>
<td>AtCiC-e</td>
<td>CP: 10.1</td>
<td>Thylakoid</td>
<td>tll0145</td>
<td>(Marmagne et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M: 13.9</td>
<td></td>
<td>244 (4e-65), 34% identity</td>
<td>(Teardo et al., 2005)</td>
</tr>
<tr>
<td>AT1G55620</td>
<td>AtCiC-f</td>
<td>CP: 0</td>
<td>Outer envelope membrane</td>
<td>tll0145</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M: 8.5</td>
<td></td>
<td>239 (1e-63), 35% identity</td>
<td>(Teardo et al., 2005)</td>
</tr>
</tbody>
</table>

Table 2. Ion Channels from Arabidopsis with Predicted and/or Proven Localization to Chloroplast Membranes.

Consensus values are those reported at the Aramemnon site (CP, chloroplast; M, mitochondria; SP, secretory pathway). Only references related to either the first description of the channel or to its localization are indicated. For homology to cyanobacterial channels, blast values and percentages of identities of amino acid sequences between the bacterial and Arabidopsis proteins are shown. n.d., not determined.
biochemical studies. Among the different biochemical approaches, proteomic studies of purified organellar membranes (Peltier et al., 2004; Ferro et al., 2010; Salvi et al., 2011; Simm et al., 2013) and suborganelle fractions (Tomiziolli et al., 2014; Yin et al., 2015) have been instrumental for the localization of proteins in the cell.

Mass spectrometry (MS) is a valid alternative for channel protein localization, even though, despite technical improvement regarding sensitivity of detection, only a few organelle-located channel proteins have been identified so far in plants (Teardo et al., 2005; Hoogenboom et al., 2007; Chang et al., 2009; Yin et al., 2015). This is likely due to very low abundance. Instead, numerous transporters of chloroplasts and mitochondria have been identified by MS (for reviews, see e.g., Millar et al., 2005; Finazzi et al., 2015). In the case of the mammalian system, assembly of an experimentally validated (by subtractive proteomics and GFP-tagged protein localization) database, named MitoCarta (Pagliarini et al., 2008), has revolutionized the field leading to the discovery of new ion channels and ion/metabolite transporters (e.g., Baughman, 2011; De Stefani et al., 2011; Herzig et al., 2012). Similarly, these approaches are expected to lead to the identification of further ion channels/transporters in plant chloroplasts and mitochondria as well. In addition, the use of a novel MS strategy in living cells, successfully applied to mammalian organelles (e.g., Rhee et al., 2013), might also be useful in plants in order to reduce ambiguities of localization due to contamination by other compartments and to multiple localization within the cell (Drissi et al., 2013). This method relies on a genetically organelle-targeted enzyme that modifies only nearby proteins (e.g., by biotinylation), which can be subsequently purified and identified by MS.

Chloroplast Ion Channels versus Cyanobacterial Channels

An ancestral photoautotrophic prokaryote related to cyanobacteria is considered as the ancestor of chloroplasts in plants and algae via an endosymbiotic event (Martin et al., 1998; Keeling, 2013). After this organism was engulfed by the host cells, a large set of genes essential to plastid function have been transferred from the ancestral plastid genome to the nucleus (Leister, 2003), leading to the necessity of a protein targeting machinery into the plastids. Genes encoding for ion channels and transporters of bioenergetics organelles are all encoded by the nucleus. Although phylogenetic profiling is useful in finding nuclear-encoded chloroplast proteins of endosymbiont origin, the physiological functions of orthologous genes may be different in chloroplasts and cyanobacteria (Ishikawa et al., 2009) and, vice versa, the same task might be fulfilled by unrelated proteins (Bayer et al., 2014). Nonetheless, experimental evidence indicates that, for example, some of the protein and solute/ion-conducting machineries share common structure and function (Bolter et al., 1998; Balsera et al., 2009b; Day et al., 2014). As to ion channels, many of the channel-forming proteins predicted to be located to chloroplast show homology with one or more cyanobacterial protein with presumed or proven ion channel function (see Table 3 and e.g., Hamamoto and Uozumi, 2014). For example, the glutamate receptor GLUR3.4 of Arabidopsis displays considerable amino acid sequence similarity to GluR0 of the completely sequenced model organism Synechocystis (Chiu et al., 2002; Teardo et al., 2011). GluR0 (sll1257) represents a very interesting evolutionary link between K+ channels and glutamate receptors, since it has a typical conserved selectivity filter sequence of potassium channels; on the other hand it is gated by glutamate (Chen et al., 1999). Indeed, many K+ channels in the Arabidopsis proteome share homology with this “ancient” channel protein (Schwacke et al., 2003). The mechanosensitive channels MSL2/3 of the envelope membrane (for recent review, see Hamilton et al., 2015) represent another example, being similar to bacterial MscS. The Synechocystis thylakoid potassium channel SynK (Zanetti et al., 2010) and the thylakoid two-pore potassium channel AtTPK3 (Zanetti et al., 2010; Carraretto et al., 2013) do not seem to share a common origin (Pfeil et al., 2014), while they conserve the function of regulating photosynthesis (see below). A cyanobacterial origin was expected for the chloride channel family members CIC-e and CIC-f, shown to be located to thylakoids (Teardo et al., 2005; Marmagne et al., 2007) and to chloroplast (Teardo et al., 2005) or Golgi (Marmagne et al., 2007), respectively. However, phylogenetic analysis did not confirm this hypothesis (Pfeil et al., 2014). In addition, in the absence of clear comparative functional studies, it is difficult to establish if the Synechocystis CIC and GluR0 and the chloroplast CIC-e/-f and GLR3.4 share the same function in the two types of organisms; although the cyanobacterial CIC (sll0855) has been shown to work as an electrogenic H+/Cl− antiporter with stoichiometry of 2/1 (Jayaram et al., 2011), AtCIC-e has been linked to nitrate transport (Marmagne et al., 2007). Both CIC-e and CIC-f are predicted to function as channels rather than transporters based on their amino acid sequence identities, in particular by substitution of a conserved Glu, implicated in proton/chloride antiporter activity (Zifarelli and Pusch, 2010). Interestingly, the expression of a probable chloride channel (sll1864) increased four times upon inhibition of photosystem II function (Hihara et al., 2003), but no other information is available about this protein. For GluR0, its function has not been studied in cyanobacteria to our knowledge, while for the two chloroplast-located GLR members, AtGLR3.4 and AtGLR3.5, clear-cut evidence for their channel-forming ability and selectivity has still to be obtained. Expression of AtGLR3.4 in mammalian cells results in calcium-permeable activity (Vincill et al., 2012) and inner envelope vesicles purified from spinach chloroplasts containing members of family 3 of AtGLRs exhibited a calcium-permeable activity that was sensitive to GLR inhibitors (Teardo et al., 2010); thus selectivity as well as functional impact of GluR0 and of Arabidopsis homolog GLRs in the respective organisms might well be different.

Unfortunately, even though an excellent and near-complete list of putative potassium channels of different families has been published already 10 years ago in a comprehensive review about prokaryotic potassium channels (Kuo et al., 2005), including those of cyanobacteria, only very few studies have addressed electrophysiological characterization and/or physiological function of these channels. Collaborative work between our groups has led to the identification and characterization in a heterologous expression system of SynK, a six-membrane-spanning voltage-dependent potassium channel (Zanetti et al., 2010). In vivo functional studies showed later that this channel plays a crucial role in regulating photosynthetic activity and
light responses (Checchetto et al., 2012) (see more details below). Checchetto et al. (2013a) have also characterized SynCaK, a calcium-dependent potassium channel the lack of which leads to increased resistance to zinc, present in the environment as a pollutant. An additional potassium channel, KirBac6.1 (slr5078), was found to be important for Synechocystis growth (Paynter et al., 2010). The protein encoded by slr1270, which resides in the outer membrane of cyanobacteria, has recently been shown to function as a potassium-selective ion channel (Agarwal et al., 2014). In addition, a proton-gated pentameric ligand-gated cation channel from Gloeobacter violaceus (GLIC) was biophysically characterized, revealing a single channel conductance of solely 8 pS (Bocquet et al., 2009; Velisetty and Chakrapani, 2012).

Another set of characterized cyanobacterial channels are members of the mechanosensitive channel superfamily. The mechanosensitive large-conductance non-specific channel MscL of cyanobacteria (slr0875) is implicated in osmotic down-shock response (Nanatani et al., 2013) and in cold and heat stress (Bachin et al., 2015). Interestingly, MscL has been proposed to allow calcium efflux from cyanobacteria upon temperature stress (Nazarenko et al., 2003). The peak of the mscL expression at the beginning of subjective night suggested that MscL might respond to stretch activation of the plasma membrane due to the decomposition of photosynthetic materials produced in day time (Nanatani et al., 2013, 2015). A member of the cyclic nucleotide-gated (CNG) channels (part of the mechanosensitive channels of the small conductance (MscS) superfamily but universally considered as a non-mechanosensitive channels) from Synechocystis has been proved to respond to mechanical stress (Malcolm et al., 2012).

Water flux across the membranes is dependent on the intra- or extra-cellular osmotic changes due to the concentrations of solutes. Since the first identification of water channels, aquaporins, in human cells and Arabidopsis thaliana (Preston et al., 1992; Maurel et al., 1993), the physiological importance of the specialized water-conducting pore has been established. Plant cells contain more than 30 copies in their genome (Maurel et al., 2008). Some aquaporin homologs are localized in organelles (for a recent review, see Beebo et al., 2013); the tobacco aquaporin,

Table 3. Proteins with Predicted/Proven Ion Channel Function in Synechocystis. Only references where channel function has been investigated are listed. Cyanobase database (http://genome.microbedb.jp/cyanobase/) was used in part to obtain information about putative channel proteins.

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Molecular function (hypothesized or proven)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>slr0498</td>
<td>SynK, thylakoid-located potassium channel</td>
<td>(Zanetti et al., 2010; Checchetto et al., 2012)</td>
</tr>
<tr>
<td>sll0993</td>
<td>KchX, potassium channel</td>
<td>(Berry et al., 2003; Matsuda and Uozumi, 2006; Checchetto et al., 2013a)</td>
</tr>
<tr>
<td>slr5078</td>
<td>Kirbac6.1, similar to potassium channel protein</td>
<td>(Paynter et al., 2010)</td>
</tr>
<tr>
<td>sll0536</td>
<td>Probable potassium channel protein</td>
<td>(Berry et al., 2003)</td>
</tr>
<tr>
<td>Slr1270</td>
<td>Potassium channel</td>
<td>(Agarwal et al., 2014)</td>
</tr>
<tr>
<td>slr1257</td>
<td>Glutamate receptor (Glur0), potassium channel gated by glutamate</td>
<td>(Chen et al., 1999)</td>
</tr>
<tr>
<td>slr1575</td>
<td>Probable potassium efflux system mechanosensitive ion channel</td>
<td></td>
</tr>
<tr>
<td>slr0855</td>
<td>Putative voltage-gated chloride channel</td>
<td>(Jayaram et al., 2011)</td>
</tr>
<tr>
<td>sll1864</td>
<td>Probable chloride channel protein</td>
<td>(Hihara et al., 2003)</td>
</tr>
<tr>
<td>slr0617</td>
<td>Unknown protein putative Cl⁻ channel</td>
<td>(Sato et al., 2007)</td>
</tr>
<tr>
<td>slr2057</td>
<td>apqZ, water channel protein</td>
<td>(Akai et al., 2011; Azad et al., 2011; Hagemann, 2011)</td>
</tr>
<tr>
<td>ssi2749</td>
<td>Hypothetical protein with similarity to water channel NIP4; 2</td>
<td></td>
</tr>
<tr>
<td>slr0875</td>
<td>MscL, large-conductance mechanosensitive channel</td>
<td>(Nanatani et al., 2013; Nazarenko et al., 2003)</td>
</tr>
<tr>
<td>slr0639</td>
<td>mechanosensitive ion channel homolog</td>
<td>(Kwon et al., 2010)</td>
</tr>
<tr>
<td>sll0590</td>
<td>Unknown protein putative mechanosensitive ion channel</td>
<td></td>
</tr>
<tr>
<td>slr1099</td>
<td>Unknown protein putative mechanosensitive ion channel</td>
<td></td>
</tr>
<tr>
<td>sll0985</td>
<td>Mechanosensitive ion channel MscS</td>
<td>(Malcolm et al., 2012)</td>
</tr>
<tr>
<td>slr0109</td>
<td>Unknown protein putative mechanosensitive ion channel MscS</td>
<td></td>
</tr>
<tr>
<td>slr0510</td>
<td>Hypothetical protein, putative mechanosensitive ion channel</td>
<td></td>
</tr>
</tbody>
</table>
NtAQP1 mediates CO₂ transport in the plasma membrane and in the inner chloroplast membrane (Jehlein et al., 2008; Kaldenhoff et al., 2014). It has to be mentioned, however, that further work would be useful to exclude with certainty that the presence of some of the AOPs in the organellar membranes is due to contamination. Synechocystis PCC 6803 possesses a single gene encoding an aquaporin, aqpZ (slr2057) (Fujimori et al., 2005). The AqpZ facilitated water flux across the plasma membrane (Akai et al., 2011; Pisareva et al., 2011). The AqpZ functions in response to the osmolarity oscillations (Shapiguzov et al., 2005; Azad et al., 2011; Akai et al., 2012) and the aqpZ mutant was sensitive to glucose in the medium, indicating that AqpZ is needed for photomixotrophic growth (Ozaki et al., 2007; Akai et al., 2011). The expression of aqpZ was regulated by an intrinsic biological clock, and the peak of the expression corresponded to the early subjected night. This might be coordinated with the circadian rhythm of the daily glucose metabolism supplied by carbon fixation.

Thus, homologs of at least some of the above channels seem to be functionally present in chloroplasts (see Table 2) and may help our understanding of the molecular identity as well as of the function of the organelle-located channels. It also has to be mentioned that, in the case of cyanobacterial GLIC, GluR0, and ClC, resolution of their crystal structure greatly helped understanding of the gating mechanisms of homolog channels in higher organisms (Mayer et al., 2001; Bocquet et al., 2009; Hilf and Dutzler, 2009; Jayaram et al., 2011).

In summary, a combination of multiple strategies might help to discover what proteins give rise to channel activities in the chloroplast membranes. But why is it so important to clarify this issue? Without knowing the molecular identity of chloroplast-localized channels, final genetic proof demonstrating their function cannot be obtained, even though important cellular and physiological processes seem to be regulated by these channels. Here, we focus on three aspects in which chloroplast channels are emerging as important players.

**Emerging Roles of Ion Channels in Chloroplasts and Cyanobacteria: Regulation of Photosynthesis**

Photosynthesis, the most important bioenergetic process on Earth, takes place in thylakoid membranes of chloroplasts and cyanobacteria, and converts light energy into chemical energy, ultimately supplying ATP and NADPH for autotrophic growth. Photosynthesis leads to the generation of a proton motive force (pmf), which comprises a proton gradient (ΔρH) and an electric field (ΔΨ). ΔΨ is generated by chloroplast lumen acidification following plastoquinol (PQH₂) oxidation by the cytochrome b₆f complex and water oxidation by photosystem II (PSII). In parallel, H⁺ uptake for plastoquinone (PQ) reduction by PSII and cyt b₆f, as well as for NADP reduction in the stroma, makes the compartment basic in the light. ΔΨ is produced by charge separation in the two photosystems (PSII and PSI) and by the activity of the cytochrome b₆f complex, the catalytic cycle of which (the so-called modified Q cycle) (Crofts et al., 1983), leads to electron flow across the transmembrane part of this complex. ΔΨ is initially a localized dipole field (positive on the luminal and negative on the stromal), but it is rapidly delocalized (time of delocalization ca. 0.1 μs (Witt, 1979) by ion ion flux across the hydrophilic compartments of the chloroplast (the stroma and the lumen). Conversely ion flux between these two compartments (i.e., across the thylakoid membranes) leads to relaxation of the pmf. This mostly stems from H⁺ translocation by the ATP synthase-ATPase CF₁-F₀ complex (Joliot and Delosme, 1974). This enzyme translocates H⁺ and thus modifies ΔΨ and ΔρH at the same time. CF₁-F₀ activity can modulate the size but cannot change the relative composition of the pmf. Therefore, the existence and implication of other ion channels/pumps in the regulation of the pmf via ion exchanges was conceived since the formulation of the chemiosmotic theory to explain the finding that, although the pmf is generated by a similar process in chloroplasts and mitochondria, it is mostly composed of a ΔρH in the photosynthetic organelle and by a ΔΨ in the respiratory organelle (see, e.g., review by Bernardi, 1999; Finazzi et al., 2015). Indeed, ion channels could modify the ΔΨ/ΔρH ratio by varying the electric field without affecting the proton gradient.

Cl⁻ and/or K⁺ channels were proposed in the past as possible candidates for such a role (Schonkecht et al., 1988; Tester and Blatt, 1989). The latter possibility was recently confirmed by the finding that some members of the TPK channel (Carraretto et al., 2013) and of the KEA K⁺/H⁺ antipporter (Armbuster et al., 2014; Kunz et al., 2014) families modulate photosynthesis. In particular, TPK3 from Arabidopsis thaliana (Carraretto et al., 2013) and its cyanobacterial counterpart (SynK; Checchetto et al., 2012), mediate K⁺ fluxes in the light, thereby controlling ΔΨ and therefore the pmf composition. Activity of the recombinant AtTPK3 channel is regulated by protons and calcium (Carraretto et al., 2013). Silenced plants lacking AtTPK3 grown at 40 μmol photons m⁻² s⁻¹ showed no difference with respect to WT plants, but when the light intensity was increased to 90 μmol photons m⁻² s⁻¹, they exhibited a decreased rosette size and an increased anthocyanin content (Figure 1A). This phenotype reflects the observed higher ΔΨ and lower ΔρH due to the lack of counterbalancing flux of positively charged potassium ions. This altered pmf partitioning results in reduced CO₂ assimilation, reduced...
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growth, and in a deficient nonphotochemical dissipation (NPQ) of excess absorbed light, since NPQ onset is controlled by ΔpH (see below). Conversely, K+/H+ exchangers belonging to the KEA family balance the ΔpH and ΔΨ during transient light shifts and in the dark, when the K+ and H+ gradients have to recover to the situation preceding illumination. The case of both AtTPK3 and AtKEA transporters illustrate that, in accordance with their chloroplast localization, their lack results in alteration of organelle morphology, which leads to an associated phenotype detectable at the whole-organism level. Whether the observed chloride channel activity corresponds to CIC-e has still to be established, but Arabidopsis plants lacking CIC-e do not display significant changes in photosynthetic electron flux (Marmagne et al., 2007).

What are the consequences of the ion regulation of the pmf on photosynthesis? While ATP synthesis can be equally fueled by ΔpH and ΔΨ, the rate of photosynthetic electron flow mostly depends on the pH gradient. Indeed, the slowest step of electron transport (oxidation of PQH2 by the cyt b6f complex) is inhibited by an acidic luminal pH, leading to the so-called photosynthetic control. In the light, changes in the ΔΨ/ΔpH ratio (even under constant pmf conditions) could therefore trigger changes in the rate of electron flow, without any modification in the rate of the CF0-F1 enzyme. This could affect the balance between NADPH (which depends on photosynthetic electron flow) and ATP (which only depends on CF0-F1 turnover ratio), ultimately modifying carbon assimilation. These effects and their consequences on light utilization and growth were experimentally verified in cyanobacterial and Arabidopsis mutants. Electron flow turned out to be faster in the absence of potassium channels (i.e., a higher ΔΨ and lower ΔpH) using time-resolved redox spectroscopy of the b6f complex (Cucchetto et al., 2012, 2013b). Consequences on ATP synthesis were also assessed, measuring membrane permeability by the electrochromic shift of photosynthetic pigments, i.e., a modification of their absorption spectrum caused by changes in the transthylakoid electric field (Witt, 1979). This approach was, however, only possible in plants (Carraretto et al., 2013), as in cyanobacteria the light-harvesting pigments are not embedded in the thylakoid membranes but rather contained in protein complexes exposed to the stroma. Therefore these pigments are no longer sensitive to changes in ΔΨ. Overall, a reduced photosynthesis was evaluated, as shown by chlorophyll fluorescence-based assessments of photosynthesis (Cucchetto et al., 2012; Carraretto et al., 2013).

Besides consequences on electron flow and carbon assimilation, which are common to cyanobacteria and plants, a modified composition of the pmf turned out to diminish light acclimation capacity in plants via an impaired NPQ response (Carraretto et al., 2013; Armbruster et al., 2014). The NPQ acronym (non-photochemical quenching of absorbed energy) refers to the increased thermal dissipation of light energy, which is observed when its intensity overcomes the utilization capacity of photosynthesis. NPQ is observed in both cyanobacteria and plants, but it is only in plants and algae that this process is directly controlled by the pmf via ΔpH-induced conformational changes in specific protein effectors modulating thermal dissipation (Niyogi and Truong, 2013). This additional regulation of photosynthesis by proton and ion fluxes, which is specific to photosynthetic eukaryotes, likely triggered an altered thylakoid membrane structure, which was observed in A. thaliana TPK3 mutants (see Figure 1B) (but not in Synechocystis lines lacking SynK), possibly reflecting the larger complexity of light-harvesting regulation in eukaryotic photosynthesis. Cation concentration might also affect thylakoid structure by counteracting negative charges as consequence of (reversible) phosphorylation, which leads to repulsion and destabilization of the thylakoid structure (Fristedt et al., 2009). In addition, during illumination, due to H+ pumping by the ATPase of the inner envelope membrane, the membrane potential across this membrane reaches ~100 mV (inside negative). This H+ extrusion is balanced by K+ influx across cation-selective channels of unknown identity of the inner envelope (Heiber et al., 1995).

Overall, these new results have opened a novel, exciting field of investigation: the molecular regulation of photosynthesis by ion homeostasis. Several tasks remain to be achieved to complete these studies. Besides chloride and potassium ions, other cations including magnesium, manganese, and calcium could have major roles in regulating (directly or indirectly) photosynthetic efficiency. Indeed, magnesium is also a well-established candidate for counterbalancing proton entry into the lumen (Hind et al., 1974) and changes in stromal Mg2+ concentration are known to affect chloroplast photosynthetic capacity by controlling H+ movement across the envelope and activation of RuBiCo (Berkowitz and Wu, 1993). Mn and calcium are necessary for the correct function of the oxygen-evolving complex (Kessler, 1955; Boussac et al., 1989). Calcium also plays a crucial role in the regulation of Calvin-cycle enzymes and recent data highlight its role in the control of cyclic electron flow as well (Terashima et al., 2012; Hochmal et al., 2015). Thus, research should focus first in identification of the molecular players involved in the flux of these ions (Nouet et al., 2011). Moreover, the pathways allowing their entry to the correct site of action within the chloroplasts should be addressed to complement their biochemical and electrophysiology studies in vitro. Finally, in vivo studies are needed to establish the link between the role of each ion in photosynthesis. Further studies should clarify to what extent the different ion channels/transporters are involved in the regulation of pmf by comparing single and double/triple mutants, and whether their relative contribution depends on specific environmental conditions (e.g., continuous or intermittent light stress).
Impaired organelar Ca\textsuperscript{2+} handling on plant physiology has been suggested by CAS and PPF1 (Wang et al., 2003; Nomura et al., 2008; Petroutsos et al., 2011; Huang et al., 2012). The mutation of the putative chloroplastic Ca\textsuperscript{2+} sensor CAS or of the transporter PPF1 led to impaired stomatal movement and impaired plant growth. Today, it is known that chloroplasts have a significant buffering capacity for Ca\textsuperscript{2+} ions, resulting in extremely low (150 nM) free [Ca\textsuperscript{2+}] (Aldakkak et al., 2010) under resting conditions. Importantly, this concentration can be actively regulated: light-dependent depletion of cytosolic Ca\textsuperscript{2+} in the vicinity of chloroplasts has been observed in green algae, suggesting that an active Ca\textsuperscript{2+} uptake machinery is present on the envelope membranes, which is regulated by light/dark transitions and/or photosynthesis (Sai and Johnson, 2002; Dodd et al., 2010). Interestingly, the bacterial pathogen-associated molecular pattern inducers flagellin peptide and chitin both induced a rapid Ca\textsuperscript{2+} transient in the cytoplasm, following by a long-lasting increase in the stromal free Ca\textsuperscript{2+} level (Nomura et al., 2012), suggesting that chloroplast calcium fluxes influence plant immunity and plant stress response.

However, despite intensive research during the last decade, the fundamental of chloroplast Ca\textsuperscript{2+} dynamics remain unanswered. In order to fully understand the impact of Ca\textsuperscript{2+} dynamics on plant physiology, identification of the molecular players, including calcium-transporting entities, is of utmost importance. Electrophysiological studies suggested the existence of voltage-dependent Ca\textsuperscript{2+} uptake activity in the inner envelope membrane of pea (Pisum sativum) chloroplasts (Pottosin et al., 2005) (see Table 1). For the calcium import across the inner envelope membrane (presuming that the outer membrane is freely permeable to calcium due to the presence of porins; but see Bolter and Soll, 2001), the most promising candidates include GLR3.4 (work is under way in our laboratories to clarify this point) and MSL2/3 channels (see above), a Ca-ATPase like protein (ACA1) (Huang et al., 1993), as well as HMA1 P-type ATPase, shown be located in the envelope membrane (Ferro et al., 2010). The nature of the metal ion passing the membrane through this latter transporter is however still debated (for recent review, see Hochmal et al., 2015). Both GLR and MSL orthologs are present in cyanobacteria (see above), but a Ca\textsuperscript{2+}-ATPase seems to be responsible for the ability to take up calcium in these prokaryotes (Berkelman et al., 1994). A further possibility is represented by one the six homologs of the mammalian mitochondrial uniporter (MCU), which displays an ambiguous N-terminal sequence, possibly allowing targeting to both mitochondria and chloroplasts (Stael et al., 2012). However, the localization, channel activity, and the permeability for calcium of members of the AtMCU family have not been described up to now (except for one member, found in mitochondria by the proteomic approach; Wagner et al., 2015). At present, it is difficult to understand whether the fast-activating cation channel (FACC), recorded directly in native pea chloroplast envelope membrane (Pottosin et al., 2005), might correspond to one of these entities. Since the described channel has a large conductance (from 40 to 200 pS in 250 mM KCl), it is unlikely to arise from ATPase action. The channel is rather unselective, therefore the calcium uniporter, which at least in mammals has a strict selectivity and a tiny single channel conductance (6 pS in 100 mM calcium) (Kirichok et al., 2004; De Stefani et al., 2011) can likely be excluded. Whether GLR or MSL gives rise to this activity might be addressed in future studies either by comparing the electrophysiological activity of envelope vesicles obtained from WT and mutant plants lacking the respective channels, or by pharmacological inhibition of FACC using GLR inhibitors, previously shown to affect photosynthetic efficiency (Teardo et al., 2011).

Since calcium is required for the function of the oxygen-evolving complex located on the luminal side of the thylakoid membrane, calcium must cross this membrane as well. Calcium concentration in the lumen is estimated to be rather high (total of 15 mM) (Loro et al., 2012), indeed the thylakoid lumen has long been postulated as the chloroplast calcium store releasing calcium to the stroma. Based on measurements of ion fluxes, the existence of a thylakoid localized Ca\textsuperscript{2+}/H\textsuperscript{+} antipporter was hypothesized more than 10 years ago (Ettinger et al., 1999). The authors provided evidence that both light and ATP hydrolysis are coupled to Ca\textsuperscript{2+} transport through the formation of a trans-thylakoid pH gradient and the activity of the antipporter facilitates the light-dependent uptake of Ca\textsuperscript{2+} by chloroplasts and by the lumen, leading to reduced stromal Ca\textsuperscript{2+} levels. The identity of this transporter still has to be understood. In cyanobacteria, a Ca\textsuperscript{2+}/H\textsuperscript{+} antipporter SynCax has been shown to locate to the plasma membrane and affect salt tolerance (Waditee et al., 2004). Although in Arabidopsis several Cax transporters show clear predicted plastidial targeting (AtCax1, AtCax3, AtCax4), all of them are recognized as vacuole transporters (Mei et al., 2009; Cho et al., 2012). Post-Floral-specific gene 1 (PPF1) is another candidate for mediating calcium flux across the thylakoid membrane since it gives rise to calcium current when expressed in a human cell line, and its expression level in Arabidopsis correlates with the calcium storage capacity of chloroplasts (Wang et al., 2003). PPF1 shows 75% identity with Alb3, a thylakoid protein that is part of the YidC/Alb3/Oxa1 protein family required for membrane insertion of some thylakoid proteins (Hennon et al., 2015). Recent structural studies revealed that YidC does not form a transmembrane channel (Kumazaki et al., 2014). In light of this discovery, it seems worthwhile to revisit the channel-forming ability of PPF1 in a heterologous system other than mammalian cells and/or in an in vitro transcription/translation system. This latter tool has the advantage of avoiding contamination by membrane proteins and avoiding the appearance of channel activity due to eventual heteromerization with endogenous channel subunits. Indeed, this system has recently been used with success to prove channel activity of various proteins displaying biophysical/structural characteristics similar to those found for the same proteins expressed in, for example, Escherichia coli or Pichia pastoris (Deniaud et al., 2010; De Stefani et al., 2011; Braun et al., 2014).

In summary, to provide further insight into the physiological relevance of organellar Ca\textsuperscript{2+} signaling, it will be crucial to identify the proteins that are responsible for calcium transport, i.e., channels and transporters. It is clear that the combination of the molecular players and the elicitors of calcium signaling in organelles together with newly generated detection systems for measuring chloroplast (and possibly sub-chloroplast) Ca\textsuperscript{2+} concentrations in intact plants should provide fruitful grounds for further discoveries. In this respect, the construction of chloroplast-targeted genetically encoded aequorin-based calcium probes represents a milestone (Mehlmer et al., 2012).
Molecular Plant

Emerging Roles of Ion Channels in Chloroplasts: Plastid Division

Plastid division is a fundamental aspect of the physiology of plant cells (Osteryoung and Pyke, 2014). Today, it is well recognized that the machinery required for this process shares some similarity to the fission-fusion machinery of bacteria as well as plant and mammalian mitochondria (Jarvis and Lopez-Juez, 2013). In this latter system, beside directly affecting mitochondrial metabolism, defects in the fission-fusion machinery have been linked to severe pathologies (Kasahara and Scorrano, 2014). Plastid division consists of the assembly of the division machinery at the division site, the constriction of envelope membranes, membrane fusion, and then separation of the two new organelles (Aldridge et al., 2005). The division site in chloroplasts is established by the FtsZ ring and the external dynamin-like ARC5/DRP5B ring, which are connected to each other via ARC6, PARC6, PDV1, and PDV2. The evolutionarily conserved components of the Min system confines FtsZ-ring formation to the plastid midpoint, thereby controlling the position of the division machinery. The two well-studied mechanosensitive channels of the inner envelope membrane, MSL-2 and MSL3, not only function to relieve plastid osmotic stress (Wilson et al., 2014) but also co-localize with the plastid division protein AtMinE and, as a result, plants lacking both channels have fewer and larger chloroplasts than the WT. The authors suggest that these channels might directly influence division site selection, may cause altered stromal ion homeostasis, and/or impede the constriction of the FtsZ ring (Hamilton et al., 2015). Whether and how exactly the lack of these two channels affects chloroplast metabolism and photosynthetic efficiency is an interesting question that awaits answer. Independently, the use of chloroplasts isolated from plastid division-deficient Arabidopsis mutants might, in principle, represent a solution to the so far unresolved technical difficulty of performing patch clamp on these tiny organelles. However, osmotic imbalance across the envelope membranes might be an issue to be resolved in order to achieve seal formation.

MITOCHONDRIAL ION CHANNELS

Electrophysiology and Molecular Players

Mitochondria are crucial bioenergetic organelles for providing ATP and the metabolites required to sustain cell growth. These organelles also have a specialized metabolic role in plants, since they participate in photosynthesis and thus impact on photosynthesis (Linka and Weber, 2005; Araujo et al., 2014). Our knowledge about plant mitochondrial ion channels is rather restricted when compared with the animal field (Szabo and Zoratti, 2014) and with respect to chloroplast ion channels, except for the mitochondrial voltage-dependent anion channel (VDAC) (for review, see e.g., Homble et al., 2012; Takahashi and Tateda, 2013) and the channels formed by components of the protein import machinery (Carrie et al., 2010; Schleiff and Becker, 2011). Indeed, only very few electrophysiological studies exist on channels other than VDAC, using mainly the lipid bilayer system employing membrane vesicles. Table 4 summarizes the channel activities observed in different species. These studies employ species other than Arabidopsis (Koszela-Piotrowska et al., 2009; Jarmuszkiewicz et al., 2010; Matkovic et al., 2011), even though protocols yielding highly purified mitochondria from this model plant exist (e.g., Millar et al., 2005; Sweetlove et al., 2007; Lee et al., 2013; Konig et al., 2014). For example, in potato inner membrane vesicles, activities displaying different conductance and kinetic behavior have been recorded. These currents were ascribed to an ATP-regulated potassium channel (mitoKATP channel), to a large-conductance Ca²⁺-insensitive iberiotoxin-sensitive potassium channel, to a (DIDS) (4,4’-diisothiocyanostilbene-2,2’-disulfonic acid)-sensitive chloride channel (Matkovic et al., 2011), and to a large-conductance, calcium-activated, iberiotoxin-sensitive potassium channel (Koszela-Piotrowska et al., 2009). For this latter channel, the authors provided evidence that, as expected, activation of the channel by Ca²⁺ and the activator NS1619 stimulated resting respiratory rate and caused partial mitochondrial membrane depolarization (due to entry of positively charged cations into the matrix) while the opposite effects can be observed upon inhibition by iberiotoxin. In the animal system, it is well established that ion channels in the mitochondrial inner membrane with a ΔΨ of approximately −180 mV (negative inside) can drastically influence mitochondrial bioenergetic efficiency by altering ΔΨ and the production of reactive oxygen species, and as a consequence, cell function (Bernardi, 1999; Szewczyk et al., 2006, 2009; Szabo and Zoratti, 2014). Likely the same applies also to plant cells. Interestingly, in mammalian mitochondria, data suggesting a structural and functional coupling between the large-conductance calcium-activated BKCa potassium channel and respiratory chain complexes has been obtained (Bednarczyk et al., 2013). Whether this is the case also for plant mitochondria is still unknown, due to the technical difficulty of performing electrophysiological measurements directly on mitochondrial membranes and to uncertain protein composition of the observed channel.

To our knowledge, only one study deals with patch clamping of mitochondria (swollen mitochondria devoid of the outer membrane) obtained from wheat germ (De Marchi et al., 2010) where the success rate of obtaining high-resistance seals was less than 5%. Arabidopsis mitochondria are far too small for patch clamping (Szabo et al., unpublished results), which prevents taking advantage of genetic tools. The above study identified current carried by an ATP-sensitive potassium channel, in accordance with previous bioenergetic experiments indicating the presence of such activity in wheat mitochondria (Pastore et al., 1999; Trono et al., 2014).

Regarding targeting predictions, the same issues discussed for chloroplasts apply to mitochondria. Table 5 gives a list of the predicted mitochondria-located proteins among the known Arabidopsis channels. Among the proteins identified by proteomics, beside VDAC, other channel-forming proteins are rarely found (Qin et al., 2009). Nonetheless, at least some hypotheses have been put forward on what proteins mediate the activities observed either by electrophysiology or by classical bioenergetics. For example, the CLCt chloride channel of tobacco cells has been located to the IMM by biochemical tools (Lurin et al., 2000) and CIC from Zea mays seems to account for plant inner membrane anion channel (PIMAC) activity observed by classical bioenergetics (Tampieri et al., 2011). For the plant BK channel, based on biochemical indications, the authors proposed that an AtKC1-like channel might be involved (Koszela-Piotrowska et al., 2009). AtKC1 is...
<table>
<thead>
<tr>
<th>Name</th>
<th>Localization</th>
<th>Conductance</th>
<th>Selectivity/voltage dependence</th>
<th>Pharmacology</th>
<th>Method of detection</th>
<th>Proposed function/identity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anionic channels</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>VDAC homolog</td>
<td>OM</td>
<td>3, 7, or 4, 0 nS for two isoforms, respectively</td>
<td>Large conductance state: slightly anionic Low-conductance state: cationic Bell-shaped voltage dependence with maximal open probability at 0 mV</td>
<td>None</td>
<td>Planar lipid bilayer (BLM) with VDAC proteins purified from bean mitochondria</td>
<td>Metabolite transport across OE</td>
<td>(Abrecht et al., 2000)</td>
</tr>
<tr>
<td>VDAC3</td>
<td>OM</td>
<td>500 pS in 300 mM KCl</td>
<td>Cationic and anionic open states; tendency to close at positive voltages, open up to ±60 mV</td>
<td>None</td>
<td>AtVDAC3 from Arabidopsis expressed in a cell-free system or E. coli studied in BLM</td>
<td>Metabolite transport across the OE</td>
<td>(Berrier et al., 2015)</td>
</tr>
<tr>
<td>MitoCl channel</td>
<td>IM</td>
<td>117 pS in 50/450 mM KCl</td>
<td>Slightly anionic; no activity at negative voltage</td>
<td>Inhibited by 200 μM DIDS</td>
<td>BLM with isolated IM vesicle from potato</td>
<td>Correspond to PIMAC?</td>
<td>(Matkovic et al., 2011)</td>
</tr>
<tr>
<td><strong>Cationic channels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large-conductance BK channel</td>
<td>IM</td>
<td>From 500 to 615 pS in 50/450 mM KCl</td>
<td>Cationic</td>
<td>Activated by 300 μM calcium, inhibited by iberiotoxin (IC_{50} 170 nM)</td>
<td>BLM with isolated IM vesicle from potato</td>
<td>Regulation of respiratory rate and membrane potential in mitochondria</td>
<td>(Koszela-Piotrowska et al., 2009)</td>
</tr>
<tr>
<td>K(ATP)</td>
<td>IM</td>
<td>150 pS in 150 mM K-gluconate</td>
<td>Cationic; activated by negative voltage (in pipette)</td>
<td>Inhibited by 1 mM ATP</td>
<td>Patch clamp on mitoplasts from wheat germ</td>
<td>Regulation of respiratory rate and membrane potential in mitochondria</td>
<td>(De Marchi et al., 2010)</td>
</tr>
<tr>
<td>K(ATP)</td>
<td>IM</td>
<td>164 pS in 50/450 mM KCl</td>
<td>Cationic</td>
<td>Inhibited by 1 mM ATP</td>
<td>BLM with isolated IM vesicle from potato</td>
<td>Regulation of respiratory rate and membrane potential in mitochondria</td>
<td>(Matkovic et al., 2011)</td>
</tr>
<tr>
<td>Large-conductance calcium-insensitive potassium channel</td>
<td>IM</td>
<td>312 pS in 50/450 mM KCl</td>
<td>Cationic, more active at positive voltage</td>
<td>Insensitive to 300 μM calcium, inhibited by iberiotoxin (IC_{50} 150 nM)</td>
<td>BLM with isolated IM vesicle from potato</td>
<td>Regulation of respiratory rate and membrane potential in mitochondria</td>
<td>(Matkovic et al., 2011)</td>
</tr>
</tbody>
</table>

Table 4. Ion Channel Activities Recorded in Plant Mitochondrial Membranes.
### Table 5. Ion Channels from Arabidopsis with Predicted and/or Proven Localization to Mitochondrial Membranes

<table>
<thead>
<tr>
<th>Access number</th>
<th>Name</th>
<th>Predicted consensus value</th>
<th>Observed localization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Cationic channels</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT4G18290</td>
<td>AtKAT2, Potassium channel</td>
<td>M: 8.0, SP: 6.7</td>
<td>Plasma membrane</td>
<td>(Nieves-Cordones et al., 2014)</td>
</tr>
<tr>
<td>AT4G22200</td>
<td>AtAKT2/ATK3, Potassium channel</td>
<td>M: 1.3, SP: 3.9</td>
<td>Plasma membrane</td>
<td>(Very and Sentenac, 2002) (Held et al., 2011)</td>
</tr>
<tr>
<td>AT5G46360</td>
<td>AtKCO3, Potassium channel</td>
<td>M: 5.4, CP: 2.7, SP: 5.5</td>
<td>Vacuole</td>
<td>(Voelker et al., 2006; Rocchetti et al., 2012)</td>
</tr>
<tr>
<td>AT2G26650</td>
<td>AtAKT1, Potassium channel</td>
<td>M: 5.5, CP: 4.0, SP: 4.3</td>
<td>n.d. in Arabidopsis; endomembrane localization in <em>Lilium longiflorum</em></td>
<td>(Safarian et al., 2015)</td>
</tr>
<tr>
<td>AT1G15990</td>
<td>AtCNGC7, Cyclic nucleotide-gated channel</td>
<td>M: 5.2, CP: 0.6, SP: 1.8</td>
<td>Plasma membrane</td>
<td>(Tunc-Ozdemir et al., 2013a)</td>
</tr>
<tr>
<td>AT4G30360</td>
<td>AtCNGC17, Cyclic nucleotide-gated channel</td>
<td>M: 1.8, SP: 1.0</td>
<td>Plasma membrane</td>
<td>(Ladwig et al., 2015)</td>
</tr>
<tr>
<td>AT4G01010</td>
<td>AtCNGC13, Cyclic nucleotide-gated channel</td>
<td>M: 7.8, CP: 2.7</td>
<td>Thylakoid membrane</td>
<td>(Yin et al., 2015)</td>
</tr>
<tr>
<td>AT3G48010</td>
<td>AtCNGC16, Cyclic nucleotide-gated channel</td>
<td>M: 15.1, CP: 2.4</td>
<td>Plasma membrane</td>
<td>(Tunc-Ozdemir et al., 2013b)</td>
</tr>
<tr>
<td>AT2G46440</td>
<td>AtCNGC11, Cyclic nucleotide-gated channel</td>
<td>M: 7.5, SP: 7.0</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>AT2G46450</td>
<td>AtCNGC12, Cyclic nucleotide-gated channel</td>
<td>M: 5.1, SP: 7.0</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>AT5G14870</td>
<td>AtCNGC18, Cyclic nucleotide-gated channel</td>
<td>M: 12.7, CP: 0.6, SP: 3.4</td>
<td>Plasma membrane</td>
<td>(Frietsch et al., 2007)</td>
</tr>
<tr>
<td>AT5G11210</td>
<td>AtGLR2.5, Glutamate receptor</td>
<td>M: 6.2, CP: 4.5, SP: 1.6</td>
<td>n.d.</td>
<td>(Li et al., 2013)</td>
</tr>
<tr>
<td>AT1G24540</td>
<td>AtGLR3.3, Glutamate receptor</td>
<td>M: 7.8, SP: 21.3</td>
<td>Plasma membrane</td>
<td></td>
</tr>
<tr>
<td>AT2G32390</td>
<td>AtGLR3.5, Glutamate receptor</td>
<td>M: 13.4, SP: 2.4</td>
<td>Mitochondria</td>
<td>(Teardo et al., 2015)</td>
</tr>
<tr>
<td>AT4G00290</td>
<td>AtMSL1, Mechanosensitive channel</td>
<td>M: 17.5, CP: 6.9</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>AT1G57610</td>
<td>Putative mitochondrial calcium uniporter 1</td>
<td>M: 17.6, CP: 1.6</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>AT5G42610</td>
<td>Putative mitochondrial calcium uniporter 5</td>
<td>M: 14.5</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Anionic channels</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT3G49920</td>
<td>AtVDAC5, Voltage-dependent anion channel</td>
<td>M: 4.5, SP: 4.2</td>
<td>Mitochondrial outer membrane</td>
<td>(Lee et al., 2009)</td>
</tr>
</tbody>
</table>

(Continued on next page)
Ion Channels in Plant Bioenergetic Organelles

Table 5. Continued

<table>
<thead>
<tr>
<th>Access number</th>
<th>Name</th>
<th>Predicted consensus value</th>
<th>Observed localization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT5G57490</td>
<td>AtVDAC4 Voltage-dependent anion channel</td>
<td>M: 4.8 CP: 1.4 SP: 3.0</td>
<td>Mitochondrial outer membrane</td>
<td>(Robert et al., 2012)</td>
</tr>
<tr>
<td>AT5G37610</td>
<td>AtVDAC6 Putative voltage-dependent anion channel</td>
<td>M: 6.6 SP: 13.1</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>AT5G15090</td>
<td>AtVDAC3 Voltage-dependent anion channel</td>
<td>M: 7.3</td>
<td>Most abundant in plasma membrane</td>
<td>(Robert et al., 2012)</td>
</tr>
<tr>
<td>AT5G67500</td>
<td>AtVDAC2 Voltage-dependent anion channel</td>
<td>M: 5.8 SP: 1.6</td>
<td>Mitochondrial outer membrane, plasma membrane</td>
<td>(Robert et al., 2012)</td>
</tr>
<tr>
<td>AT3G01280</td>
<td>AtVDAC1 Voltage-dependent anion channel</td>
<td>M: 5.7 SP: 0.6</td>
<td>Mitochondrial outer membrane</td>
<td>(Pan et al., 2014)</td>
</tr>
<tr>
<td>AT1G55620</td>
<td>AtClC-f Voltage-dependent anion channel</td>
<td>M: 8.5 CP: 0</td>
<td>Chloroplast outer envelope membrane</td>
<td>(Teardo et al., 2005)</td>
</tr>
</tbody>
</table>

Consensus values are those reported at the Aramemnon site (CP, chloroplast; M, mitochondria; SP, secretory pathway). All proteins harboring mitochondrial targeting sequences are listed. References given for localization concern only studies in Arabidopsis unless otherwise specified. n.d., not determined.

However, a silent shaker-type subunit (Reintanz et al., 2002), therefore another so far unidentified subunit must be also involved in the formation of plant BK. And what is the molecular composition of the well-studied mitochondrial ATP-dependent K⁺ channel (KATP) (Petrussa et al., 2008; Pastore et al., 2013)? This is an important question that awaits answer, even in the mammalian system for which several different possibilities have been proposed (for recent review, see e.g., Szabo and Zoratti, 2014). However, none of the proposed proteins (Kir6.1, Kir6.2, ROMK) have plant counterparts, except the ABC transporters AtATM1-3 of mitochondrion, which show a high degree of sequence homology with the sulfonylurea receptors SUR1 and SUR2A (De Marchi et al., 2010).

Hot Topics: Composition of the Mitochondrial Permeability Transition Pore in Plants

The permeability transition pore (PTP) is a channel giving rise to an increase in permeability of the inner mitochondrial membrane under specific pathophysiological conditions and has been characterized mainly in mammalian cells (Bernardi et al., 2015). PTP can be activated by different insults including high matrix calcium concentration and oxidative stress, leading to swelling of mitochondria and dissipation of energy (Zoratti and Szabo, 1995). Plant mitochondria can also undergo permeability transition (Arpagaus et al., 2002; Petruzza et al., 2004; Vianello et al., 2012) and plant PTP mediates DNA import into mitochondria (Koulitchenko et al., 2003) but is also involved in nitric oxide-induced cell death (Saviani et al., 2002).

Over the last 50 years, several different hypotheses envisioned VDAC, the adenine nucleotide carrier, the benzodiazepine receptor, cyclophilin D, and other proteins as possible components of the mammalian PTP. The recent discovery that, under conditions of oxidative stress in the presence of calcium, dimers of the F-ATP synthase can be turned into a channel whose electrophysiological properties match those of PTP, opened a completely new perspective to the field (Giorgio et al., 2013). This aspect seems to be conserved in different species such as Drosophila and yeast (Bernardi et al., 2015), but whether the F-ATP synthase forms the PTP in plants as well still has to be clarified. In this respect, it is interesting to note that the thylakoid membrane, which also contains this ATP-producing machinery, harbors a high-conductance channel resembling PTP (Hinnah and Wagner, 1998). What could be the physiological role of such channel in the thylakoids? May this channel open only upon strong oxidative stress and increased calcium concentration in chloroplasts? And if yes, what would be the consequence for the thylakoid ultrastructure? Can we exploit knockout Arabidopsis plants lacking different subunits of the F-ATP synthase to obtain genetic proof in favor of the above hypothesis?

In addition, it has to be mentioned that a recent work proposes that mitochondrial spastic paraplegia 7 (SPG7), a nuclear-encoded mitochondrial metalloprotease (m-AAA) that interacts with cyclophilin D, VDAC1, and with a paraplegin-like protein and has homologs in plants, is essential for PTP complex formation (Shanmughapriya et al., 2015; but see Bernardi and Forte, 2015), rather than the ATP synthase.

Thus, all these open questions point to an exciting future in which a combination of genetics, electrophysiology, bioenergetics, and plant physiology will hopefully provide answers.

Hot Topics: Mitochondrial Ion Channels and Calcium Signaling

Plant mitochondria have been known to contain an uptake system for calcium for five decades (Hanson et al., 1965), the functional properties of which have been thoroughly characterized (Dieter and Marme, 1980; Zottini and Zannoni, 1993). A molecular basis of the regulation of calcium import into plant mitochondria has been lacking, even though data
obtained in vivo using calcium sensors such as aequorin probe and probes of the Cameleon family revealed differential Ca\(^{2+}\) dynamics between the cytosol and mitochondria (Sai and Johnson, 2002; Logan and Knight, 2003; Mehlmer et al., 2012; Loro et al., 2012, 2013). In the mammalian system, following the discovery and initial characterization of MCU (Baughman, 2011; De Stefani et al., 2011), several additional proteins such as EMRE, MCUR1, and MCUb (a dominant negative MCU isoform) as well as MICU2 and MICU3 were reported to be essential components and/or regulators of the mammalian MCU complex (MCUC; for recent reviews, see e.g., De Stefani et al., 2015; Foskett and Philipson, 2015).

In *Arabidopsis thaliana*, six proteins that can be identified as putative MCU channel proteins are present, since they are homologs of human MCU counterparts sharing the transmembrane domains, the pore-loop domain, and the conserved DVME (Asp-Val-Met-Glu) signature sequence (Stael et al., 2012). Five of the six *Arabidopsis* MCU isoforms are predicted to localize to the mitochondria (Schwacke et al., 2003), but so far our recent study has identified only one isoform, At1g57610, in mitochondria by proteomics (Wagner et al., 2015). Figure 2 shows mitochondrial localization of another isoform, At2g23790, when expressed in *Arabidopsis* using endogenous promoter (E.T., L.C., et al., unpublished results). Thus, these results suggest that bioinformatic prediction regarding localization of the MCU homologs are also correct also for the other isoforms, even though experimental proof is still lacking. Besides the MCU isoforms, other homologs of mammalian MCUC components in plants include two homologs of MCUR1 and one of MICU1. However, a convincing case has recently been made for MCUR1 to act as a cytochrome oxidase assembly factor rather than a direct and essential component/regulator of MCUC (Paupe et al., 2015). Instead, homologs of EMRE are not present. Indeed, EMRE is required for calcium uptake only in the case of mammalian MCU, but not of MCU derived from fungi (Kovacs-Bogdan et al., 2014). Thus, the plant homologs of MCU and MICU1 represent good candidates for involvement in mitochondrial calcium homeostasis, regulation, and signaling, but formal proof for the ability of AtMCU proteins to form calcium-selective channels is still lacking. As to the role of AtMICU1, recent data prove that this protein is indeed able to bind calcium and plays an active role in the regulation of mitochondrial calcium uptake since its lack increases both the steady-state calcium level and stimulus-induced calcium uptake into mitochondria (Wagner et al., 2015), suggesting that AtMICU1 has a function similar to that observed for MICU2 in the mammalian system (Patron et al., 2014).

In addition to members of the putative AtMCU family, the mitochondria-targeted splicing isoform of AtGLR3.5 might contribute to maintenance of mitochondrial calcium homeostasis. Because the previously studied members of the plant iGLRs of family 3 are permeable to Ca\(^{2+}\) (Teardo et al., 2015) measured calcium dynamics with the genetically encoded calcium probe Cameleon targeted to mitochondria in WT and knockout plants and found a significant difference in

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**Figure 2. An Arabidopsis Isoform of the Mammalian Mitochondrial Calcium Uniporter Is Efficiently Targeted to Mitochondria in Arabidopsis Plants.**

(A) At2g23790 full locus was expressed in fusion with EGFP in *Arabidopsis* leaves. An Ar laser (488 nm) was used to excite GFP; the fluorescence signal was recovered from 505 to 525 nm for GFP and from 680 to 720 nm for chlorophyll autofluorescence. Images were collected using a Leica TCS SP5 II confocal system mounted on a Leica DMi8000 inverted microscope and LAS AF software (Leica Microsystems). Green organelles prevalently localize to organelles resembling mitochondria regarding both size (see B) and motility (see Supplemental Movies 1 and 2). Partial localization to chloroplasts (blue color; detection due to chlorophyll autofluorescence) can be observed in both representative images. (B) Confocal microscopy image from *Arabidopsis* plants stably expressing GFP fused to the presequence of the mitochondrial β subunit of the F1-ATP synthase (see Duby et al., 2001; Zottini et al., 2008) identify mitochondria. Comparison suggests that the organelles identified in the images in (A) are mitochondria.
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Figure 3. Lack of the Mitochondria-Located Isoform of Glutamate Receptor AtGLR3.5 Affects Mitochondrial Morphology.

Transmission electron microscopy images from leaves of WT and knockout 6-week-old plants showing swollen mitochondria with decreased electron density and disorganization toward the center of their matrix in the absence of AtGLR3.5. Changes in ultrastructure are less evident in 3-week old mutant plants with respect to WT (not shown). Since expression of AtGLR3.5 increases with aging, the function of this putative calcium-permeable channel for mitochondrial physiology seems to be prevalent in a more advanced state of development and/or under stress conditions like those linked to aging or wounding. For further details, see text and Teardo et al. (2015).

4-week-old plants challenged with wounding. In accordance with the mitochondrial localization of one isoform of this putative glutamate receptor, plants lacking AtGLR3.5 displayed an altered mitochondrial ultrastructure (Figure 3), in particular a part of the mitochondria displayed enlargement and partial loss of cristae. Knockout plants underwent anticipated senescence. The exact link between reduced mitochondrial calcium uptake, mitochondrial swelling, and the anticipated onset of senescence in the mutant plants has still to be understood, but in any case this work provides the first indication in favor of the importance of mitochondrial calcium homeostasis for plant physiology.

In summary, we foresee a stimulating period in this field, which has to face challenges related to redundancy of the predicted calcium transport systems into mitochondria. Therefore, it will be important to understand whether the different proteins cooperate and/or ensure a tissue- and stimulus-specific change in calcium dynamics.

FUTURE PERSPECTIVES

Ion channels greatly contribute to maintain the daily active organelles, chloroplasts and mitochondria. The function of the ion channels must play a central role in the energy conversion and electrochemical potential preservation through light perception and carbon metabolism across the organelle membranes. Since the endosymbiotic event, the mechanisms regulating organelle ion channels have likely been developed to coordinate biological events of the whole cell. Information gained in cyanobacteria about the structure/function of their easily accessible PM-located ion channels will likely be useful for discovering the function of chloroplast channels.

Technical improvements in proteomic techniques (increased sensitivity, preparation of highly purified membranes) may help us to identify more and more organelle-located channel proteins. Once the localization of a given protein is validated in intact cells (e.g., using tagged proteins), its channel function can be proved either by exploiting the innovative in vitro translation/translation system for expression and reconstitution of active membrane proteins (Berrier et al., 2004; Ezure et al., 2014) or by using purified membrane vesicles. Application of the sophisticated electrophysiological patch clamp technique to native membrane of isolated Arabidopsis organelles would be of great advantage, but for the moment this task seems very difficult to achieve. In any case, independently of the channel activity, an indication for the in vivo function and physiological role of the putative channel might be obtained by comparison of WT and mutant organisms lacking a given protein that likely forms the channel. In this respect, design of ion-specific probes that can be targeted to organelles are really helpful (see e.g., Mehlmer et al., 2012; Loro et al., 2012; Hong-Hermesdorf et al., 2015). However, the above approach might lead to misleading results, since the function of a missing channel can be overtaken by, for example, another member of the same family. This problem, arising from redundancy of proteins with structural similarities and likely fulfilling the same task, does not have to be under-estimated. Emerging techniques allowing multiple genetic manipulation (e.g., CRISPR-Cas9) in diverse organisms including plants might be of great help in this respect. Finally, elucidation of the regulation of plant chloroplast and mitochondrial ion channels by post-translational modification such as phosphorylation also represents a major challenge for the future. Understanding the role, if any, of ion channels in the energetic coupling between chloroplasts and mitochondria (see e.g., Bailleul et al., 2015) would also be an interesting topic for future studies. A combined diversified approach will certainly lead to a considerable increase in our knowledge of the mechanisms of organelle function mediated by ion channels.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Molecular Plant Online.

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