An update on the regulation of photosynthesis by thylakoid ion channels and transporters in *Arabidopsis*

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In natural variable environments, plants rapidly adjust photosynthesis for optimal balance between light absorption and utilization. There is increasing evidence suggesting that ion fluxes across the chloroplast thylakoid membrane play an important role in this regulation, by affecting the proton motive force, and consequently photosynthesis and thylakoid membrane ultrastructure. This minireview presents an update on the thylakoid ion channels and transporters characterized in *Arabidopsis thaliana* as being involved in these processes, as well as an outlook at the evolutionary conservation of their functions in other photosynthetic organisms. This is a contribution to shed light on the thylakoid network of ion fluxes and how they help plants to adjust photosynthesis in variable light environments.

\textbf{Abbreviations} – Best, bestrophin-like protein; CCHA1, chloroplast-localized calcium proton antiporter 1; CLC, chloride channel; Cyt, cytochrome; $\Delta \Psi$, electric field; $\Delta \varphi$, $\text{H}^+$ concentration gradient; LHC, light-harvesting complex; ECS, electrochromic shift; ETR, electron transport rate; KEA3, potassium efflux antiporter 3; NPQ, nonphotochemical quenching; PHT4;1, phosphate transporter 4;1; PAM71, PHOTOSYNTHESIS AFFECTED MUTANT 71; PMF, proton motive force; PS, photosystem; TAAC, thylakoid ATP/ADP carrier; TPK3, two-pore potassium channel 3; VCCN, voltage-dependent chloride channel.

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Introduction

Electron transfer along the photosynthetic complexes located in the chloroplast thylakoid membrane is coupled with a vectorial transfer of protons (H+) from the stroma to the thylakoid lumen, generating a H+ concentration gradient (ΔpH). The transfer of H+ occurs at the level of water splitting in photosystem (PS) II and of plastoquinol oxidation by the Cytochrome b6f (Cyt b6f) complex (Witt 1979). Besides H+ pumping into the thylakoid lumen, the asymmetry between the electron donors and acceptors of the different photosynthetic complexes leads to the generation of an electric field (ΔΨ) – negative on the stromal side in the light. Indeed, charge separation in PSI and PSII as well as electron flow in the so-called “low potential chain” of Cyt b6f (Crofts et al. 1983) promote a net accumulation of negative charges on the stromal side of the membrane, and of positive charges on the lumenal side. The resulting electric field rapidly delocalizes on the membrane surface, due to ion redistribution, creating a stable ΔΨ (Witt 1979). The sum of the ΔΨ and the ΔpH represents the electrochemical H+ gradient, ΔµH+ (or proton motive force, PMF), which feeds ATP synthesis for CO₂ assimilation and other metabolic activities.

Although both components of the PMF equally promote ATP synthesis (Witt 1979), they also play specific roles. The ΔpH through the luminal H⁺ concentration modulates the rate of electron transport (ETR), process known as the “photosynthetic control” (Hope 1993). The ΔpH also triggers deexcitation of excess energy within the light harvesting complexes (LHCs) via nonphotochemical quenching (NPQ; Li et al. 2009). On the other hand, the ΔΨ is required for metabolite and protein transport across membranes (Jarvis and Robinson 2004), and is proposed to modulate PSII photodamage by enhancing the rate of charge recombination and singlet oxygen production in PSII (Davis et al. 2016). Eventually, the ΔΨ modifies the spectral features of photosynthetic pigments embedded in the thylakoid, resulting in the so-called electrophoresic shift (ECS; Witt 1979).

Because of these different roles of the ΔpH and ΔΨ, their relative contribution to the PMF has to be properly adjusted, to avoid misregulation of photosynthesis. It is commonly accepted that mitochondria and chloroplasts generate PMF by the same phenomenon, i.e., coupling between H⁺ and electron flow. It is also well established that in mitochondria, the PMF is almost entirely made up of the ΔΨ, whereas in chloroplasts the PMF is made up mostly of ΔpH likely due to fluxes of counterions (Cl⁻, K⁺ and Mg²⁺) by unclear mechanism (e.g., Vredenberg and Bulychev 1976, Van Kooten et al. 1986). To study the mechanism, one needs to quantify the ΔpH and ΔΨ in a noninvasive way in intact systems.

Several approaches have been developed in the past to achieve this goal. The first one consists in comparing the rate of a pH-sensitive reaction in a relatively intact system (e.g., isolated thylakoids; Rumberg and Siggel 1969) with its absolute pH dependence in the same system, under conditions where the pH can be controlled (i.e., after thylakoid membrane permeabilization). This comparison has led to the estimation of a maximum ΔpH in thylakoid membranes of about 3 pH units. The same approach in vivo
has been subsequently used by Finazzi and Rappaport (1998) to estimate the value of the \( \Delta \text{pH} \) in the dark. Because this technique requires a rather long manipulation, it is therefore only suitable to measure a steady-state PMF. This is the case for the PMF created in the dark at the expense of the hydrolysis of mitochondrial ATP. Other approaches can be employed to evaluate the PMF induced in the light, including the ECS (Witt 1979, Sacksteder et al. 2000, Bailleul et al. 2010), which is a fast, noninvasive technique, accessible to commercially available instruments. A typical recording of the ECS changes during a light-to-dark transition is presented in Fig. 1. This trace is indicative of the changes in the \( \Delta \Psi \), which has a complex profile. The fast ECS relaxation phase (a) almost certainly reflects H\(^+\) flux through the ATP synthase (Maiwald et al. 2003). This relaxation phase is followed by a slower ECS increase (b), which has been interpreted as charge redistribution across membranes, leading to a partial membrane repolarization (Cruz et al. 2001). On a longer time scale, an equilibrium point is seen, where the ECS signal becomes relatively constant (c). This point has been interpreted as the value where an equilibrium state is achieved between both components of the PMF (the \( \Delta \text{pH} \) and \( \Delta \Psi \)). Thus, the ratio between the amplitude of the reversible (d) and irreversible (e) ECS changes has been taken as a signature of the relative contribution of the two PMF components (Kramer et al. 2004).

Recently, analysis of mutants of the model plant *Arabidopsis thaliana* has suggested possible molecular candidates that could catalyze fluxes of ions different from H\(^+\) to modulate the PMF composition. In the sections below, we will briefly review the nature of the ion transport proteins in the thylakoid membrane, and list the different phenotypes of mutants associated to a modification of their activity at the level of the PMF, photosynthesis, and membrane ultrastructure (Table 1 and Fig. 2). Ion fluxes occurring across the chloroplast envelope may also affect the PMF and photosynthesis, and are discussed in a recent review by Pottosin and Shabala (2016).

**Thylakoid ion channels, transporters and pumps**

Proteins belonging to ion channels, transporters and pumps have been identified and characterized in the thylakoid membrane of *Arabidopsis thaliana*. Previously, reviews on this topic were published by Spetea and Schoefs (2010), Finazzi et al. (2014), Pfeil et al. (2014) and Carraretto et al. (2016). Given the very fast development of this field and the fact that the number of such proteins in thylakoids is continuously increasing, this minireview with the aim at placing the pieces of the puzzle together might be stimulating for further research.

**Ion channels**

Channels transport ions down their concentration gradient without consuming energy and are the fastest among transport proteins. Indeed, the rate of ion transport through channels can reach \( 10^6 \) ions per channel per second, or even more. Multiple evidence for such activities in thylakoids has been provided in the past,
as reviewed by Pottosin and Dobrovinskaya (2015) and Carraretto et al. (2016). It was only in the recent years that genes have been annotated with functions that may correspond to the reported activities. The two-pore K⁺ channel TPK3 (Carraretto et al. 2013) and two bestrophin-like anion channels AtBest1 and AtBest2 (Duan et al. 2016) that were renamed voltage-dependent chloride channels VCCN1 and VCCN2 (Herdean et al. 2016b) have been localized to the thylakoid membrane in Arabidopsis. AtBest1/VCCN1 is the major form in Arabidopsis leaves, whereas AtBest2/VCCN2 is mostly expressed in flowers (Duan et al. 2016, Herdean et al. 2016b). Electrophysiological experiments in planar lipid bilayers have revealed TPK3 as a K⁺ selective channel regulated by Ca²⁺ and pH (Carraretto et al. 2013). A similar approach indicated the activity of VCCN1 as sensitive to voltage, with preference for Cl⁻ versus NO₃⁻ (Herdean et al. 2016b). Whether these activities correspond to those previously observed in intact thylakoids using the patch-clamp electrophysiological technique, still awaits an answer. Direct comparison of the recombinant channel activities with those recorded in the native membrane is difficult due to the use of different species and lipid environment in the various studies.

The CLCe member of the Cl⁻ channel/transporter CLC family was also localized to thylakoids (Marmagne et al. 2007, Lv et al. 2009). No electrophysiological studies are available to assess if CLCe is a Cl⁻ selective channel or H⁺/Cl⁻ transporter, similarly to some other CLC-family members. Also, the substrate specificity and its mode of regulation are unknown. Based on phenotypic characterization of Arabidopsis mutants, CLCe was initially suggested to function in NO₃⁻ export to the lumen (Monachello et al. 2009), but more recent evidence is consistent with a role in Cl⁻ homeostasis in the chloroplast (Herdean et al. 2016a). Our attempts however to record single channel activity of CLCe expressed in a heterologous system were unsuccessful (Szabo et al., unpublished data), possibly due to a very low conductance of CLCe. Moreover, some of the data presented by Marmagne et al. (2007) and Lv et al. (2009) also suggest that this channel may be an envelope protein, and thus future work is required to clarify its location within the chloroplast and its overall function.

**Ion transporters**

Secondary transporters work using the concentration gradient of a co-transported ion and may operate as uniporters, antiporters or symporters. Four ion transporters belonging to various families have been localized to the thylakoid membrane of Arabidopsis, namely the K⁺/H⁺ exchanger KEA3, the PHOTOSYNTHESIS AFFECTED MUTANT 71 (PAM71), the thylakoid ATP/ADP carrier (TAAC), and the Pi transporter PHT4;1.

The function of KEA3 in K⁺/H⁺ exchange has been attributed based on the presence of a C-terminal K⁺ transport/nucleotide-binding (KTN) motif conserved in all K⁺ transporters (Armbuster et al. 2014) and on the characterization of several homologues such as the Escherichia coli KefC (Fujisawa et al. 2007)
and the Arabidopsis KEA2 (Aranda-Sicilia et al. 2012). The Arabidopsis KEA3 was localized to the thylakoid membrane, where it was suggested to function in regulation of luminal pH (Armbruster et al. 2014, Kunz et al. 2014). Experiments using overexpressing KEA3 variants with modified C-terminus demonstrated that KEA3 activity is regulated by the C-terminus harboring the KTN domain (Armbruster et al. 2016). While topology experiments in the same study revealed that the C-terminus faces the lumen, experiments reported by Wang et al. (2017) suggest its exposure to the stroma, where it could sense changes in NADPH levels. Although this later topology makes more sense from the point of KEA3 activity regulation, further experimental clarification is required.

Beside K\(^+\), divalent cations such as Ca\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\) are also transported across the thylakoid membrane of chloroplasts, where they are required as cofactors for enzymes, signaling, water oxidation and thylakoid organization (Finazzi et al. 2014). PAM71 was localized to the thylakoid membrane in Arabidopsis (Schneider et al. 2016). It belongs to the conserved UPF0016 family of membrane proteins, including well-studied yeast and human Ca\(^{2+}\)/H\(^+\) antiporters (Demaegd et al. 2013, Demaegd et al. 2014). PAM71 suppressed sensitivity to high Mn\(^{2+}\) of a Ca\(^{2+}\)/Mn\(^{2+}\)-ATPase \textit{pmr1} mutant in the yeast \textit{Saccharomyces cerevisiae}. This finding together with the partially restored photosynthesis by high Mn\(^{2+}\) in the Arabidopsis loss-of-function mutants indicated a function of PAM71 in Mn\(^{2+}\) uptake into the thylakoid lumen (Schneider et al. 2016). This function is not surprising since several cation exchangers in Arabidopsis (e.g., vacuolar CAX2, CAX4 and CAX5) also catalyze Mn\(^{2+}\) transport, most likely via a Mn\(^{2+}\)/H\(^+\) antiport mechanism (Edmond 2009). Wang et al. (2016) characterized the transporter encoded by the same Arabidopsis gene as a chloroplast-localized Ca\(^{2+}\)/H\(^+\) antiporter (CCHA1) based on its ability to complement the Ca\(^{2+}\)-sensitive phenotype of the Ca\(^{2+}\)/H\(^+\) antiporter \textit{gdt1A} mutant in yeast. The same study described an altered homeostasis of Ca\(^{2+}\) and pH in the chloroplast of the Arabidopsis \textit{ccha1} mutants together with an increased sensitivity of the plant growth to high Ca\(^{2+}\) and Mn\(^{2+}\). Electrophysiological experiments are needed to assess the possibility of a broad permeability of PAM71/CCHA1 to divalent cations (see e.g., Accardi and Miller 2004).

TAAC belongs to the mitochondrial carrier family and is localized to the thylakoid membrane where it transports stromal ATP in exchange for luminal ADP (Thuswaldner et al. 2007). TAAC has been proposed to supply ATP for the nucleotide-dependent reactions in the thylakoid lumen, which play critical role in the repair of PSII during light stress in plants (Yin et al. 2010, Spetea and Lundin 2012). However, TAAC was also localized to the chloroplast envelope and found to use additional substrates such as phosphoadenosine 5\(^\prime\)phosphosulfate (Gigolashvili et al. 2012). The envelope-localized protein has been named PAPST1, and is proposed to play role in sulfur metabolism, including the biosynthesis of thiols, glucosinolates, and phytosulfokines (Gigolashvili et al. 2012).

PHT4;1 belongs to the PHT4 family of Pi symporters and was localized to the thylakoid membrane.
and to the chloroplast envelope (Pavon et al. 2008, Ferro et al. 2010). Yeast complementation experiments revealed a H⁺-dependent mechanism of Pi transport (Guo et al. 2008), however, assays in *Escherichia coli* indicated PHT4;1 as a high-affinity Na⁺-dependent Pi transporter (Pavon et al. 2008, Ruiz-Pavon et al. 2010). More recently, the envelope PHT4;4 transporter was found to preferentially function in ascorbate rather than Pi transport based on uptake assays in proteoliposomes (Miyaji et al. 2015). Although no such experiments were performed for PHT4;1, the same report proposed that PHT4;1 may be the thylakoid ascorbate transporter. However, the wild type levels of luminal ascorbate reported by Karlsson et al. (2015) in mutants lacking PHT4;1 is not consistent with this hypothesis.

**Ion pumps**

Primary transporters (pumps) require energy (e.g., light, ATP hydrolysis) to transport ions. The chloroplast ATP synthase (CF₀F₁) is a H⁺ pump that synthesizes ATP on the thylakoid membrane using H⁺ transported into the lumen by the photosynthetic electron flow. In addition, the thylakoid membrane harbors the Cu⁺-pumping ATPase PAA2 (Abdel-Ghany et al. 2005). Its function is to deliver Cu⁺ to the plastocyanin, a Cu⁺-binding protein involved in electron transfer between Cyt b₆f and PSI at the luminal side of the thylakoid membrane. The function and physiology of PAA2 have been recently reviewed by Aguirre and Pilon (2015).

**Thylakoid ion fluxes in regulation of the proton motive force**

**K⁺, Cl⁻ and H⁺ ion fluxes**

Efflux of cations (K⁺ and Mg²⁺) and influx of anions (Cl⁻) are required to counterbalance part of the light-induced H⁺ pumping across the thylakoid membrane, and are therefore expected to affect the size and composition of the PMF. These factors modulate electron transfer, NPQ and apparently, the H⁺ conductivity of the ATP synthase by a still unknown mechanism.

TPK3 and VCCN1 are channels mediating K⁺ and Cl⁻ fluxes, respectively, across the thylakoid membrane. Based on the ECS analyses described above, *Arabidopsis* mutants silenced in the TPK3 gene or knockouts of the *VCCN1* gene displayed an increase in ∆Ψ at the expense of ∆pH (Carraretto et al. 2013, Herdean et al. 2016b). However, while the magnitude of PMF was not affected and the apparent H⁺ conductivity of the ATP synthase was slightly reduced in TPK3-silenced lines (Carraretto et al. 2013), elevated levels of these parameters have been reported in the *vccn1* mutants (Herdean et al. 2016b). ECS analyses indicated a wild-type profile for the PMF size, partitioning and H⁺ conductivity in the *vccn2* mutants and a profile in the *vccn1vccn2* double mutant resembling the one of *vccn1* (Herdean et al. 2016b). Duan et al. (2016) analyzed ECS of an *atbest1atbest2* mutant (named *atbest* in their study) and came to similar conclusions about the importance of AtBest1/VCCN1 in the regulation of the PMF. No genes
coding for Mg\(^{2+}\) transport proteins have yet been reported in thylakoids, and no \(tpk3\)\(vccn1\) double mutants have been analyzed thus far to verify a possible synergy between the two channels in the regulation of the PMF.

\textit{Arabidopsis clce} knockout mutants displayed very mild increase in the PMF size and partitioning in favor of \(\Delta \Psi\) under some conditions (Herdean et al. 2016a). Mutants deficient in KEA3 did not affect the PMF size, but they displayed increased PMF partitioning to \(\Delta p\)H (Armbruster et al. 2014, Kunz et al. 2014). This pattern indicates a function of KEA3 in efflux of H\(^+\) from the lumen, which needs validation using sensors of luminal pH. Nevertheless, the mechanism is probably similar to that of nigericin, since this eliminates the \(\Delta p\)H by exchanging K\(^+\) with H\(^+\). Interestingly, the PMF partitioning phenotype of \(kea3\) mutants is observed only in low light conditions (90–160 μmol photons m\(^{-2}\) s\(^{-1}\); Armbruster et al. 2014), whereas in the case of \(clce\) and \(vccn1\) mutants, this is observed regardless of light intensity (Duan et al. 2016, Herdean et al. 2016a, Herdean et al. 2016b). Conversely, the phenotype of \(tpk3\)-silenced lines was only seen in plants grown in light at 90 μmol photons m\(^{-2}\) s\(^{-1}\) (Carraretto et al. 2013). Overall, these observations suggest different roles of K\(^+\) and Cl\(^-\) ion channels and transporters in modulating the composition of the PMF.

**Fluxes of other ions**

Mutants deficient in transport of divalent cations or Pi across the thylakoid membrane have also been reported to display altered PMF partitioning. ECS analyses of the \(pam71\) mutants indicated an enhanced \(\Delta \Psi\) only in low and moderate light conditions (Schneider et al. 2016). This effect was attributed to the enrichment of Ca\(^{2+}\) ions inside the lumen of the mutants, as indicated by ECS experiments with divalent cation ionophores together with radioactive transport assays. Despite larger PMF size, the \(pam71\) mutants displayed reduced ATP synthase conductivity, possibly due to reduced water oxidation yielding less H\(^+\) in the lumen (Schneider et al. 2016). Remarkably, severe PSII and growth effects were observed and attributed by the authors to the lower Mn\(^{2+}\) content of the mutant PSII relative to the wild type. However, alternative explanation in the light of the recent finding by Davis et al. (2016) could be that the elevated \(\Delta \Psi\) may have enhanced PSII photodamage impacting growth. Wang et al. (2016) found that mutants of the same transporter (named CCHA1 in their study) were sensitive to pH changes in the growth media, and found increased pH and higher Ca\(^{2+}\) concentration in the cytosol relative to the wild type. These observations are not fully consistent with the analyses by Schneider et al. (2016), which pointed to enrichment of Ca\(^{2+}\) and a less acidic pH in the thylakoid lumen in the light. Therefore, further studies are requested to solve the question of the mutant phenotype.

The \(pht4;1\) knockout mutants displayed reduced ATP synthase H\(^+\) conductivity likely due to limited availability of Pi in the stroma, resulting in a higher H\(^+\) concentration in the lumen with respect to the wild
type (Karlsson et al. 2015). The evidence for this mechanism is came from the restored wild type levels of H\(^+\) conductivity when plants were grown in high Pi conditions. In these mutants, a higher PMF partitioning to the ΔpH than in the wild type was observed in the first minutes of illumination. These results could be explained by either localization of PHT4;1 in the envelope or in the thylakoid membrane, when co-transporting Pi and H\(^+\) into the stroma. The differences in PMF partitioning disappeared later on during illumination suggesting that activation of the ATP synthase could be achieved thanks to Pi supplied to the stroma by low-affinity chloroplast envelope Pi transporters such as the triose-Pi/Pi translocator and PHT2;1.

The PMF partitioning has not been measured in the taac knockout mutants. The ΔΨ could be altered by the electrogenic ATP\(^4\)/ADP\(^3\) exchange unless it is compensated by other ions (e.g., Pi). TAAC-mediated nucleotide transport and associated ionic alterations most likely occur in the first minutes of illumination, and may be activated by the electric field across thylakoids in an analogous mechanism to that described for the ADP/ATP carrier in the mitochondrial inner membrane (Klingenberg 2008).

**Thylakoid ion fluxes in regulation of photosynthesis**

K\(^+\), Cl\(^-\) and H\(^+\) ion fluxes
In line with the importance of PMF and pH homeostasis for regulation of photosynthesis, all so far characterized mutants with altered PMF partitioning also displayed altered ability to modulate the proportion of light used for ETR and of the light dissipated via NPQ. Some mutants appear disturbed in low-to-high light transition, others in high-to-low light or light-to-darkness transition, as reviewed below. *Arabidopsis* lines silenced for the TPK3 gene displayed reduced NPQ (both in the induction and the steady state) as well as reduced ETR with respect to wild-type plants (Carraretto et al. 2013). The mechanism behind is related to accumulation of K\(^+\) in the lumen, which enhanced the ΔΨ component at the expense of the ΔpH, a condition that is unfavorable for NPQ induction and results in photoinhibition.

The kea3 mutants displayed elevated NPQ in low- and high light and slower relaxation of NPQ following high-to-low light transition, also resulting in reduced ETR (Armbruster et al. 2014, Kunz et al. 2014). Thus, a role in photosynthetic acclimation following transitions from high light to low light was proposed for KEA3 (Armbruster et al. 2014). The proposed mechanism is that KEA3 imports K\(^+\) into the lumen in exchange for H\(^+\), and thus prevents acidification of the thylakoid lumen, which normally triggers NPQ. This is similar to the action of nigericin, which could rescue the NPQ phenotype of kea3 mutants.

The C-terminus containing the KTN domain of KEA3 regulates its activity upon transitions from high light to low light and keeps it inactive under constant high light conditions (Armbruster et al. 2016). However, as discussed above, the exact function of this domain and its topology remain to be elucidated. In this context, recent data from Kromdijk et al. (2016) have shown that acclimation to fluctuating light

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conditions via NPQ changes affect the biomass capacity of plants.

The vccn1/atbest mutants displayed slower NPQ induction than the wild type at all tested light intensities and also upon repetitive low-to-high light transitions, most likely by a mechanism involving a reduced ΔpH component of the PMF, however without significantly altering the ETR (Duan et al. 2016, Herdean et al. 2016b). Nevertheless, when grown in the field with natural fluctuations in light, the atbest mutant was photoinhibited, as indicated by reduction in both NPQ and ETR (Duan et al. 2016). In principle, the lower ΔpH could be the factor responsible for the observed increased susceptibility to photoinhibition in atbest and also in the TPK3-silenced mutants (Carraretto et al. 2013, Duan et al. 2016) due to a diminished NPQ, which is activated by luminal acidification. However, the same finding can be alternatively explained based on the recent proposal that the ΔΨ component of the PMF can enhance PSII photodamage (Davis et al. 2016). Indeed, ECS data indicate that the ΔΨ is larger in both mutants when compared to the wild type (Carraretto et al. 2013, Duan et al. 2016).

The clce mutants displayed higher steady state of NPQ than the wild type at all tested light intensities without altering the induction phase, and despite a reduced PMF partitioning to the ΔpH (Herdean et al. 2016a). Slower NPQ relaxation and lower ETR in the clce mutants than in the wild type were observed upon transition from light to dark, suggesting involvement of this transport protein in achieving a “resting state” of the light-dependent photosynthetic reactions. Dark-adapted clce mutants displayed a modified profile of the fast OJIP kinetics of chlorophyll a fluorescence around the I-peak (Marmagne et al. 2007, Herdean et al. 2016a). This effect was demonstrated to be associated with altered Cl− but not nitrate homeostasis in dark-adapted chloroplasts (Herdean et al. 2016a). Remarkably, the above-described photosynthetic phenotype was not always observed (Herdean and Spetea, unpublished data; Allorent and Finazzi, unpublished data), suggesting that it depends on some still not fully understood experimental factors such as the age of the plants.

**Fluxes of other ions**

Mutants deficient of PAM71/CCHA1 were retarded in growth due to reduced PSII activity at the water-oxidizing complex (Schneider et al. 2016, Wang et al. 2016). They also displayed reduced NPQ levels, which could be partly due to reduced proportion of PSII-LHCII supercomplexes and partly due to reduced ΔpH (Schneider et al. 2016).

The taac mutants displayed faster induction of NPQ at all tested light intensities, but indistinguishable steady state as compared to the wild type (Yin et al. 2010). In the same study, pre-treatment with nigericin eliminated the differences in NPQ induction, suggesting a faster acidification of the lumen in the mutants upon illumination. ECS measurements are needed to verify that PMF partitioning to ΔpH is higher in the mutants. When grown in standard light conditions, the mutants were smaller in size.
than the wild type plants, and the differences become more pronounced when grown in high light conditions (Yin et al. 2010). The authors explained the reduced growth based on a defective PSII repair cycle at the disassembly steps, which demand lumenal ATP supplied by TAAC (Spetea and Lundin 2012). In a recent study, Cruz et al. (2016) used a dynamic environmental photosynthesis imager and revealed patchy phenotypes for the taac mutants when grown in fluctuating light conditions. The authors hypothesized that in such conditions TAAC may alter the stromal ATP levels, and in this way modulates the activity of the ATP synthase. This possibility is interesting and worthy of further investigations.

The pht4;1 mutants displayed reduced growth, which was associated with limited access of the ATP synthase to stromal Pi, impacting carbon fixation and sugar accumulation in leaves (Karlsson et al. 2015). Therefore, PHT4;1 was proposed as a local Pi supplier to the ATP synthase in thylakoids during the photosynthetic reactions in the first minutes of illumination. The envelope-located PHT4;1 could also regulate the ATP synthase before the activation of other envelope Pi transporters. The pht4;1 mutants displayed wild-type levels of ETR and faster induction of NPQ, but did not differ in the steady state from the wild type (Karlsson et al. 2015).

**Thylakoid ion fluxes in regulation of the membrane ultrastructure**

**K⁺, Cl⁻ and H⁺ ion fluxes**

Ion transport defects have been shown to alter thylakoid ultrastructure, which is sensitive to its ionic environment. The stacking is regulated by a mechanism involving electrostatic screening of negative charges on the stroma-exposed surface and on the LHCII antenna (see e.g., Chow et al. 2005).

Alterations in the thylakoid ultrastructure have been reported for the various mutants with defects in K⁺ or Cl⁻ transport. The TPK3-silenced mutants displayed a swollen lumen (Carraretto et al. 2013), whereas the vccn1 thylakoid grana were longer and had an altered (“banana-like”) shape, possibly due to changed ion homeostasis (Herdean et al. 2016b). Duan et al. (2016) did not analyze the ultrastructure of thylakoids from the atbest1 mutants, but reported a larger thylakoid lumen in the atbest (atbest1atbest2) double mutant. The clce chloroplasts and thylakoid network had a peculiar bow-like shape leaving a larger thylakoid-free stromal space in dark-adapted plants, but were indistinguishable from the wild type in light-adapted plants (Herdean et al. 2016a). The kea3 chloroplasts did not exhibit morphological defects, but those in the kea1kea2kea3 triple mutant were fewer and had reduced number of thylakoids as compared to the double mutants of envelope-localized homologous K⁺/H⁺ antiporters KEA1 and KEA2 (Kunz et al. 2014).

**Other ion fluxes**

Much less dramatic changes in thylakoid organization were reported in mutants defective in other ion fluxes.
The \textit{taac} thylakoid structure was not distinguishable from the wild type in plants exposed to standard growth light, but higher grana stacks were observed in high light conditions due to impaired PSII cycle (Yin et al. 2010). The \textit{ph4;1} thylakoid organization did not appear affected in the transmission electron microscopy analyses, however, small-angle neutron scattering revealed smaller lamellar repeat distance of thylakoids, attributed to a tighter stacking and/or narrower lumen in the likely altered ionic environment (Karlsson et al. 2015). The \textit{pam71} mutants did not differ from the wild type in organization of chloroplast and thylakoid membranes (Schneider et al. 2016). However, Wang et al. (2016) found fewer and smaller chloroplasts with shorter distance between the grana stacks for mutants of the same gene (\textit{ccha1}). The reasons behind the discrepancy between the results of the two studies are not clear.

**Conservation of thylakoid ion fluxes in photosynthetic organisms**

The so far characterized proteins mediating ion fluxes across thylakoids appear to play critical roles in regulating photosynthesis in response to changes in the natural environment of land plants such as \textit{Arabidopsis}. Environmental conditions have greatly changed throughout evolution, and various types of photosynthetic organisms grow in very different environments (land/aquatic) and with distinct pattern of perturbations. Therefore, it is expected that if homologues of genes for ion fluxes are present in algae and cyanobacteria, their functions may not necessarily be the same, and their absence in either of these could suggest a specific invention to cope with the fluctuations in the particular environment. With few exceptions, no cyanobacterial or algal homologues have been characterized, so that most available information comes from phylogenetic analyses of the \textit{Arabidopsis} proteins (Fig. 2).

The cyanobacterium \textit{Synechocystis} sp. PCC 6803 has the K$^+$ channel SynK, which has a similar physiological role as the \textit{Arabidopsis} TPK3 (Zanetti et al. 2010, Checchetto et al. 2012). No algal homologues of TPK3 have been characterized thus far although putative sequences in green algae and diatoms have been revealed by phylogenetic analyses (Pfeil et al 2014). For KEA3, homologues do exist in the available sequenced genomes of green, red and brown algae, diatoms and cyanobacteria, but not in glaucophytes (Pfeil et al. 2014).

Phylogenetic analyses revealed candidates for \textit{CLCe}-like genes in green algae, diatoms, cryptophytes and cyanobacteria (Pfeil et al. 2014) and for \textit{VCCN}-like genes even in red algae (Herdean et al. 2016b). Similar type of analyses highlighted TAAC homologues in some green algae, implying conserved functions, but not in cyanobacteria, red algae, brown algae or diatoms (Spetea et al. 2011). Phylogenetic analyses have been also performed for PHT4;1 and revealed homologues sequences in green and red algae but not in cyanobacteria, brown algae, diatoms or glaucophytes (Pfeil et al. 2014).

PAM71 is conserved in cyanobacteria, green algae and red algae. The \textit{Chlamydomonas} homologue CGDL1 and the \textit{Synechocystis} homologue MnX have been characterized as having function in Mn$^{2+}$...
transport and providing PSII water-oxidizing complex with manganese (Schneider et al. 2016, Brandenburg et al. 2017). The Cu\(^+\)-ATPase PAA2 is present in green algae, but is absent in cyanobacteria, red algae, glaucophytes and diatoms. Cyanobacteria have another Cu\(^+\)-ATPase PacS (Kanamaru et al. 1994). Among algae, some use plastocyanin for electron transport, whereas others have replaced it with Cyt \(_{c_6}\) (Peers and Price 2006), and thus may not require a system for copper delivery to the thylakoid lumen. Characterization of the various homologues of ion flux components in cyanobacteria and algae should be focus of future studies, as working with more simple organisms may reveal new functions and also facilitate understanding of those conserved throughout evolution from bacteria to plants.

**Conclusion and hypotheses**

Based on the phenotypic analyses of various (mostly single) mutants reviewed above, it appears that an increasing number of thylakoid ion channels/transporters are needed to properly regulate the PMF, photosynthesis and membrane ultrastructure in variable light environments. Experiments using higher-order loss-of-function mutants will be useful tools to understand functional interconnections between these proteins.

To rationalize the role of different ion channels/transporters in modulating the PMF, one can start from a parallel between the changes in the thylakoid membrane potential during the light-to-dark transition (Fig. 1), and the modification of the membrane potential occurring in neurons during an action potential (Sadava et al. 2014).

In neurons, exposure to a stimulus induces a membrane depolarization from the resting state (negative \(\Delta\Psi\)). This triggers a fast ion flux via Na\(^+\) channels, which collapses the \(\Delta\Psi\) (from negative to positive) until the achievement of the equilibrium potential for Na\(^+\). At this stage, the Na\(^+\) channels close, while K\(^+\) channels open, generating an inverse current that brings the \(\Delta\Psi\) back to the resting state, and leads to a transient period of hyperpolarization (Sadava et al. 2014). The activity of these two ion channels with different activation threshold and kinetics is therefore sufficient to promote a regulation of the membrane potential.

In thylakoids, the ECS kinetics indicates a similar behavior, suggesting that at least two ion transport components could regulate this PMF. The first one is the H\(^+\)-efflux ATP synthase CF\(_{0}\)F1, while several candidates mediating counterion fluxes (see above) could play the role of the second component.

Overall, the parallel between the two systems reinforces the notion that the regulation of the PMF in vivo follows similar rules as in other well-characterized biological processes. Yet, as mentioned in the Introduction, several questions remain open as to the precise regulation of the PMF by ion fluxes. Additional research on ion fluxes in plants, algae and cyanobacteria will certainly provide a more precise scenario of this process in the future, explaining the regulation of a fundamental step in the conversion of

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sunlight into chemical energy during photosynthesis.

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Figure legends

Fig. 1. Kinetics of the electrochromic shift (ECS) in *Arabidopsis thaliana* leaves exposed to saturating light (1100 µmol photons s\(^{-1}\) m\(^{-2}\)). White box: light ON; black box: light OFF. (a) ECS relaxation during the light-to-dark transition. (b) Slow ECS recovery in the dark, promoting the attainment of a pseudo equilibrium state (c) where a reversible (d) and an irreversible (e) ECS signal can be detected.

![Figure 1](image1)

Fig. 2. Current picture of ion channels, transporters and pumps in the thylakoid membrane of *Arabidopsis thaliana*. Proteins with homologues in green algae, diatoms, red algae and cyanobacteria are represented in green, brown, red and blue, respectively. Predicted homologues proteins are framed with broken lines. Proteins with at least one homologue experimentally characterized in a non-plant organism are framed with continuous line.

![Figure 2](image2)
Table 1. Characterized thylakoid ion transport proteins in *Arabidopsis* and the effects measured in mutants on the proton motive force (PMF), non-photochemical quenching (NPQ), electron transport rate (ETR), and thylakoid ultrastructure. \(\uparrow\) increase; \(\downarrow\) decrease.

<table>
<thead>
<tr>
<th>Type of transport protein</th>
<th>Ion flux across thylakoids</th>
<th>Regulator</th>
<th>Effect on PMF</th>
<th>Effect on NPQ</th>
<th>Effect on ETR</th>
<th>Effect on thylakoid ultrastructure</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-pore K⁺ channel</td>
<td>K⁺ efflux</td>
<td>pH, Ca²⁺</td>
<td>(\Delta \Psi)/(\Delta \rho)H</td>
<td>(\downarrow) Induction, steady state</td>
<td>(\downarrow)</td>
<td>Swollen lumen</td>
<td>Carraretto et al. (2013)</td>
</tr>
<tr>
<td>K⁺/H⁺ exchanger KEA3</td>
<td>K⁺ influx, H⁺ efflux</td>
<td>C-terminal KTN domain</td>
<td>(\Delta \rho)H/(\Delta \Psi)</td>
<td>(\downarrow) Relaxation</td>
<td>(\downarrow)</td>
<td>No visible effects</td>
<td>Kunz et al. (2014), Armbruster et al. (2014, 2016), Wang et al. (2016a)</td>
</tr>
<tr>
<td>Cl⁻ channel CLCe</td>
<td>Cl⁻ influx/efflux</td>
<td>n.d.</td>
<td>(\uparrow) PMF, (\Delta \Psi)</td>
<td>(\uparrow) Steady state/Relaxation</td>
<td>(\downarrow)</td>
<td>Bow-like arrangement of thylakoids and large thylakoid-free stroma in darkness, no effect in standard light</td>
<td>Marmagne et al. (2007), Herdean et al. (2016b)</td>
</tr>
<tr>
<td>¹AtBest1/²Voltage-dependent Cl⁻ channel VCCN1</td>
<td>Cl⁻ influx</td>
<td>Voltage</td>
<td>(\uparrow) PMF, (\Delta \Psi)</td>
<td>(\downarrow) Induction</td>
<td>none</td>
<td>¹Swollen lumen in <em>atbest</em> mutant ²Longer grana in darkness, longer and bended in the light</td>
<td>¹Duan et al. (2016) ²Herdean et al. (2016a)</td>
</tr>
<tr>
<td>Thylakoid ATP/ADP carrier TAAC</td>
<td>ATP influx, ADP efflux</td>
<td>n.d.</td>
<td>(\uparrow) (\Delta \rho)H</td>
<td>(\uparrow) Induction</td>
<td>none</td>
<td>No effect in standard light, higher grana stacks in high light</td>
<td>Thuswaldner et al. (2007), Yin et al. (2010)</td>
</tr>
<tr>
<td>Phosphate transporter PHT4:1</td>
<td>Pi, H⁺ (Na⁺) influx</td>
<td>n.d.</td>
<td>(\uparrow) (\Delta \rho)H</td>
<td>(\uparrow) Induction</td>
<td>none</td>
<td>Tighter grana stacking and/or narrower lumen</td>
<td>Guo et al. (2008), Pavon et al. (2008), Karlsson et al. (2015)</td>
</tr>
<tr>
<td>³Photosynthesis affected mutant PAM71/⁴Chloroplast localized Ca²⁺/H⁺ antiporter</td>
<td>³Mn²⁺ influx, ⁴Ca²⁺ influx, H⁺ efflux?</td>
<td>n.d.</td>
<td>(\uparrow) PMF, (\Delta \Psi)</td>
<td>(\downarrow) Steady state</td>
<td>(\downarrow)</td>
<td>³None ⁴Fewer and smaller chloroplasts, shorter distance between grana stacks</td>
<td>³Schneider et al. (2016) ⁴Wang et al. (2016b)</td>
</tr>
</tbody>
</table>