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Review

Tansley review

Chloroplast ion homeostasis – what do we know and where should we go?

Author for correspondence:
Hans-Henning Kunz
Email: kunz@lmu.de

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Hans-Henning Kunz[1], Ute Armbruster[2,3], Susanne Mühlbauer[1], Jan de Vries[4] and Geoffry A. Davis[1,5]

[1]Plant Biochemistry, Biology, LMU Munich, Großhadernerstr. 2-4, 82152, Planegg-Martinsried, Germany; [2]Institute of Molecular Photosynthesis, Heinrich Heine University Düsseldorf, 40225, Düsseldorf, Germany; [3]CEPLAS – Cluster of Excellence on Plant Sciences, Heinrich Heine University Düsseldorf, 40225, Düsseldorf, Germany; [4]Department of Applied Bioinformatics, Institute for Microbiology and Genetics, Goettingen Center for Molecular Biosciences (GZMB), Campus Institute Data Science (CIDAS), University of Goettingen, Goldschmidtstr. 1, D-37077, Göttingen, Germany; [5]Department of Life Sciences, Imperial College London, London, SW7 2AZ, UK

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Summary

Plant yields heavily depend on proper macro- and micronutrient supply from the soil. In the leaf cells, nutrient ions fulfill specific roles in biochemical reactions, especially photosynthesis housed in the chloroplast. Here, a well-balanced ion homeostasis is maintained by a number of ion transport proteins embedded in the envelope and thylakoid membranes. Ten years ago, the first alkali metal transporters from the K^{+} EFFLUX ANTIPORTER family were discovered in the model plant Arabidopsis. Since then, our knowledge about the physiological importance of these carriers and their substrates has greatly expanded. New

insights into the role of alkali ions in plastid gene expression and photoprotective mechanisms, both prerequisites for plant productivity in natural environments, were gained. The discovery of a Cl^- channel in the thylakoid and several additional plastid alkali and alkali metal transport proteins have advanced the field further. Nevertheless, scientists still have long ways to go before a complete systemic understanding of the chloroplast's ion transportome will emerge. In this Tansley review, we highlight and discuss the achievements of the last decade. More importantly, we make recommendations on what areas to prioritize, so the field can reach the next milestones. One area, laid bare by our similarity-based comparisons among phototrophs is our lack of knowledge what ion transporters are used by cyanobacteria to buffer photosynthesis fluctuations.

I. Introduction

Cellular life relies on inorganic nutrients in just the right amounts. This was demonstrated through the work on plants by Carl Sprengel and Justus von Liebig in the mid of the 19th century. Their work led to Liebig's law of the minimum, which states that plant growth is dictated by the scarcest resource such as the least abundant nutrient in the soil (Liebig, 1840). This profound awareness was an inspiration to the founder of this review series Sir Arthur George Tansley. In his first textbook for students – Elements of Plant Biology (Tansley, 1922), Tansley advised an experimental setup of water cultures (Fig. 1). Students should 'observe the effects of leaving out each of the essential elements in the mixed solution of salts used to feed the plant'. Soon after, Tansley defined today's all-encompassing concept of life on earth: the ecosystem (Tansley, 1935): 'In an ecosystem the organisms and the inorganic factors alike are components which are in relatively stable dynamic equilibrium'. Tansley had realized the necessity to understand nutrient cycling and the physiological relevance of nutrient ions at every biological scale. As molecular biologists, we interpret and expand his ideas down to the cellular, organellar, and suborganellar level. In this way, Tansley's vision continues to inspire today's scientists as many aspects remain unclear.

Plants take up mineral nutrient ions primarily from the soil via the root system and distribute them throughout the plant body employing the vascular tissue. Between tissues, that is distinct cell types but also cell organelles, strikingly different mineral levels exist (Salt, 2004; Giehl *et al.*, 2023). The inorganic ion requirements of an organelle are a reflection of the biochemical pathways active within the compartment. Of special interest for this article is photosynthesis and its hosting organelle, the chloroplast. The involved membrane-bound and soluble protein complexes are well-known. Most proteins rely, directly or indirectly, on bound metal cofactors and anions, respectively (Fig. 2). Hence, improving plant and algae photosynthetic efficiency requires detailed knowledge of (1) the mineral ion composition of the chloroplast, (2) the suborganellar metal distribution, and (3) how ion gradients necessary to fuel biochemical processes become established.

In this article, we will briefly review mineral ions of relevance and their respective transport mechanisms in Arabidopsis chloroplasts. We focus on the function and evolution of plastid alkali and alkaline earth metals transport proteins, as well as Cl^- channels, which in concert maintain the organellar ion homeostasis. Chloroplasts contain three different membranes: outer envelope (OE), inner envelope (IE), and thylakoid. The OE contains pores that can function as high conductance channels (Goetze *et al.*, 2015; Barth *et al.*, 2022). Since their specificity remains debated, we will start our journey at the highly selective IE

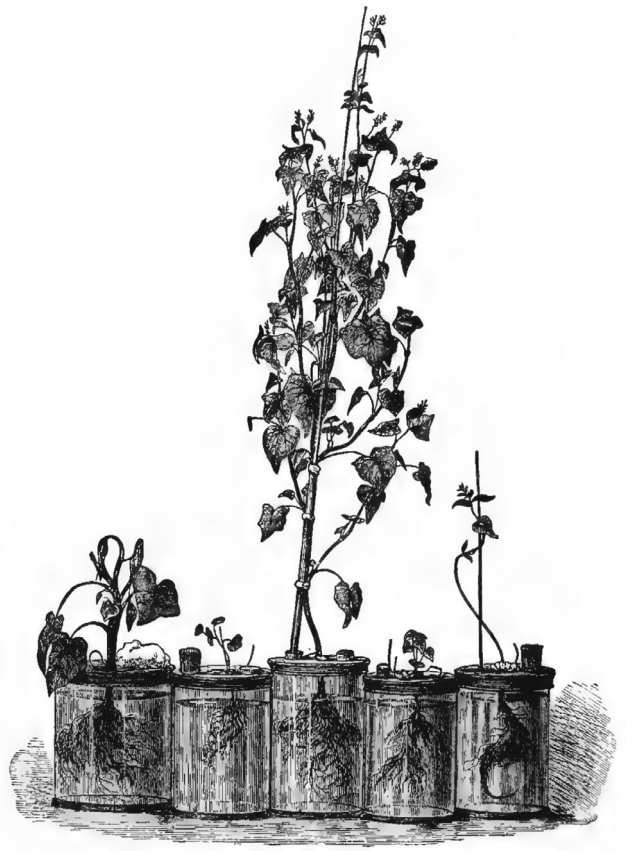

FIG. 13.—Water cultures of Buckwheat (after Nobbe). 1, plant grown in complete solution; 2, without potassium; 3, with sodium instead of potassium; 4, without calcium; 5, without nitrogen.

Fig. 1 Illustration from Tansley's book – Elements of Plant Biology (Tansley, 1922). Buckwheat plants grown in water cultures to test the effects of nutrient starvation. Cultures from left to right are: without calcium, sodium instead of potassium, complete solution (control), without potassium, without nitrogen.

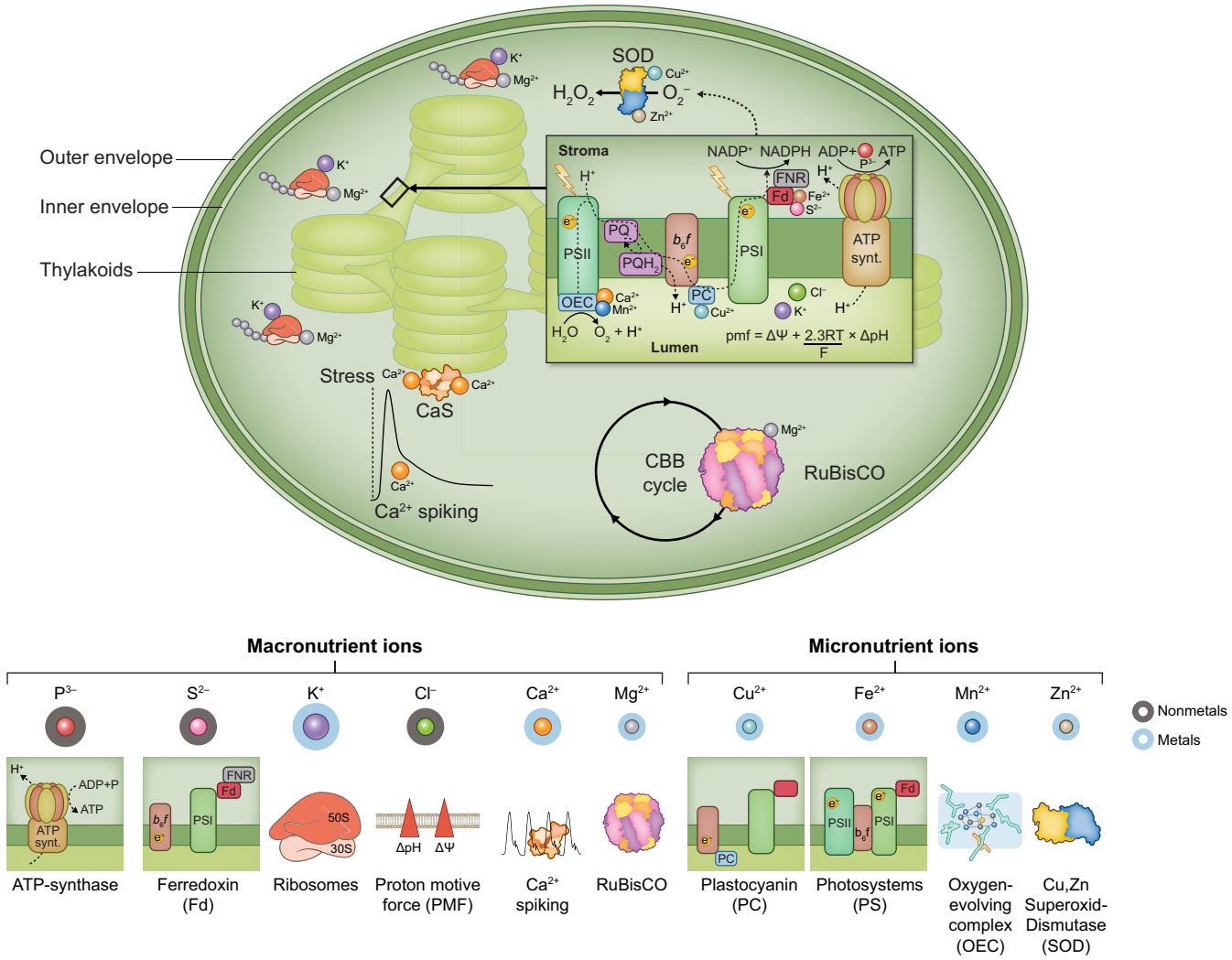

Fig. 2 Nutrient ion demand and binding to selected proteins involved in photosynthesis and located in the chloroplast. Δψ and ΔpH represent the differences in electric field (in volts) and pH, respectively, between the lumenal and stromal. ATP Synt, ATP synthase; b_6f, cytochrome b_6f complex; CaS, calcium sensor; CBB, Calvin–Basham–Benson; F, Faraday's constant; Fd, ferredoxin; FNR, ferredoxin-NADP$^+$ reductase; PC, plastocyanin; *pmf*, proton motive force; PQ, plastoquinone; PQH_2, plastoquinol; PSI, photosystem I; PSII, photosystem II; R, universal gas constant; SOD, superoxide dismutase.

membrane and continue at the thylakoid membrane where the light-dependent reactions of photosynthesis take place.

II. Alkali and alkaline earth metal ion flux across the inner envelope membrane

Ions of alkali and alkaline earth metals fulfill a plethora of photosynthesis-related functions (Fig. 2). While several transport proteins remain unknown in the IE, substantial progress has been made in the last years. We discuss the now-known ion carriers grouped by their respective substrates while briefly introducing the physiological relevance of each metal type. An extensive overview on all currently known chloroplast alkali and alkaline earth metal transport proteins, including those for the counterion Cl^-, was compiled for the model plant *Arabidopsis thaliana* (Table 1; Fig. 3).

III. Mg^{2+} exchange across the plastid inner envelope membrane

Mg^{2+} represents the central ion in the porphyrin structure of chlorophyll molecules and consequentially is crucial for light energy capture. Mg^{2+} chelation into chlorophyll precursors takes place in the stroma (Wang & Grimm, 2021). Several other stromal enzymatic reactions such as in CO_2 fixation rely on Mg^{2+} or MgATP as cofactors. Lastly, the stacking of thylakoid membranes depends on Mg^{2+} (Barber, 1980). It follows that plastid Mg^{2+} import is critical for photosynthesis in many ways. MAGNESIUM TRANSPORTER 10 (AtMGT10), an IE protein, was described as the first plastid Mg^{2+} carrier (Sun *et al.*, 2017). Members of the MGT family were characterized mostly through yeast and *Escherichia coli* mutant complementation (Li *et al.*, 2001; Ishijima *et al.*, 2015). Recently, reconstitution of AtMGT10 into liposomes followed by dye-based

Table 1 Molecular characteristics of currently known plastid alkali and alkaline earth metal transport proteins.

AGI	Protein name	Calc. monomeric size incl. TP (kDa)	Complex size (kDa)	(Suggested) Substrate	CP membrane loc	Localization method	Main reference
At1g01790	KEA1	128.3	600–800	K^+/H^+	IE	GFP, immunoblot, MS	Kunz *et al*. (2014)
At4g00630	KEA2	126.2	250 + 700	K^+/H^+	IE	GFP, immunoblot, MS	Kunz *et al*. (2014)
At4g04850	KEA3	83.8	100 + 250	K^+/H^+	Thy	GFP, immunoblot, MS	Kunz *et al*. (2014)
At4g35440	ClCe	75.6	NA	Cl^-/H^+	Thy	GFP, MS	Marmagne *et al*. (2007)
At3g61320	VCCN1/Best1	46.5	NA	Cl^-	Thy	GFP, immunoblot	Herdean *et al*. (2016)
At2g45870	VCCN2	46.3	NA	Cl^-	Thy	GFP, immunoblot	Herdean *et al*. (2016)
At5g02940	PEC1	92.1	600–1000?	K^+ (& Ca^{2+}?)	IE	GFP, *in vitro* uptake, MS, immunoblot	Völkner *et al*. (2021)
At5g43745	PEC2	92.2	700–1000?	K^+ (& Ca^{2+}?)	IE	GFP, *in vitro* uptake, MS, immunoblot	Völkner *et al*. (2021)
At3g19490	NHD1	61.4	100–130	Na^+/H^+	IE?, Thy?	GFP, MS	Furumoto *et al*. (2011)
At1g64150	BiCAT1/ PAM71	39.1	NA	Ca^{2+}, Mn^{2+}	Thy	GFP, MS	Schneider *et al*. (2016)
At4g13590	BiCAT2/CMT1	37.9	NA	Ca^{2+}, Mn^{2+}	IE	GFP, MS	Eisenhut *et al*. (2018)
At5g66650	cMCU	36.8	NA	Ca^{2+}	IE?	GFP	Teardo *et al*. (2019)
At5g22830	MGMT10	51.1	600–700	Mg^{2+}	IE	GFP, MS	Li *et al*. (2001)
AtCg00530	YCF10	27.4	NA	K^+/H^+	IE	–	Harada *et al*. (2019)
At4g31040	DLDG1	51	NA	K^+/H^+	IE	GFP	Harada *et al*. (2019)
At3g13070	MGR8	73.8	250–300 + 500	Mg^{2+}	IE	GFP	Zhang *et al*. (2022)
At1g55930	MGR9	72.9	NA	Mg^{2+}	IE	GFP	Zhang *et al*. (2022)
At5g10490	MSL2	74.43	800	Cl^-/K^+	IE	GFP, MS	Haswell & Meyerowitz (2006)
At1g58200	MSL3	74.2	800	Cl^-/K^+	IE	GFP, MS	Haswell & Meyerowitz (2006)

All complex sizes were collected as previously discussed (Schröder *et al*., 2022). For localization data, we consulted SUBA and PeptideAtlas (Hooper *et al*., 2017; van Wijk *et al*., 2021). ?, ambiguous result; AGI, Arabidopsis Genome Initiative; CP, chloroplast; GFP, green fluorescent protein; IE, inner envelope; MS, mass spectrometry; NA, no information available; Thy, thylakoid; TP, transit peptide.

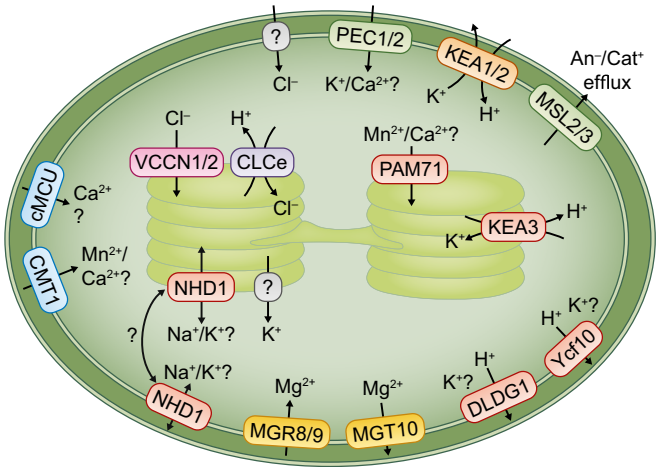

Fig. 3 Overview of currently known and unknown plastid ion transport mechanisms. Protein names and locations as based on work in *Arabidopsis thaliana*. Question marks (?) denote unknown genes, localization, or substrates.

Mg^{2+} uptake using mag-fura-2 fluorescence was successful (Ishijima *et al*., 2021). *Mgt10* loss-of-function mutants exhibit defects in chloroplast development and lower photosynthesis rates. Since leaf and plastid Mg^{2+} contents were elevated, AtMGT10 was assigned the role of a plastid Mg^{2+} exporter. Recently, Magnesium Release 8 (MGR8) and MGR9 isoforms were discovered as Mg^{2+} uptake mechanisms into Arabidopsis chloroplasts. Corresponding *mgr8mgr9* loss-of-function mutants are extremely pale with underdeveloped chloroplasts. Mutant plastids but not leaves contained lower Mg^{2+}. The function of both carriers was verified through a rescue assay of Mg^{2+}-uptake-deficient Salmonella and *E. coli* cells (Zhang *et al*., 2022; Dukic *et al*., 2023).

IV. Ca^{2+} dynamics in plastids

Ca^{2+} fulfills many important roles in the chloroplast. Besides its relevance for photosynthesis (Hochmal *et al*., 2015), Ca^{2+} is an important cellular signaling molecule (He *et al*., 2021; Resentini *et al*., 2021). The Ca^{2+} sensor CaS, a protein in the chloroplast, has emerged as a central player affecting Ca^{2+} signals in the cytosol and plastids (Weinl *et al*., 2008). Changes in growth light, temperature, osmotic pressure, and pathogen attack all trigger distinct Ca^{2+} transients in both compartments (Costa *et al*., 2018). For many years, no plastid Ca^{2+} transport candidates were known (Stael, 2019). That situation has changed. Initially, the CHLOROPLAST MITOCHONDRIAL CALCIUM UNIPORTER (cMCU), a dual-targeted channel shared between mitochondria and guard cell plastids, was described (Teardo *et al*., 2019). The cMCU-facilitated Ca^{2+} flux was shown in heterologously expressing *E. coli* cells and

electrophysiological bilayer experiments on recombinant cMCU. The mechanism by which cMCU gets exclusively targeted into guard cell plastids remains unknown. The cMCU discovery was followed by putative CALCIUM/MANGANESE CATION TRANSPORTER 1/BIVALENT CATION TRANSPORTER 2 (CMT1/BICAT2), a Mn^{2+} uptake mechanism in the envelope but, as indicated by complementation of a yeast mutant and uptake studies in BICAT2 expressing *E. coli* cells, potentially also Ca^{2+} (Eisenhut *et al.*, 2018; Frank *et al.*, 2019). Different from wild-type (WT) looking *cmcu* loss-of-function mutants, *cmt1/bicat2* mutants are highly compromised.

V. Na^+ transport in leaf plastids

The relevance for Na^+ in chloroplasts is unclear and may be species-dependent. As shown already in Tansley's water culture experiment, Na^+ cannot substitute the role of K^+ for plant cell function (Fig. 1). The leaves of plants sensitive to soil salinity (glycophytes), such as *A. thaliana*, contain K^+ to Na^+ at a ratio of 46 to 1 mg g^{-1} dry weight (Salt, 2004). It seems therefore reasonable to assume that Na^+ levels in leaf plastids of glycophytes are fairly low. However, the only data on plastid Na^+ contents date back to the 1980s and were recorded in non-model species. The reported values range from three times more K^+ than Na^+ in spinach and sugar beet chloroplasts to equal K^+ to Na^+ amounts in pea plastids (Demmig & Gimmler, 1983; Robinson & Downton, 1984; Schröppel-Meier & Kaiser, 1988). Curiously, Na^+-dependent carriers from the Bile Acid Sodium Symporter (BASS) family are common in plant plastids. Hence, Na^+ may act as a micronutrient required as a co-substrate for some transporters (Maathuis, 2014). Generally, salt tolerance is frequently found in C_4 lineages, which may suggest higher Na^+ requirements in leaves to drive pyruvate uptake to fuel the CO_2 concentrating mechanism (Bromham & Bennett, 2014). C_4 plants exhibit high rates of Na^+-dependent pyruvate uptake into mesophyll chloroplasts (Ohnishi *et al.*, 1990). The carrier BASS2 from *Flaveria trinervia* was characterized as a Na^+-dependent pyruvate importer in the plastid IE. The transport activity of BASS2 *in vitro* was dependent on the presence of co-reconstituted NHD1, a plastid putative Na^+/H^+ exchanger (Furumoto *et al.*, 2011). NHD1 was reported in the IE and therefore suggested to limit stromal Na^+ accumulation (Barrero-Gil *et al.*, 2007; Cosentino *et al.*, 2010; Müller *et al.*, 2014). In Arabidopsis, NHD1 was detected in the thylakoid membrane proteome suggesting an overlooked additional role for this protein and maybe Na^+ in chloroplasts (Tomizioli *et al.*, 2014). The original *nhd1* loss-of-function allele carries a deletion, which affects several loci in close proximity. Additionally, mutant lines lacking NHD1 exhibit growth defects under dynamic light stress (Lopez *et al.*, 2022), a behavior typical for mutants with defects in thylakoid ion carriers (Schneider *et al.*, 2019; Li *et al.*, 2021). Hence, the exact membrane localization of NHD1 needs to be revisited.

VI. K^+ efflux across the plastid inner envelope membrane

Under physiological growth conditions, K^+ is the most abundant nutrient in the plant body (Wang & Wu, 2013). Cytosolic K^+ levels range from 100 to 120 mM, while chloroplasts may hold similar K^+ amounts (Robinson & Downton, 1984). A small pH gradient across the envelope membrane in light and the abundance of negatively charged macromolecules inside the organelle results in a Donnan effect and concomitant K^+ influx (Demmig & Gimmler, 1983; Bernardi, 1999). The influx is balanced by IE membrane K^+/H^+ exchangers, which expel K^+ ions to avoid osmotic damage to the organelle. In Arabidopsis, K^+ EFFLUX ANTIPORTER 1 (KEA1) and KEA2 fulfill this role. Their simultaneous loss affects stromal pH, the organelle's osmotic potential, and plastid size distribution; *kea1kea2* mutant plants possess more enlarged but also significantly higher numbers of small plastids (Kunz *et al.*, 2014; Aranda-Sicilia *et al.*, 2016; Aranda Sicilia *et al.*, 2021).

The small plastids constitute proplastids, which develop into photosynthesizing chloroplasts through coordinated gene expression from the nuclear and the plastid genome. Halted chloroplast development in *kea1kea2* mutants is visibly manifested by its delayed greening phenotype. A mechanistic explanation for the defect in K^+/H^+ exchangers and organellar biogenesis was long lacking (Sze & Chanroj, 2018). Recently, it was shown that the lack of IE KEAs results in severe maturation defects of plastid rRNAs in *kea1kea2* plants (DeTar *et al.*, 2021). It was hypothesized that the lack of K^+/H^+ exchange across the IE membrane skews the plastid ion homeostasis so that RNA-protein binding and processing in the stroma becomes ineffective. In line with this observation, *kea1kea2* mutants exhibit altered secondary structures of stromal mRNAs and decreased plastid gene expression (PGE). Disturbed PGE delays chloroplast development to minimize damage to the plant (DeTar *et al.*, 2021). The discovery of a functional link between plastid ion transport and PGE will benefit our understanding of the PGE machinery and the biochemical environment it requires to function *in vivo*.

In the meantime, two additional IE proteins of interest have been discovered: DAY-LENGTH-DEPENDENT DELAYED-GREENING 1 (DLDG1) and its plastome-encoded homolog YCF10 (ATCG00530) (Harada *et al.*, 2019). Both proteins possess a CemA-like proton extrusion protein-like domain and are homologous with PxcA (*slr1596*) and PxcA-like (*sll1685*) from the cyanobacterial model organism *Synechocystis*. In cyanobacteria, PxcA and PxcA-like take part in H^+ translocation across the plasma membrane (Inago *et al.*, 2020). DLDG1 but not YCF10 expressed in *E. coli* showed low K^+/H^+ and Ca^{2+}/H^+ exchange. It was therefore proposed that DLDG1 may function as a K^+-dependent H^+ extruder across the chloroplast envelope membranes. As the authors state: The exact nature of the transporter activity mechanism remains unclarified (Trinh *et al.*, 2021). Curiously, *dldg1* loss-of-function mutants exhibit delayed greening resembling *kea1kea2* mutants. The phenotype may be directly linked to loss of DLDG1 or points toward a requirement of DLDG1 function for IE KEA activity, for example by building up ΔpH across the IE. Genes encoding a K^+-stimulated ATP-dependent H^+ pump as described in pea chloroplast IE vesicles have never been found in Arabidopsis (Shingles & McCarty, 1994). DLDG1 alone or in combination with YCF10 may fulfill the role of H^+ extrusion across the IE albeit by a different mechanism as originally suggested.

Plastids can also release ions through activation of mechanosensitive ion channel 2 (MSL2) and MSL3 (Haswell & Meyerowitz, 2006). Especially, the lack of MSL2 affects plant development. Although the mutants are as green as WT plants, their leaf shape is altered. Microscopy suggests that primarily epidermal plastids are affected in size, shape, and division (Wilson *et al.*, 2014). Electrophysiology on the Arabidopsis homolog MSL1, which localizes to mitochondria, suggests that MSL members function as unspecific ion channels with preference for anions (Cl^-/ K^+) (Lee *et al.*, 2016). It is unknown whether MSL2/3 and KEA1/2 functions overlap.

VII. K^+ and cation influx into plastids

While the function of plastid K^+ efflux can be explained by IE KEA activity, genes encoding for ion influx mechanisms remained unknown for a long time. Electrophysiology studies on pea and spinach chloroplasts gave insights into cation channel conductivities in the IE (Mi *et al.*, 1994; Heiber *et al.*, 1995; Pottosin *et al.*, 2005). However, the lack of genetic tools prevented the identification of the respective genetic loci. Recently, it was shown that two distant homologs from the Pollux/Castor family, nuclear cation channels with K^+, Na^+, and Ca^{2+} conductivity (Charpentier *et al.*, 2008; Kim *et al.*, 2019), exist in the plastid IE membrane (Völkner *et al.*, 2021). Although electrophysiology studies are missing, the now-called plastid envelope channel (PEC) proteins are capable of rescuing K^+ uptake-deficient yeast mutants. *pec1pec2* loss-of-function plants are indistinguishable from WT plants. Interestingly, the mutants have almost no stress-induced Ca^{2+} transients in the stroma. Further investigations will help to unveil the role of plastids in Ca^{2+} signaling. The WT appearance of *pec1pec2* loss-of-function mutants suggests that other cation importers are present and/or that the role of PECs is highly specific, for example in shaping stress-triggered Ca^{2+} signaling. Future work should focus on understanding the mechanistic link between PEC activity and the dampened stromal Ca^{2+} signals observed in loss-of-function mutants. For now, an indirect involvement, through activating the known envelope Ca^{2+} transport proteins (cMCU, CMT1/BICAT2), has been suggested (Völkner *et al.*, 2021). However, a detailed understanding of PEC proteins' substrate spectrum should be of highest priority.

VIII. Ion flux across the thylakoid membrane and function in photosynthesis

In eukaryotes, photosynthesis can be subdivided into directly light-dependent reactions in the chloroplast thylakoid membrane (Figs 2, 4a) and light-independent reactions, that is CO_2 fixation in the stroma. In the first part, light drives proton-coupled electron transfer from photosystem II (PSII) via the cytochrome b_6f complex (b_6f), and then plastocyanin toward photosystem I (PSI). At the stromal side of PSI, electrons are transferred to ferredoxin (Fd) and subsequently to $NADP^+$, finalizing linear electron flow (LEF). Here, the thylakoid-bound copper/zinc-superoxide dismutase (Cu/ZnSOD) detoxifies reactive oxygen species emerging from the photosystems. Ion transport sustains the functionality of the photosynthetic apparatus by providing essential components of the electron transport chain to the thylakoid lumen, namely Mn^{2+}, Ca^{2+}, and Cl^-, required in the oxygen-evolving complex (OEC) of PSII, $Fe^{2+/3+}$, as part of PSII, b_6f, and PSI, and $Cu^{+/2+}$, the redox-cofactor of electron carrier plastocyanin (PC), which connects the b_6f complex with PSI. For further information on Fe^{2+} and Cu^{2+} transport, we refer to excellent reviews by others (Aguirre & Pilon, 2015; Schmidt *et al.*, 2020).

IX. Mn^{2+} and Ca^{2+} transport enable function of the oxygen-evolving complex

PSII requires Mn^{2+} and Ca^{2+} at the lumen side of the thylakoid membrane, which form the manganese cluster (Mn_4CaO_5) of the OEC that catalyzes the oxidation of water. The thylakoid carrier PAM71/BICAT1 has homology with putative Ca^{2+}/H^+ exchangers from yeast and human. It is required for efficient PSII function and plant growth. Lower PSII activity in *pam71/bicat1* loss-of-function mutants was explained by decreased Mn^{2+} import into the lumen of isolated thylakoids (compared with total chloroplast loading). By contrast, relative Ca^{2+} loading was higher in mutants. Exogenous supply of Mn^{2+}, but not Ca^{2+}, restored PSII function in *pam71/bicat1* mutants. An independent study reported a function of the protein in Ca^{2+} transport into the thylakoids, arguing that PAM71/BICAT1 constitutes a Mn^{2+}/Ca^{2+} transporter with dual-specificity *in planta* (Frank *et al.*, 2019).

PSII is essential for photosynthesis and PSII-deficient mutants cannot grow under photoautotrophic conditions (Meurer *et al.*, 1998). PSII functionality is strictly dependent on its cation cofactors. Since *pam71/bicat1* loss-of-function mutants exhibit only mild growth impairments, additional unknown thylakoid ion transport proteins seem to also facilitate Mn^{2+}/Ca^{2+} transport into the thylakoid lumen partially compensating the loss of PAM71/BICAT1.

X. Function of thylakoid ion transport proteins in the regulation of photosynthesis

Upon illumination, the rapid movement of electrons in both PSII and PSI from the lumenal to the stromal side of the membrane generates a membrane potential ($\Delta\Psi$) due to the low electrical capacitance of the thylakoid membrane (*c.* 0.6 μF cm^{-2}; Junge & Witt, 1968) (Fig. 4b). This electric potential is dissipated by the movement of K^+ and Cl^- ions across the thylakoid, converting the electric potential into an ion gradient (Cruz *et al.*, 2005a).

Simultaneously, H^+ are released into the thylakoid lumen by PSII and cytochrome b_6f. After transferring enough H^+ to overcome the 0.03 M per pH unit buffering capacity of the thylakoid lumen, the H^+ gradient (ΔpH) component of the proton motive force (pmf) is established (Junge *et al.*, 1979). In addition to ΔpH, total $\Delta\Psi$ contributes to the pmf in varying degrees depending on light conditions (Cruz *et al.*, 2005b). Both pmf components are capable of driving ATP synthesis (Hangarter & Good, 1982). Nevertheless, the pmf in chloroplasts is predominately stored as ΔpH, in part to avoid $\Delta\Psi$–mediated PSII damage (Davis *et al.*, 2016).

The relative size of pmf components matters since ΔpH also has photoprotective implications (Kramer *et al.*, 2003). Stress conditions,

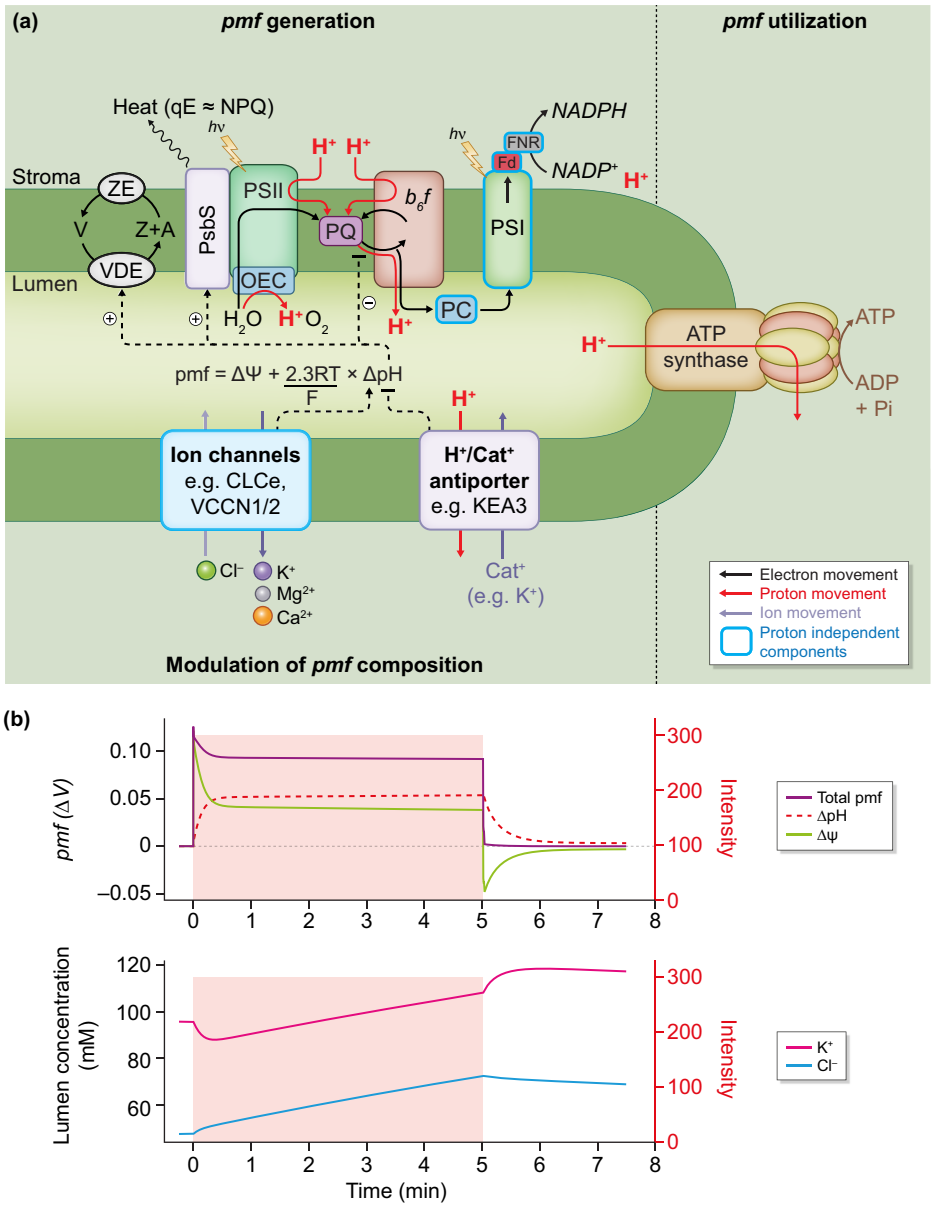

Fig. 4 Generation and dynamics of the proton motif force (pmf) across the thylakoid membrane. (a) Overview of the linear electron transfer chain components. Note, cyclic electron flow was left out for simplicity. (b) Model of pmf dynamics and its components ΔpH, and ΔΨ after the onset of illumination (red background) according to established (Li *et al*., 2021). Note that pmf changes are shown as differences relative to the dark non-zero pmf (ΔV). Changes in ΔpH, and ΔΨ are linked to the rapid influx of Cl^- and an efflux of K^+ ions from the thylakoid lumen (Davis *et al*., 2017). Δψ and ΔpH represent the differences in electric field (in volts) and pH, respectively, between the lumenal and stromal.
A, antheraxanthin; Cat^+, cation; Fd, ferredoxin; L, lumen; NPQ, nonphotochemical quenching; PC, plastocyanin; PQ, plastoquinone; qE, energy-dependent quenching; S, stroma; V, violaxanthin; VDE, violaxanthin de-epoxidase; Z, zeaxanthin; ZE, zeaxanthin epoxidase.

which result in a high lumen acidification trigger, decreased b_6f turnover (photosynthetic control) and energy-dependent nonphotochemical quenching, that is heat dissipation of absorbed light (qE from here on referred to as NPQ). When illumination changes, for example after a sudden light intensity drop, photoprotection limits LEF. Therefore, fine-tuning photoprotection has been suggested as a biotechnological approach to improve photosynthetic efficiency in crop plants for over a decade (Zhu *et al*., 2010). Indeed, engineering higher amounts of proteins involved in the violaxanthin cycle and the PsbS subunit of PSII led to faster NPQ inactivation kinetics and significantly higher yields in field-grown transgenic tobacco and soybean (Kromdijk *et al*., 2016; De Souza *et al*., 2022). Not all plant species respond similarly to this strategy (Garcia-Molina & Leister, 2020; Lehretz *et al*., 2022). Hence, the feasibility of this approach remains debated (De Souza *et al*., 2023; Leister, 2023; Sinclair *et al*., 2023). One alternative is to manipulate thylakoid ion flux, which affects NPQ kinetics via pmf partitioning (Davis *et al*., 2017).

The search for thylakoid ion transport proteins started decades ago, when patch-clamp studies revealed the presence of cation and anion permeable channels in this membrane (Schönknecht *et al*., 1988; Tester & Blatt, 1989; Pottosin & Schönknecht, 1996). Almost 20 yr later, the first thylakoid ion transport protein KEA3 and its gene were discovered in the model plant Arabidopsis (Armbruster *et al*., 2014; Kunz *et al*., 2014). Thus far, direct *in vivo* flux measurements could not be realized. Therefore, the main concepts about KEA3's physiological role have been deduced from spectroscopic measurements of NPQ as a proxy for ΔpH and the electrochromic shift (ECS) signal for insights into ΔpH and ΔΨ. The ECS is based on an absorbance shift of carotenoids at 515 nm, which is proportional to the membrane potential (ΔΨ). This approach has drawn criticisms (Johnson & Ruban, 2014; Wilson *et al*., 2021).

Some discrepancies seem related to the measuring conditions and different wavelengths utilized to deconvolute the ECS signal. In the simplified two-wavelength deconvolution, the 515 nm ECS signal is contaminated by a spectral overlap with the 535 nm qE signal and 505 nm zeaxanthin absorbance. This can be resolved by employing a three-wavelength deconvolution to consider the spectral overlap between xanthophyll absorbance (505 nm), ECS (515 nm), and qE (535 nm (Cruz *et al.*, 2001; Takizawa *et al.*, 2007)). However, this method does not discriminate the ion(s) moved or directionality, but only a charge difference.

The three-wavelength and by now even six-wavelength ECS approaches were used to characterize *kea3* loss-of-function mutants (Armbruster *et al.*, 2014; von Bismarck *et al.*, 2023). When *kea3* plants transition from high into a low light phase, the lack of K^+/H^+ exchange results in delayed NPQ inactivation (Fig. 5a). This has been widely interpreted as an inability to convert some ΔpH into ΔΨ. Conversely, genetic manipulations that render KEA3 more active speed up NPQ shutoff because less ΔpH is maintained in the mutants. This led to better growth rates under fluctuating light (Armbruster *et al.*, 2016; Wang *et al.*, 2017).

Other pmf regulators of interest are the thylakoid BESTROPHIN-LIKE PROTEIN/voltage-dependent Cl^- channels (VCCN1 and VCCN2) (Duan *et al.*, 2016; Herdean *et al.*, 2016). Electrophysiology of reconstituted VCCN1 in a lipid bilayer system confirmed its activity with Cl^- ions. The spectroscopy of *vccn1* loss-of-function mutants suggests a role in dissipating ΔΨ to enable timely NPQ activation, for example when plants transition from low into high light phases (Fig. 5b). In line with this observation, pmf in *vccn1* mutants is mostly constituted of ΔΨ. The *in vivo* relevance of VCCN2 is less clear as no mutant phenotype has emerged. Another Cl^- channel called CLCe was found in the thylakoid membrane (Marmagne *et al.*, 2007). However, similar to *vccn2* plants, *clce* loss-of-function mutants did not exhibit clear changes in NPQ and pmf dynamics, respectively (Dukic *et al.*, 2019; Li *et al.*, 2021).

To comprehend the links between light changes, the role of thylakoid counter ion flux, and photosynthesis, a computational model was established (Davis *et al.*, 2017). By now, this model has been tested and refined through the input of experimental spectroscopic data collected from Arabidopsis WT and ion transport mutants (Li *et al.*, 2021; Lyu & Lazár, 2023). The data obtained from respective single and higher order loss-of-function mutants have established VCCN1 and KEA3 as independently regulated modulators of the pmf, and with that electron transport and energy dissipation in chloroplasts (Davis *et al.*, 2017; Li *et al.*, 2021).

XI. Short- and long-term light acclimation effects on KEA3 and VCCN1

The relationship between short- and long-term light acclimation and VCCN1 and KEA3, respectively, were characterized to untangle their roles under natural conditions. Short-term light stress of 6 h did not trigger a transcriptional response of *VCCN1*, *KEA3*, and other plastid ion transport genes (Gollan *et al.*, 2023). By contrast, a long-term study revealed a strong effect of VCCN1 on NPQ induction in plants acclimated to low growth light and a weak effect in those acclimated to high growth light. The opposite effect was found for KEA3 (von Bismarck *et al.*, 2023). An explanation for the different requirements of respective thylakoid

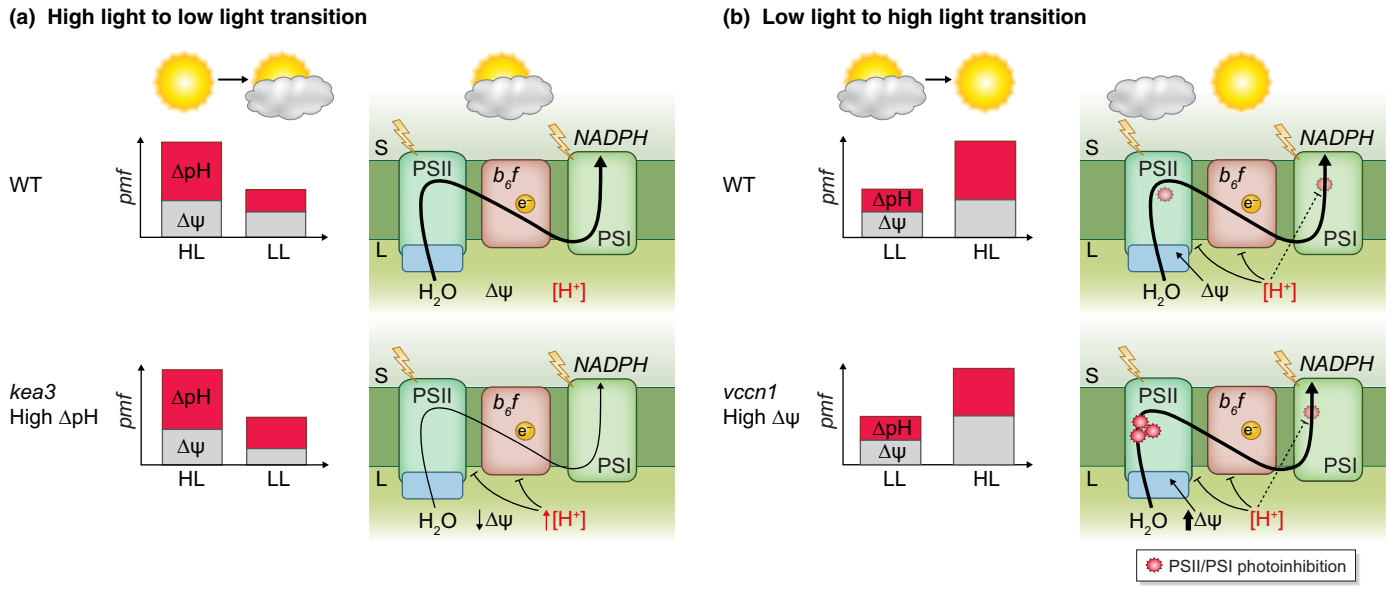

Fig. 5 Dynamics of the proton motive force (pmf) and its contributors ΔpH and ΔΨ across the thylakoid membrane. (a) High light to low light transition. In the wild-type (WT) relative pmf contribution is shifted toward ΔΨ for rapid shutdown of photoprotection. In the absence of the thylakoid K^+/H^+ exchanger KEA3, this conversion is hampered and photoprotection remains high. (b) Low light to high light transition. In WT plants, the relative contribution of ΔΨ is decreased to allow for rapid activation of photoprotection preventing photosystem II (PSII)/photosystem I (PSI) damage by photoinhibition. Plant mutants defective in the Cl-channel VCCN1 cannot activate photoprotection in time. High level of ΔΨ result in PSII damage.

ion transport types is that overall photosynthetic capacity increases during light acclimation with growth light intensities. Measurements of a KEA3-dependent effect on thylakoid $\Delta\Psi$ as well as the activity of the chloroplast ATP synthase suggest that both proteins become almost simultaneously activated after transition into higher light intensities. Synchronization of both proteins might be realized via partially overlapping regulation through nucleotide pools, that is ATP/ADP for the ATP synthase and ATP/ADP and NADPH/$NADP^+$ for KEA3. Thus, KEA3 activity in high light may be directly regulated in response to photosynthetic capacity.

XII. Regulatory mechanisms of thylakoid transport proteins KEA3 and VCCN1

The regulation of KEA3 occurs on the stromal side via a so-called K^+ transport and NAD-binding (KTN) domain in the C terminus (Wang *et al.*, 2017). The domain has a binding pocket for ATP and ADP, but also binds NADP(H) *in vitro* at an independent, so far unknown binding site. Over the last years, a sophisticated mechanism has been unveiled (Uflewski *et al.*, 2022). KEA3's C terminus senses the chloroplast energy state by monitoring the stromal phosphorylation potential in a pH-dependent manner in addition to the redox potential. The synergistic interplay allows for precise flux regulation via KEA3 in tight coordination with photosynthetic activity. After transition from low to high light, increased stromal pH, elevated ATP, and a high NADPH/$NADP^+$ ratio inactivate KEA3. By contrast, KEA3 is activated upon high to low light transition. This coincides with decreased stromal pH, possibly ATP and NADPH/$NADP^+$.

Less is known about regulatory mechanisms governing VCCN1 activity. Some insights have emerged from the protein structure of VCCN1's homolog from apple (Hagino *et al.*, 2022). VCCN1's regulation takes place in an N-terminal region where GgBestrophin1, an animal homolog from chicken, carries a Ca^{2+} clasp. Importantly, plant VCCNs lack this Ca^{2+}-binding motif (Duan *et al.*, 2016; Herdean *et al.*, 2016). Initially, a voltage-gated regulation was suggested. However, electrophysiological studies found only low voltage-dependency of VCCN1 and thus $\Delta\Psi$ seems an unlikely activator (Hagino *et al.*, 2022). A secondary signal on the stromal side derived from the light-induced pmf generation may be the unknown actor. Whatever the signal is, experimental evidence on truncated VCCN1 variants strongly suggests that the regulatory element resides in the stromal N-terminal extension of VCCN1. Thus, research should focus first on resolving the regulatory mechanism at this site.

XIII. A similarity-based approach to identify new direction to research plastid ion flux

Greater understanding of chloroplast ion transporters has primarily come from work in Arabidopsis. Often, these findings are used to infer ion transport in other photosynthetic organisms, but experimental evidence is sparce for ion movements, transporter presence/absence, and localization. A comparison of known chloroplast ion transport proteins from *A. thaliana* across photosynthetic lineages illustrates the challenge in presuming that work from the model plant is directly applicable to other organisms (Fig. 6).

Homologs of the known chloroplast transport proteins ClCe, VCCN1/2, DLDG, and PEC are found in some cyanobacteria, but the majority of known Arabidopsis chloroplast ion transporters lack homologs in cyanobacteria. For instance, the basal cyanobacterium *Gloeobacter kilaueensis*, which belongs to a clade that branches sister to all other cyanobacteria and possesses no internal membranes, only has distant PEC1 and MGR8/9 homologs. These findings suggest that chloroplast ion transport proteins have largely evolved after the initial primary endosymbiotic event and have been coopted from host genes – or that the signal/xenologs have been lost from cyanobacteria (or are not captured by the analyzed diversity). The former idea is substantiated by the canonical plant plastid ion transport genes, which emerged in the nuclear genome of the glaucophyte alga *Cyanophora paradoxa*.

Most of the known Arabidopsis plastid ion transport protein homologs can be identified in genomes throughout the eukaryotic phototrophs, with KEA3, NHD1, VCCN1, and ClCe being highly conserved. Indeed, all four members are present in Glaucophytes as evidence from the *C. paradoxa* genome suggests. On the other end of the evolutionary spectrum, that is in the Stramenopile *Phaeodactylum tricornutum*, the importance of KEA3 in regulating NPQ and pmf partitioning, akin to Arabidopsis, was documented in loss-of-function mutants (Seydoux *et al.*, 2022). Together, this indicates a general importance of Cl^- and K^+ flux in the regulation of the thylakoid pmf and osmotic potential in phototrophic eukaryotes' chloroplasts. One exception is Norway spruce (*Picea abies*), which barely contains chloroplast ion transport homologs known from Arabidopsis. Therefore, ion transport in conifer chloroplasts may differ from other green lineage organisms. Gymnosperms generally lack a plastid NDH complex (Braukmann *et al.*, 2009). The degree of PGR5-dependent cyclic electron flow (CEF) varies greatly among species (Yang *et al.*, 2020). The relationship between CEF and thylakoid ion flux is a highly interesting subject for future studies.

Generally speaking, it appears that ion transport proteins identified in Arabidopsis thylakoid membranes are more conserved throughout photoautotrophic eukaryotes than carriers in the envelope membranes. This may reflect the importance of thylakoid ion flux for pmf dynamic fine-tuning and electron transfer protein assembly. It further highlights the need to investigate the only exception, that is gymnosperms. Curiously, NHD1 is also present in the earliest branching eukaryotes along with KEA3, and consistently appears with other confirmed thylakoid ion transport proteins in land plants (KEA3, VCCN1/2, and ClCe), potentially providing additional evidence for a role of NHD1 in thylakoid membranes.

Lastly, a few more observations are noteworthy: First, envelope proteins KEA1/2, PEC1/2, and MGT10 are strongly conserved in all green algae but absent from red algae. Second, MSL2/3, the plastid MSL-type, show a similar distribution but exist in many red algae. Third, cMCU is neither conserved among green or red algae nor in plants pointing to a highly specific function in Arabidopsis guard cell plastids, which may deserve more research.

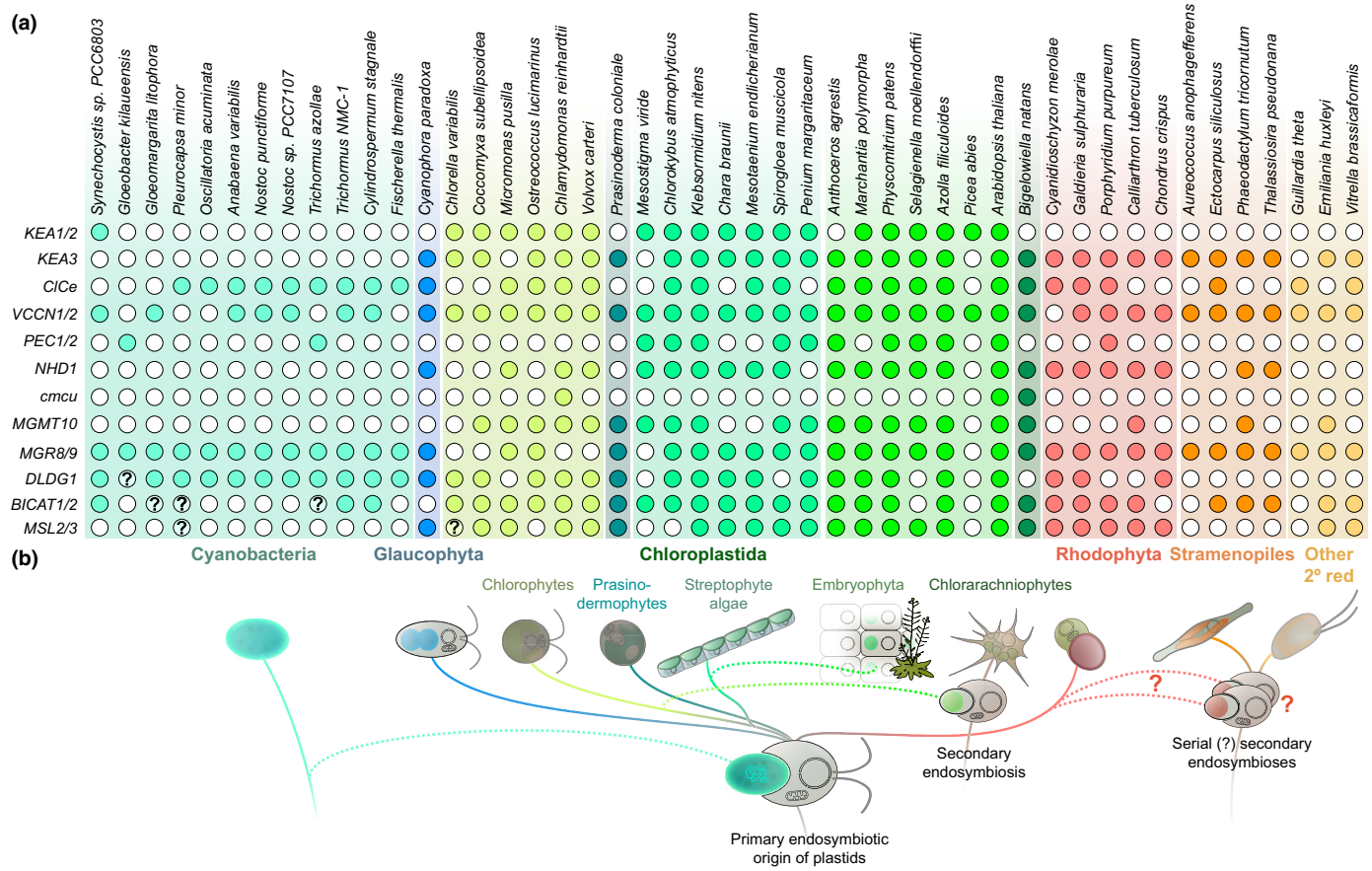

Fig. 6 Similarity of chloroplast ion transport proteins across selected phototrophic species. (a) Distribution of chloroplast ion transport proteins characterized in the model plant Arabidopsis across photosynthetic eukaryotes. Homologous proteins were identified in the protein data of genome-sequenced organisms by sequence similarity searches (BLASTP). For this, sequences yielding reciprocal hits that passed an *e* value cutoff of 10^{-7} were manually investigated. In the case of eukaryotes, the search was limited to nuclear genomes. A core set of proteins of the thylakoid membrane are found across all phototrophic eukaryotes. Strikingly, cyanobacteria show little overlap with the canonical plastid ion transportome. (b) Evolutionary context between the organisms used here. Dashed lines indicate likely endosymbiotic events. (?) denotes an ambiguous result.

XIV. Outlook and recommendations to the field

With the discovery of first loci encoding for plastid ion transport proteins about a decade ago, our view on the organelle's ion transportome has improved substantially. Nevertheless, several aspects in our understanding remain preliminary. Significant obstacles prevent scientists from utilizing the wealth of carriers and channels to engineer plants, algae or cyanobacteria with improved photosynthesis. Here, we discuss four directions to advance the field in the next decade (Fig. 7).

1. The need for a chloroplast ionome and chloroplast-specific genomic tools

Elemental analyses coupled to genetics has shaped our understanding of the plant ionome, that is the sum of metal ions found in different plant tissues under a given growth condition (Baxter, 2010). This approach enabled the discoveries of many ion transport proteins and their regulatory components (Whitt *et al.*, 2020). Interestingly, the same strategy has barely produced candidate genes encoding plastid proteins. Two possible explanations are as follows: (1) imbalances in the plastid ion homeostasis do not affect total tissue element levels to a discernible degree and (2) functional genetic redundancy, that is a lack of observable phenotypes in single loss-of-function mutants, as reported for KEA1/2, PEC1/2, and MGR8/9, is common among plastid proteins.

A more in-depth understanding of plastid ion homeostasis should be grounded in an accurate numerical framework of the total elemental makeup of leaf plastids. About 40 yr ago, a handful of studies were carried out to determine the elemental composition of chloroplasts isolated from pea, spinach, etc. (Demmig & Gimmler, 1983; Robinson & Downton, 1984). Scientists keep referencing the original data because metal ion contents of plastids from *A. thaliana* are rarely reported. This prevents targeted searches for proteins involved in establishing chloroplast ion homeostasis through elemental analysis of plant mutants. Also, photosynthesis models would benefit from elemental information. Hence, an Arabidopsis plastid ionome under standard physiological conditions and its contributing proteins needs to be

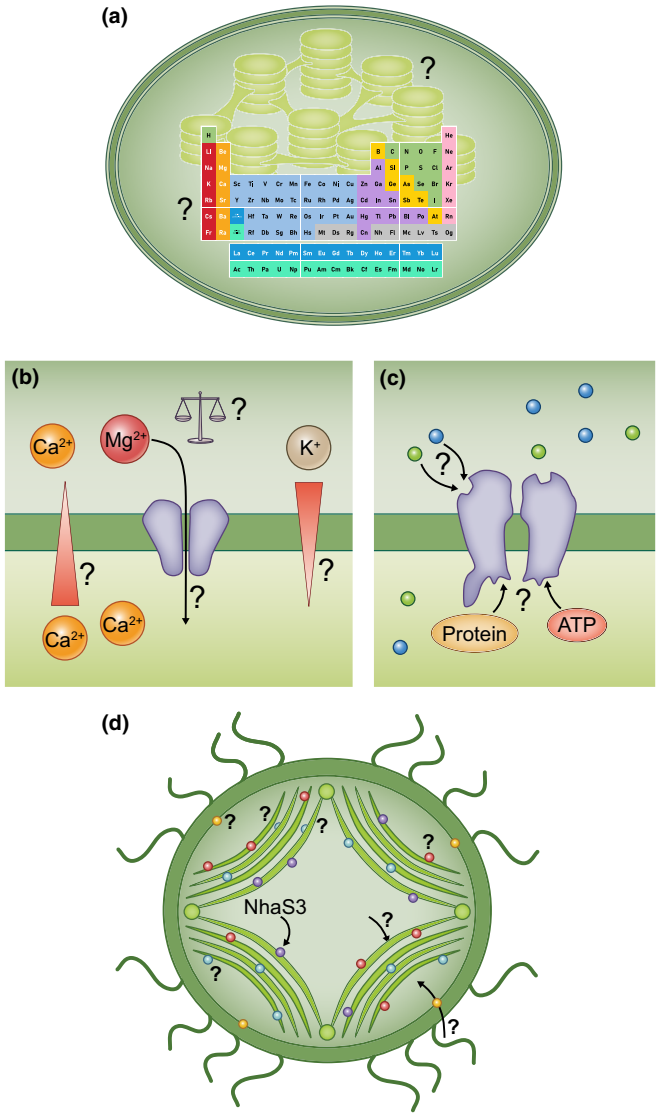

Fig. 7 Four research areas to advance understanding of plastid ion transport. (a) Build a numeric understanding of the plastid ionome and plastid-specific genomic tools. (b) Improve knowledge on transport protein substrates and protein structure. (c) Understand ion transport protein regulation. (d) Investigate ion transport in cyanobacteria with special emphasis on thylakoid flux and spectroscopy method design. Question marks (?) indicate open reseach questions detailed in the main text in the section XIV.

established in the next decade. Many mutants deficient in plastid ion carriers are pale or exhibit developmental defects. Therefore, different means of normalizing element data, for example chlorophyll content, plastid number, or volumes, should be tested. *In situ* WT and mutant plastid volumes can be rapidly quantified by confocal microscopy (Knoblauch *et al.*, 2024). It is unclear whether these attempts can deliver robust results across laboratories but it should be tried nevertheless.

Electrophysiology studies suggest that additional plastid transport proteins such as a thylakoid K^+ channel must exist (Schönknecht *et al.*, 1988; Tester & Blatt, 1989; Pottosin & Schönknecht, 1996). To identify new plastid ion flux mutants for instance by spectroscopy-based phenotyping, functional genetic redundancy needs be overcome. Genome-wide multi-target artificial micro-RNA and CRISPR libraries developed over the last years have the potential to achieve this (Hauser *et al.*, 2013; Hu *et al.*, 2023). Current libraries lack subcellular focus, which can cause pleiotropic phenotypes with limited information. This can be resolved through libraries, which target exclusively the plastid or other organellar proteomes of interest.

2. *In vitro* and *in vivo* substrate spectra of plastid ion transporters

To better utilize a given transport protein detailed mechanistic insights are critical. Here, the field has to innovate. Most assumptions on KEA proteins are based on *in vitro* transport experiments pioneered by the Venema group employing a KEA2 fragment without its soluble N terminus (Aranda-Sicilia *et al.*, 2012). Using a pH dye, Aranda-Sicilia and colleagues were able to show that KEA2 exchanges K^+ with H^+ in an electroneutral manner. Later, K^+ flux via all six Arabidopsis KEAs was inferred from rescue experiments of transport-deficient *E. coli* mutant strains (Tsujii *et al.*, 2019). H^+-dependent antiport in everted membrane vesicles was not detected. Tsujii *et al.* speculated that limitations in their assay are responsible for this discrepancy. We conclude that *in vitro* transport assays, which rely on pH dyes, remain limited in their quantitative information, that is substrate affinities and detailed kinetic parameters. Recently, electroneutral ion exchange was successfully assayed on a purified mammalian Na^+/H^+ exchanger and on KefC, a KEA1 homolog from *E. coli*, using solid-supported membrane-based electrophysiology (Gulati *et al.*, 2023; Matsuoka *et al.*, 2023). Solid-supported membrane may provide an avenue for detailed substrate characterization of KEAs and other plastid ion transport proteins and thus should be tried more frequently. In the case of electrogenic ion transporters and channels, patch-clamp measurements of plastid membranes as pioneered by Schönknecht, Pottosin, and colleagues could be reestablished and attempted on Arabidopsis for WT–mutant comparisons (Pottosin & Schönknecht, 1996; Pottosin & Dobrovinskaya, 2015).

In vivo, x-ray microscopy provides spatial information on metal accumulation. Although improvements have been made, not all lab-based systems offer sufficient resolution for subcellular studies (Fittschen *et al.*, 2017; Duncan *et al.*, 2022). The highest resolution still requires work at synchrotron facilities (Kopittke *et al.*, 2018). In general, these measurements rather provide static information on elemental distributions. Flux studies *in planta* have historically relied on radioisotopes such as $^{86}Rb^+$ (as a proxy for K^+) and $^{22}Na^+$. High safety requirements have limited their use mostly to root uptake and make it difficult to adapt these for organellar transport studies. Since physiological Rb^+ levels in plants are generally very low, cold Rb^+ feeding and subsequent elemental analysis may provide an interesting alternative if it can be realized on isolated organelles (DeTar *et al.*, 2022). A potential game-changer are genetically encoded ion sensors. Starting with Ca^{2+} substrates, the development has advanced tremendously. By now, K^+, Mg^{2+}, Cl^-, and several other sensors have been successfully tested in various organisms (Sadoine *et al.*, 2023). Sensors functional within the

right concentration range can be titrated and expressed in WT and mutant plant cells. Here, they can be targeted to the plastid stroma and the thylakoid lumen (Sello *et al.*, 2018). Hence, the imaging of genetically encoded ion sensors will become an increasingly relevant orthogonal avenue to elucidate plastid transport proteins and their significance directly *in planta*.

3. Structural insights into plastid ion transporters

Interestingly, as exemplified by the VCCN1/bestrophin-like channels, transport substrates can vary among homologs from different species. While the plant proteins were characterized as Cl^- channels (Herdean *et al.*, 2016; Hagino *et al.*, 2022), homologs from Chlamydomonas seem to transport bicarbonate (HCO_3^-) into the CO_2 concentrating mechanism (Mukherjee *et al.*, 2019). Given the importance of plant VCCN1 proteins in dissipating $\Delta\Psi$ to allow for timely NPQ induction, the discovery from Chlamydomonas suggests limited transferability between species. In the meantime, Cryo-EM has yielded structural information on VCCNs from plant and algae, respectively (Hagino *et al.*, 2022; Rozenberg *et al.*, 2022). Both types form pentameric complexes *in vivo*. Undoubtably, these data are highly valuable for protein engineering without specificity loss. Cryo-EM and structural predictions, for example using Alpha-fold (Jumper *et al.*, 2021), will have a strong impact on the field and may correct or confirm hypothesized transport substrates. Our survey of oligomeric states using publicly available data from *A. thaliana* (Table 1) indicates that many ion transport proteins exist as large homo- or heteromultimers *in vivo* (Schröder *et al.*, 2022). These complexes might be dynamic with regulatory implications adding another layer of complexity. This showcases that our structural understanding of many proteins of interests is in its infancy.

4. Regulation of plastid ion transport proteins

Besides controlling transport substrates, manipulating protein regulation provides another interesting path to improve plant productivity. Recent studies on KEA3 and VCCN1 yielded valuable insights. Far less is known about the regulation of the other plastid ion transport proteins discussed in this review, emphasizing the need for more research activity in this area.

KEA3 and IE KEA members are highly conserved in their transport domain and their stromal C terminus including the KTN motif. Therefore, KEA1 and KEA2 activity is expected to depend in part on similar factors as shown for KEA3, that is ATP/ADP, NADP(H) ratios, and stromal pH. However, it is unknown whether the prevailing effector levels near the IE equal those on the stromal side of the thylakoid membrane. Simultaneous activation of all plastid KEAs should not be desirable. Interestingly, all known IE KEAs carry a roughly 500 amino acid long N-terminal loop, which resides in the stroma and does not exist on thylakoid KEA members (Bölter *et al.*, 2020). The full-length version of KEA2 was inactive in liposome assays. An auto-inhibitory role for the N terminus was hypothesized (Aranda-Sicilia *et al.*, 2012). The N-terminal loop contains a coiled-coiled domain, which often acts as rigid molecular placeholder or scaffold in a broad variety of processes. Among these are interactions with the cytoskeleton, vesicle tethering, sequence-specific DNA/RNA binding, and chromosome separation (Truebestein & Leonard, 2016; Ford & Fioriti, 2020). In addition to the space-holder function, coiled-coil domains also aid in oligomerization of proteins. Indeed, IE KEAs and other ion carriers were found in high-molecular-weight fractions (Table 1). To understand the regulation of the IE exchangers, it is necessary to dissect the content of these complexes. Potential protein–protein interaction partners need to be considered, too. Proximity labeling employing stromal-targeted TurboID (TID)-fusions has now produced promising results in Arabidopsis (Wurzinger *et al.*, 2022). The method may hold the key to identify regulatory interactors of the known plastid ion transport proteins.

5. Ion flux in cyanobacteria

As the energetics of PSII remain similar between plants, algae, and cyanobacteria, the susceptibility of PSII to $\Delta\Psi$-mediated photodamage is expected to be a continuous challenge that is mitigated in part by thylakoid ion flux. Being the progenitors of chloroplasts, it could be expected that many of the ion transport proteins discovered in Arabidopsis arose from cyanobacteria. Contrarily, our analysis highlights a vast lack of (known) Arabidopsis chloroplast ion transport protein homologs in diverse cyanobacterial lineages (Fig. 6). This suggests that currently unknown ion transport proteins are to be discovered in cyanobacteria which regulate $\Delta\Psi$ at both the plasma and thylakoid membranes.

Ion flux measurements in cyanobacteria have so far relied on bulk element measurements, that is cytosol and thylakoid lumen content is represented as one batch relative to the growth medium. These experiments suggest that K^+ and Cl^- are actively accumulated by cyanobacteria (Padan & Vitterbo, 1988; Ritchie, 1991, 1992). Conversely, Na^+ is excluded from cells, possibly as part of light-dependent Na^+-coupled HCO_3^- import (Miller *et al.*, 1984; Kaplan *et al.*, 1989; Mangan *et al.*, 2016). Ca^{2+} transport and flux are less characterized in cyanobacteria, although the accumulation is likely to be species-dependent (De Wever *et al.*, 2019). In heterocyst forming cyanobacteria, Ca^{2+} fluxes have been measured in response to stress (Torrecilla *et al.*, 2000) as well as light/dark transitions (Torrecilla *et al.*, 2004).

The ion distribution and flux between the cytosol and thylakoid lumen is unknown. The proteins and ions involved in modulating thylakoid $\Delta\Psi$ will be important for understanding their biophysical importance in photosynthesis, as well as what, if any, changes have occurred throughout evolution to modulate the transthylakoid ion composition. Ion transport proteins identified in cyanobacteria may prove useful in plant plastids to specifically modulate ion responses.

6. Thylakoid ion transport in cyanobacteria

A similar role for K^+ in regulating thylakoid pmf in cyanobacteria is anticipated based on its important role in plants. A putative thylakoid K^+ transporter (SynK, *slr0498*) was identified in the model cyanobacterium *Synechocystis* PCC 6803 (Zanetti *et al.*, 2010). Although an explicit involvement in thylakoid K^+ transport

has not been shown, a SynK loss-of-function mutant displayed a light-sensitive phenotype and faster b_6f turnover kinetics than WT. SynK was proposed to be dually located to the plasma and thylakoid membranes, but subsequent subcellular proteomics failed to confirm this (Liberton *et al.*, 2016; Baers *et al.*, 2019; Wang *et al.*, 2022). SynK homologs in Arabidopsis from the tandem-pore potassium cation channel family (TPK) do not reside in chloroplasts but the plasma membrane and the tonoplast (Höhner *et al.*, 2019).

Because growth of cyanobacteria relies on light-dependent Na^+ uptake (Miller *et al.*, 1984; Kaplan *et al.*, 1989), it seems possible that Na^+ may fulfill partly the role of K^+ flux across the thylakoid membrane. Indeed, NhaS1 (*slr1727*) and NhaS3 (*sll0689*) (Mills *et al.*, 2020) from *Synechocosystis* PCC 6803 were localized to the thylakoid and showed Na^+/H^+ antiport activity in a Na^+/H^+ antiport deficient *E. coli* mutant. While total loss of NhaS3 is lethal, viable *nhaS3* knockdown mutants exhibited decreased growth under all conditions tested. *NhaS3* expression is controlled by CO_2 levels and circadian rhythms (Tsunekawa *et al.*, 2009), potentially hinting at a link to photosynthesis. Hence, NhaS3 is a good starting point to gain insights into the role of thylakoid ion flux and its role in photosynthesis.

In phototrophic eukaryotes, thylakoid $\Delta\Psi$ dynamics can be studied non-invasively via ECS (Cruz *et al.*, 2005a; Bailleul *et al.*, 2010). The existence, size, and dynamics of $\Delta\Psi$ or ΔpH in the pmf of cyanobacteria has never been shown (Belkin *et al.*, 1987; Hinterstoisser & Peschek, 1987). A hurdle in identifying *bona fide* cyanobacterial thylakoid ion transport proteins linked to pmf dynamics is the difficulty to differentiate plasma membrane vs thylakoid membrane potentials. Recently, Viola *et al.* (2019) reported an ECS signal that is absorption changes at 500–505 and 480–485 nm, in the model cyanobacteria *Synechocystis* PCC 6803 and *Synechococcus elongatus* PCC 7942. The ECS signal has yet to be characterized for steady-state parameters of cyanobacterial pmf. It is unknown whether other cyanobacteria also possess usable ECS signals. Methods to study thylakoid pmf in eukaryotes mainly measure ECS changes during a brief dark interval (Dark Interval Relaxation Kinetics or DIRK). The near total light-dependent nature of photosynthetic electron transfer allows pmf dynamics during these brief dark intervals to be studied (Cruz *et al.*, 2001). The co-localization of light-dependent and light-independent respiratory electron transfer proteins within the thylakoid of cyanobacteria requires demonstrations of the applicability of DIRK for cyanobacterial pmf measurements. Development of these signals to understand physiological changes in pmf, combined with ion transport mutant studies will accelerate our understanding of thylakoid ion transport in cyanobacteria.

XV. Conclusion

The discovery of KEA transporters in plant chloroplasts 10 yr ago initiated an influential research phase on plastid ion transport. Knowledge on thylakoid proteins KEA3 and VCCN1 has provided groundbreaking information on the molecular mechanisms that govern pmf regulation. Both proteins represent targets to improve photosynthetic efficiency in plants. At the IE membrane, several ion transport proteins show striking importance for chloroplast development. Their function is critical for the organellar ion homeostasis, which is instrumental for PGE since several components such as ribosomes, polymerases, and RNA folding rely on metal ion cofactors. Thus, through the careful phenotypic study of mutants with defective IE ion transport proteins, we will continue to learn what defines the stromal ion homeostasis and enables PGE. Several genes encoding for expected ion transport mechanisms, such as a Cl^- importer or a thylakoid K^+ channel, remain unknown. Thus, we need to overcome the technical hurdles and advance the field. This must include an understanding of thylakoid ion flux in cyanobacteria a critical clade for global CO_2 fixation.

More than a 100 yr after Tansley instructed students to study the effects of nutrient ion starvation on plant function, the research, now including the subcellular scale, remains up to date. Innovative genetic and analytical tools allow us to track nutrient ion flux on the sub micrometer scale. More importantly, we are able to utilize these results for a new understanding of photosynthesis. It is on us and the next generation of scientists to capitalize on this opportunity.

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Competing interests

None declared.

Author contributions

H-HK wrote the majority of the manuscript. GAD wrote sections focusing on cyanobacteria. UA wrote sections focusing on KEA3 regulation. JV performed similarity-based comparisons of ion transport proteins. SM designed all original figures. All authors helped in the editing of the article.

ORCID

Ute Armbruster https://orcid.org/0000-0002-8814-8207
Geoffry A. Davis https://orcid.org/0000-0002-6964-9196
Hans-Henning Kunz https://orcid.org/0000-0001-8000-0817
Susanne Mühlbauer https://orcid.org/0009-0004-8706-306X
Jan de Vries https://orcid.org/0000-0003-3507-5195

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