A voltage-dependent chloride channel fine-tunes photosynthesis in plants

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In natural habitats, plants frequently experience rapid changes in the intensity of sunlight. To cope with these changes and maximize growth, plants adjust photosynthetic light utilization in electron transport and photoprotective mechanisms. This involves a proton motive force (PMF) across the thylakoid membrane, postulated to be affected by unknown anion (Cl−/CO32−) channels. Here we report that a bestrophin-like protein from Arabidopsis thaliana functions as a voltage-dependent Cl− channel in electrophysiological experiments. AtVCCN1 localizes to the thylakoid membrane, and fine-tunes PMF by anion influx into the lumen during illumination, adjusting electron transport and the photoprotective mechanisms. The activity of AtVCCN1 accelerates the activation of photoprotective mechanisms on sudden shifts to high light. Our results reveal that AtVCCN1, a member of a conserved anion channel family, acts as an early component in the rapid adjustment of photosynthesis in variable light environments.
Chloroplasts have essential roles in harvesting and converting energy from the sun into carbohydrates, which are then used in cell metabolism. The protein machineries in the two aqueous compartments of this organelle (stroma and thylakoid lumen) are fine-tuned to the demands of the cell by changes in ion balance1. Photosynthetic electron transport in thylakoid membranes and the architecture of these membranes are highly sensitive to the concentration of ions (H⁺, K⁺, Mg²⁺ and Cl⁻) in the stroma and thylakoid lumen1,2. In natural habitats, plants experience variable light conditions, for example, shifts in light intensity and quality within seconds to minutes due to clouds, canopy architecture and leaf movement due to wind. To adjust to variable light, rapid changes in ion balance of the chloroplast occur through the regulation of ion transport3.

Ion channel activities across chloroplast envelopes and thylakoid membranes have been demonstrated, and are postulated to play critical roles in chloroplast physiology4–7. Light-induced charge separation and coupled H⁺ uptake into the thylakoid lumen generate a proton motive force (PMF), composed of the transmembrane electric-potential gradient (ΔΨ) and H⁺ concentration gradient (ΔpH). Both PMF components activate and drive ATP synthesis by chloroplast F₀F₁ ATP synthase. A high H⁺ concentration in the thylakoid lumen downregulates electron transport at the level of the cytochrome b₅/photoprotection through the dissipation of excess light as heat8. PMF partitioning into ΔΨ and ΔpH can vary with changes in the light environment, and is proposed to be a fine-tuning mechanism for photosynthesis9. More specifically, the plant stores PMF predominantly as ΔΨ in conditions of sudden increases in light intensity, which would otherwise result in damage to the photosynthetic machinery and reduction in growth10. Conversely, the plant reduces the fraction of PMF stored as ΔpH after transitions to low light intensities to downregulate NPQ and maximize photosynthesis and growth. The mechanism by which PMF is partitioned and fine-tuned to achieve rapid photosynthetic acclimation is poorly understood. A critical factor is thought to be the ionic composition of the stroma and thylakoid lumen. The reasoning is that fast movements of counterions (Cl⁻ influx, Mg²⁺ and K⁺ efflux) electrically balance H⁺ uptake into the thylakoid lumen4. Hence, these ion fluxes would rapidly adjust PMF partitioning by efficient regulation of ΔΨ.

Our recent study in Arabidopsis thaliana11 revealed that a thylakoid K⁺ channel (AtTPK3) is involved in the modulation of PMF partitioning into ΔΨ and ΔpH through K⁺ efflux from the lumen, and in this way controls utilization of light in photosynthesis. More recently, two laboratories independently localized a K⁺/H⁺ exchanger (AtKEA3) to thylakoids and showed that it modulated PMF partitioning through H⁺ efflux from the lumen, which is critical for photosynthetic acclimation after transitions from high to low light12,13. In comparison with K⁺ transport, knowledge about the Cl⁻ fluxes that counterbalance H⁺ pumping in thylakoids is still limited, even though this is necessary to fully understand the mechanism of PMF partitioning by ions. Activities of voltage-dependent Cl⁻ channels have been reported in plant and algal thylakoid membranes6,7,13. However, they have not been conclusively attributed to any specific proteins. A member of the Cl⁻ channel family CIC in Arabidopsis, named AtClCe, was localized to the thylakoid membrane14,15. This protein was initially proposed to be involved in photosynthesis16, and later in NO₃⁻ assimilation pathways17. Whether ClCe works as a Cl⁻ channel remains to be demonstrated. In this work, we describe a voltage-dependent Cl⁻ channel located in Arabidopsis thylakoids (AtVCCN1), where it functions to fine-tune PMF and allows the plant to adjust photosynthesis to variable light.

Results
AtVCCNs are thylakoid members of a conserved channel family. A T-DNA insertion mutant of AtVCCN1 gene locus (At3g61320) was annotated to have altered NPQ by the Chloroplast Phenomics 2010 project (www.plastid.msu.edu)18, which identified previously uncharacterized regulatory photosynthetic proteins19,20. This gene encodes a chloroplast-predicted membrane protein, which was annotated as ‘bestrophin-like’ in public databases. Bestrophins function as Ca²⁺-activated monovalent anion channels in mammalian membranes21.

Data extracted at ARAMEMNON database (http://aramemnon.botanik.uni-koeln.de)22 indicated that the amino-acid sequence of AtVCCN1 comprises a predicted chloroplast-targeting peptide (residues 1–40), 4–5 putative transmembrane domains and termini of 60–70 residues (Supplementary Fig. 1). Using phylogenetic analyses, we found that the protein sequence was highly conserved in land plants, and that it had homologues in cyanobacteria, red and green algae, diatoms and haplophytes (Supplementary Fig. 2, Supplementary Data 1). The fact that we found homologues at non-photosynthetic bacteria and eukaryotes (fungi and arthropods) suggests a widespread importance of the biochemical function of VCCNs. No mammalian bestrophins were found in our searches, and alignment to human BEST1 revealed only 9% identity. Three residues important for anion selectivity in bestrophins21 were conserved, but four out of five acidic residues involved in Ca²⁺ binding were missing in AtVCCN1 (Supplementary Fig. 1). This casts doubt on the previous annotation as a ‘bestrophin-like protein’, and instead suggests a family of uncharacterized anion channels. Similar to other thylakoid transport proteins such as ClCe23, an origin of this family from the host or symbiont in the endosymbiotic event in the evolution of chloroplasts could not be discerned in our analyses.

A close parologue of AtVCCN1 is the protein encoded by the A12g58760 gene, which we named AtVCCN2. Their amino-acid sequences are highly similar (76% identity; 86% similarity) and diverged during the early evolution of the Brassicaceae (c. 24–40 million years ago), but they are part of distinct clades (Supplementary Fig. 3a). AtVCCN2 also has a predicted chloroplast-targeting peptide, but we found that its transcripts were more abundant in flowers than in leaves, in contrast to AtVCCN1 transcripts that were highly abundant both in leaves and flowers (Supplementary Fig. 3b,c), which was in agreement with Genevestigator database (https://genevestigator.com)24.

For intracellular localization in Arabidopsis, we stably introduced green fluorescent protein (GFP) fusions of AtVCCN1 and AtVCCN2 into corresponding knockout mutants (vccn1-1 and vccn2-1, respectively) and overexpressed them under the control of CaMV 35S constitutive promoter. In leaf protoplasts isolated from transformed plants, the GFP fluorescence signal of both fusions colocalized with chlorophyll fluorescence of chloroplasts (Fig. 1a), thereby confirming the predicted chloroplast location. Immunoblotting of chloroplast subfractions isolated from leaves of transformed plants revealed a location for both AtVCCNs in the thylakoid membrane, more precisely in the stroma-exposed regions (Fig. 1b,c, Supplementary Fig. 4a,b). Due to the lack of specific antibodies against the two proteins, the expression level of AtVCCN1 and AtVCCN2 proteins in non-transformed wild-type (wt) plants could not be assessed. However, on the basis of the tissue expression pattern, it is likely that AtVCCN1 is the predominant form in thylakoids from wt leaves.

Electrophysiology of AtVCCN1 in planar lipid bilayers. Previous electrophysiological studies on thylakoid membranes have revealed the activity of two distinct anion channels, one with
a conductance of 110 pS in 110 mM KCl\(^+\) and another one with a conductance of 220 pS in 100 mM KCl\(^+\). We decided to study the biophysical function of AtVCCN1 in a recombinant form expressed in an \textit{in vitro} transcription/translation system previously used for the study of various ion channels\(^{25–27}\) (Supplementary Fig. 5). When inserted into a planar lipid bilayer, the AtVCCN1 protein reproducibly displayed a channel activity in Cl\(^-\) and NO\(_3^\) media (Fig. 2a, Supplementary Figs 6,7d,8a–c,9a). The channel was selective for Cl\(^-\) over K\(^+\) displaying a permeability ratio of \(P_{Cl^-}/P_{K^+} = 1.017 \pm 0.03\) (\(\pm\) indicates s.d., \(n = 3\)) as determined from the reversal potential under asymmetric ionic conditions (300 mM/100 mM KCl; Supplementary Fig. 9), and calculated according to the Goldman–Hodgkin–Katz equation\(^{28}\). The single-channel conductance of AtVCCN1 in 100 mM KCl (96.1 \(\pm\) 5.0 pS and 60.0 \(\pm\) 8.2 pS at positive and negative voltages, respectively, where \(\pm\) indicates s.d., \(n = 5\)) was higher than that in 100 mM KNO\(_3\) (28.7 \(\pm\) 12.2 pS at both voltage ranges, where \(\pm\) indicates s.d., \(n = 3\)). The channel was selective for Cl\(^-\) over NO\(_3^\) (Fig. 2a, Supplementary Figs 7c,8d), indicating a preference for Cl\(^-\) over NO\(_3^\). The channel displayed voltage dependence, being more active at positive than at negative voltages (Fig. 2a, Supplementary Figs 6,9,10). A fast-gating, flickering pattern was observed at positive voltages, whereas burst-like openings were dominant at negative voltages (Fig. 2a, Supplementary Figs 8a,10b). Furthermore, cooperative gating, that is, simultaneous opening of more than one channel and conductance sub-states (Fig. 2a, Supplementary Figs 8b,9) were observed frequently, as were changes in kinetic behaviour, that is, from slow to fast gating (Supplementary Fig. 8c). AtVCCN1 activity was blocked by general anion channel inhibitors dithiocyanatostilbene-2,2'-disulphonic acid (DIDS) and niflumic acid (Fig. 2b, Supplementary Figs 6c,7a,b,8e). Activity was affected by neither the K\(^+\) channel inhibitor tetraethylammonium (Supplementary Fig. 8f) nor by Ca\(^{2+}\) (Supplementary Fig. 7d), in accordance with the absence of Ca\(^{2+}\)-binding motifs in the AtVCCN1 sequence (Supplementary Fig. 1). Taken together, these data demonstrate that AtVCCN1 forms a voltage-dependent, DIDS-sensitive and anion-selective channel that is permeable to Cl\(^-\) and NO\(_3^\). These characteristics are similar to those of the channel recorded directly in the thylakoid membrane of \textit{Peperomia metaltica} by Schönknecht \textit{et al.}\(^7\) and in \textit{Nitellopsis obtusa} by Pottosin and Schönknecht\(^{13}\). There are also differences from the previously reported activity: AtVCCN1 is less permeable to NO\(_3^\) than to Cl\(^-\), is also active at high voltages, and displays a different gating behaviour. The use of different species with respect to the above-mentioned works, different lipid composition and/or lack of possible regulatory subunits, post-translational modification in our system might \textit{a priori} cause such differences. However, it cannot be excluded that AtVCCN1 and the proteins giving rise to the activities thus far characterized are distinct. The conductance and overall characteristics of AtVCCN1 were significantly different from those of another anion channel observed in thylakoids by Wagner and colleagues\(^6\).

PMF and photosynthetic acclimation from dark to light. In the thylakoid membrane, the above-described activity for AtVCCN1 by electrophysiology would allow immediate Cl\(^-\) influx at the onset of illumination in response to an increase in thylakoid membrane voltage, that is, the \(\Delta \Psi\) component of PMF (positive on lumenal side) created by H\(^+\) uptake. Cl\(^-\) influx would in turn contribute to the rapid reduction in \(\Delta \Psi\) resulting in higher \(\Delta \Phi\). Conversely, defects in Cl\(^-\) influx would allow the total PMF to be higher and stored more as \(\Delta \Phi\) at the expense of \(\Delta \Psi\). The altered PMF size and partitioning are expected to perturb H\(^+\) efflux through ATP synthase, electron transport and activation of NPQ, with consequences for the acclimation of the plant after transition from dark to light and in fluctuating light conditions.

To investigate the role of AtVCCN1 activity in these processes in leaves, we characterized two \textit{Arabidopsis} T-DNA insertion knockout lines (\textit{vccn1-1}, \textit{vccn1-2}) and the overexpression line used in the localization of AtVCCN1-GFP (\textit{oeVCCN1}; Supplementary Fig. 11). All genotypes grew like wt in standard conditions and displayed similar levels of photosynthetic proteins and pigments (Supplementary Fig. 12, Supplementary Table 1). To determine the total PMF size, we recorded decay of the total electrochromic shift (ECS\(_t\)) by applying brief (600 ms) dark intervals at specific time points during illumination of intact leaves. PMF size and its partitioning into \(\Delta \Psi\) and \(\Delta \Phi\) were subsequently determined from the dark interval decay kinetics of the ECS signal (Supplementary Fig. 13a,b). On transition from
dark to light, PMF reached maximal values within the first 2 min, decreased by 20% until 5 min and remained stable for the remaining illumination time in wt plants (Fig. 3a). In vccn1-1, PMF size was moderately but significantly elevated, whereas in oeVCCN1 it was strongly reduced as compared with wt. A significantly enhanced capacity to maintain high ΔΨ at the expense of ΔpH was observed in vccn1-1 at both studied time points (Fig. 3b). The residual PMF observed in oeVCCN1 consisted entirely of ΔpH, indicating the behaviour of a ‘ΔΨ uncoupler’ for the overexpressed protein. These data are consistent with our hypothesis described above, that is, that AtVCCN1 is immediately activated on illumination and dissipates part of ΔΨ to maintain a significant ΔpH across the thylakoid membrane. A complementary method using pH-sensitive acridine orange (AO) fluorescence in isolated chloroplasts indicated significantly more acidic lumenal pH in the light in oeVCCN1 than in wt (Supplementary Fig. 14), supporting the higher PMF partitioning to ΔpH in this line. In contrast to the more acidic lumenal pH, the total PMF size was lower than in wt (Fig. 3a). We do not exclude the possibility that PMF size was underestimated in oeVCCN1, for example, because of elevated accumulation of negative charges (Cl−) in the thylakoid lumen. This may have prevented the decay of the ECS signal on turning off the light during ECS measurement, which is required for estimation of PMF. This possibility does not alter the interpretation of our results for the other genotypes where decay of the ECS signal clearly takes place (Supplementary Fig. 13c,d). The observed rate was higher in vccn1-1 than in wt throughout the illumination period (Fig. 3c), and resembled the pattern observed for PMF size rather than PMF partitioning (Fig. 3a,b). This result is in accordance with the knowledge that H+ efflux through the ATP synthase can be driven by both PMF components and is dependent on the total PMF size8. A reduced rate of H+ efflux was observed in oeVCCN1 in the first minute of illumination as compared with wt (Fig. 3c). Later on during illumination, the rate increased over that of wt despite the reduced total PMF size. In control experiments, we incubated the leaves with N,N-dicyclohexylcarbodiimide (DCCD), which binds covalently to an F0-subunit, thus inhibiting H+ efflux through the ATP synthase29. DCCD reduced the rate of H+ efflux in both wt and mutants to similar levels (Supplementary Fig. 15), confirming that H+ efflux through the ATP synthase caused the observed differences between the three genotypes.

Chlorophyll fluorescence measurements were performed to determine the effects of AtVCCN1 on the regulation of photosynthesis. NPQ transiently decreased in vccn1 and increased in oeVCCN1, resulting in an adjustment of the linear electron transport measured as PSII quantum yield (ΦII; Fig. 3d,e, Supplementary Fig. 16). On the basis of these data and the electrophysiological results described above, we suggest that AtVCCN1 is involved in light-triggered voltage-dependent Cl− fluxes in thylakoids, which contribute to the counterbalancing of...
H$^+$ pumping. In this way, AtVCCN1 fine-tunes the PMF and photosynthesis on transition from dark to light.

Although less abundant than AtVCCN1, AtVCCN2 is also expressed in leaves (Supplementary Fig. 3b,c), and may share a location in the chloroplast thylakoid membrane (Fig. 1b,c). To address possible functional redundancy between the two proteins, we characterized vccn2, vccn1vccn2 and oeVCCN2 (Supplementary Figs 11,12 and Supplementary Table 1) and compared them with wt, vccn1 and oeVCCN1. We show that vccn2 displayed a wt-like pattern for the measured parameters, whereas vccn1vccn2 closely resembled vccn1 (Supplementary Figs 17,18). Interestingly, the oeVCCN2 line displayed a wt phenotype in PMF size, partitioning and rate of H$^+$ efflux in the first 2–3 min of illumination, and resembled oeVCCN1 afterwards (Supplementary Fig. 19). Pre-treatment with DCCD reduced the rate of H$^+$ efflux through ATP synthase to similar levels in all six genotypes (Supplementary Fig. 15). These data suggest that AtVCCN2 is not redundant to AtVCCN1 in terms of the regulation of PMF and photosynthesis in leaf thylakoids. However, AtVCCN2 could form a channel based on the sequence similarity (Supplementary Fig. 1). Indeed, overexpressed AtVCCN2 triggered a similar response as overexpressed AtVCCN1 but with different kinetics.

Photosynthetic acclimation from low to high light. To further investigate the role of AtVCCN1 in photosynthetic regulation, we undertook experiments under fluctuating light. As for the dark-to-light transition, vccn1-1 displayed slower NPQ induction, but without significant change in linear electron transport compared with wt (Fig. 4a,b). In oeVCCN1, NPQ was induced faster and decayed slower, resulting in less electron transport than seen in the wt. This pattern of changes in vccn1-1 and oeVCCN1 can be explained by the observation that AtVCCN1 activity favours the build-up of ΔpH (Fig. 3b), and suggests its involvement in fine-tuning photosynthesis on transitions from low to high light. On the basis of the kinetic pattern of the parameters presented in Figs 3a–e and 4a,b we propose a model for the sequence of events where AtVCCN1 plays role in the regulation of photosynthesis (Fig. 4c).

AtVCCN1 influences thylakoid ultrastructure. In addition to charge compensation of light-driven H$^+$ uptake in thylakoids, ion fluxes have been hypothesized to modulate the overall architecture of the thylakoid membrane because the formation of thylakoid stacks (grana) is known to depend on the ionic strength$^{1,2}$. An impaired anionic permeability in vccn1 would result in changes in intra-thylakoid ionic status and would alter thylakoid ultrastructure. Transmission electron microscopic (TEM) analyses revealed significantly longer grana in vccn1-1 than in wt plants in the dark-adapted state (Fig. 5, Supplementary Table 2). In light-adapted plants, vccn1-1 grana were also longer than in wt and in addition, often exhibited a curved ‘banana-like’ shape rather than the horizontal shape that was commonly observed in the other samples. The overall increased granum size and modified shape in vccn1-1 suggests significant rearrangements of the thylakoid membrane network as a result of altered ionic strength.

Figure 3 | Kinetics of proton motive force and photosynthetic acclimation on transition from dark to light. (a) Kinetics of ECS$^*$ (total electrochromic shift, a measure of total proton motive force size, PMF) in wild-type plants (wt), vccn1-1 knockout mutant and mutant overexpressing AtVCCN1 (oeVCCN1) were recorded at 650 μmol photons per m$^2$ per s after 30 min dark adaptation. (b) Fraction of PMF contributed by transmembrane electric-potential gradient (ΔΨ) and H$^+$ concentration gradient (ΔpH) at two time points in the light. (c) H$^+$ conductivity through ATP synthase (gH$^+$ ) was determined from ECS decay kinetics. (d) Kinetics for induction of non-photochemical quenching (NPQ) as a measure of photoprotective mechanisms were recorded during 10 min of illumination at 650 μmol photons per m$^2$ per s. (e) Photosystem II quantum yield (Φ$^*_$PSII) as a measure of linear electron transport was calculated from the same experiment as NPQ. Data are the means ± s.e.m. (n = 5). Where not visible, error bars are smaller than the symbols. Asterisks denote a statistically significant difference between wt and mutants (Student’s t-test, P<0.05).
Studies using non-invasive small-angle neutron scattering (SANS) on detached, deuterium oxide (D_2O)-infiltrated leaves from dark-adapted vccn1-1 indicated a shift of the Bragg peak related to periodicity of the thylakoid stacks (grana) to higher q values as compared with wt (Supplementary Fig. 20a). This shift revealed a smaller lamellar repeat distance (RD) of grana in vccn1-1 (Supplementary Table 2), which we attribute to a tighter packing than in wt. Isolated thylakoid membranes, while exhibiting larger RDs than leaves\(^{30}\), retained the small difference between the two genotypes (Supplementary Fig. 20b, Supplementary Table 2). On addition of KCl or KNO\(_3\), slight shrinkage of grana occurred in both samples, as indicated by a minor shift in the Bragg peak—this, the RD in the wt decreased to that of untreated vccn1-1 (Supplementary Fig. 20c,d).

Circular dichroism (CD) spectra on detached leaves revealed a significantly increased (~675-nm polymer- or salt-induced (psi)-type CD in vccn1-1 as compared with the wt (Supplementary Fig. 21), indicating tighter stacking of grana\(^{21}\) in the mutant, in agreement with SANS data. At the same time, vccn1-1, similar to the wt, exhibited intense psi-type bands at around (+)505 and (+)690 nm, arising from the long-range chiral order of LHCCI-PSII super-complexes\(^{32}\), suggesting that the overall macro-organization of pigment-protein complexes in grana was not perturbed by the mutation (Supplementary Fig. 21). Taken together, these data suggest the influence of AtVCCN1 on thylakoid ultrastructure in addition to its role in photosynthetic regulation.

**Discussion**

Experimental evidence has recently shown that K\(^+\) fluxes by AtTPK3 and AtKEA3 in thylakoids regulate photosynthesis by modulation of PMF composition\(^{3,11,12}\). Activities of Cl\(^-\) channels have been reported previously in thylakoids and hypothesized to play role in chloride counterbalance of H\(^+\) pumping\(^{6,7}\), and a member of the ClC chloride channel family, ClGe, has been located to thylakoids\(^{14-16}\). However, there is no experimental evidence for AtClCe being a Cl\(^-\) channel. Therefore, it can neither be excluded nor confirmed that ClCe contributes to the previously reported activities. In this study, we bring evidence that AtVCCN1 forms a voltage-dependent Cl\(^-\) channel, which regulates photosynthesis by modulation of PMF composition and plays role in chloride counterbalance of H\(^+\) pumping.

**Figure 4** Dynamics of photosynthesis and photoprotection during transitions from low to high light. (a) Induction of non-photochemical quenching (NPQ) is slowed down in vccn1-1 but approaches wild-type (wt) levels with each transition from low light (70 \mu mol photons per m\(^2\) per s) to high light (2,000 \mu mol photons per m\(^2\) per s). NPQ is induced faster in oeVCCN1 and decays slower than in wt. (b) Photosystem II quantum yield (\(\Phi_{II}\)) as a measure of electron transport is not changed in vccn1-1, but is reduced in oeVCCN1 when compared with the wt. Data are the means ± s.e.m. (n = 5). Where not visible, error bars are smaller than the symbols. Asterisks denote a statistically significant difference between wt and mutants (Student’s t-test, \(P < 0.05\)). (c) Simplified model for sequence of events in the regulation of photosynthesis by AtVCCN1. At the onset of illumination (grey bar), electron transport-coupled H\(^+\) pumping into the thylakoid lumen results in the formation of proton motive force (PMF), consisting of a major transmembrane electric-potential gradient (\(\Delta \Psi\)) and a minor pH gradient (\(\Delta pH\)). AtVCCN1 is activated by \(\Delta \Psi\) and partially dissipates it by anion influx. This phenomenon allows a rapid increase in \(\Delta pH/\Delta \Psi\) ratio, which is maintained during the remaining illumination. AtVCCN1 also fine-tunes the size of total PMF (not illustrated in this model), which in turn regulates H\(^+\) efflux from the thylakoid lumen through ATP synthase. Electron transport and activation of NPQ are modulated by the \(\Delta pH/\Delta \Psi\) ratio. After sudden shift from low light to high light (white bar), the \(\Delta pH/\Delta \Psi\) ratio is changed by an increase in \(\Delta \Psi\), but AtVCCN1 re-establishes a steady-state, resulting in rapid adjustment of photosynthesis.
to cope with rapid changes in natural habitats. AtVCCN2 is a close parologue, but is not functionally redundant to AtVCCN1 in leaves, where its expression is low.

Using electrophysiology in planar lipid bilayers we show that AtVCCN1 mediates flux of Cl\(^{-}\) in response to the applied voltage (Fig. 2, Supplementary Figs 6,7,9,10). In wt thylakoid membranes, the $\Delta \Psi$ component of PMF is the voltage that activates and regulates AtVCCN1 to transport Cl\(^{-}\) into the lumen, which results in partial dissipation of $\Delta \Psi$ (Fig. 3). A significant $\Delta \Psi$ persists across wt thylakoids under steady-state conditions, in agreement with previous work by Kramer et al.\(^{8}\) Our observations appear to differ from those in the recent report by Johnson and Ruban\(^{33}\), who suggested that $\Delta \Psi$ is completely and rapidly dissipated by ion fluxes during illumination. In our conditions, complete dissipation of $\Delta \Psi$ was observed only in oeVCCN1, most likely due to an elevated accumulation of negative charges (Cl\(^{-}\)) in the lumen of this genotype.

Through partial dissipation of $\Delta \Psi$, AtVCCN1 could also reduce the size of total PMF. Thus AtVCCN1 could be one of the factors determining the PMF size, together with the photosynthetic linear and cyclic electron transport and the H\(^{+}\) efflux through ATP synthase\(^{34}\). Overexpression of AtVCCN1 led to increased rate of H\(^{+}\) efflux through ATP synthase despite a lower PMF than in wt, without impacting plant growth under standard cultivation conditions (Fig. 3, Supplementary Fig. 11, Supplementary Table 1). Together with similar observations by Wang et al.\(^{34}\) for mutants affected in cyclic electron transport, our data suggest that even a lower PMF size could be sufficient for the activity of the ATP synthase during steady-state photosynthesis and plant growth. Overexpression of AtVCCN1 resulted in PMF consisting solely of $\Delta \phi$H (Fig. 3), and an enhanced acidification of the thylakoid lumen (Supplementary Fig. 14). These observations are in line with the higher NPQ and lower electron transport (Fig. 3, Supplementary Fig. 19). On the basis of our results, we propose that the lower luminal pH did not limit but rather stimulated the activity of the ATP synthase.

The wt-like phenotype of vccn1 mutant in all studied PMF and photosynthesis parameters (Supplementary Figs 17,18) could be explained by a possible compensation by AtVCCN1 for the function of AtVCCN2. However, the double vccn1vccn2 mutant did not display an enhanced phenotype but resembled vccn1. These findings together with the limited expression of AtVCCN2 in leaves make it unlikely that it is functionally redundant to AtVCCN1 in the regulation of PMF and photosynthesis in leaf thylakoids. The possibility that AtVCCN2 could form an anion channel as well, based on the close homology to AtVCCN1 and similar effects on PMF caused by its overexpression, requires further investigations.

Participation of AtVCCN1 in the regulation of PMF and photosynthesis ascribes this protein a function in the rapid acclimation of plants in variable light environments (Fig. 4). We propose that AtVCCN1 plays distinct roles as well as roles in common with AtTPK3 and AtKEA3 in photosynthetic acclimation. Like these K\(^{+}\) transport systems, AtVCCN1 modulates PMF partitioning into $\Delta \phi$H and $\Delta \Psi$, affecting the balance between electron transport and photoprotective mechanisms. Distinctly, AtVCCN1 is activated immediately on the transition from dark to light by the establishment of $\Delta \Psi$, and is thus an early component modulating the PMF. Activation of AtTPK3 and AtKEA3 may require a significant $\Delta \phi$H to be established by the partial dissipation of $\Delta \Psi$, for example by AtVCCN1. Furthermore, AtVCCN1 acts in photosynthetic acclimation after transitions to high light, whereas AtKEA3 activity is important for transitions to low light. Finally and distinctively from AtTPK3 and AtKEA3, AtVCCN1 alters the total PMF size and activity of ATP synthase, which may be a strategy to adjust photosynthetic carbon fixation in addition to electron transport. Our findings make AtVCCN1 an early component acting to rapidly adjust plant photosynthesis in variable light environments, and therefore it may be a target for improving photosynthetic acclimation in fields for agricultural and bioenergy production.

**Methods**

**Plant material and growth conditions.** Arabidopsis thaliana wt plants (Columbia ecotype) and mutants were grown in soil for 7–8 weeks in a growth chamber (CLF PlantMaster; Plant Climates, Wertingen, Germany) using a daily cycle of 8 h of light (150 μmol photons per m\(^2\) per s) at 20 °C and 16 h of dark at 19 °C at relative humidity of 60%. The vccn1-1 (SALK_103612) and vccn2-1 (SALK_114715) mutants were obtained from the SALK collection\(^{35}\), vccn1-2 (GABI_796C09) from the GABI-KAT collection\(^{36}\), and vccn2-2 (SK_2655) from the Saskatoon collection\(^{37}\). The double vccn1vccn2 mutant was obtained by crossing vccn1-1 and vccn2-1. Reverse transcription–PCR with gene-specific primers was used to confirm the absence of transcript in the corresponding mutants (Supplementary Fig. 11b, Supplementary Table 3). ACTIN8 (At1g94290) was used as the reference gene. AtVCCN-GFP fusions were constructed as follows. The complete coding sequence of AtVCCN1 or AtVCCN2 (excluding native stop codons), were synthesized with flanking attL1 and attL2 sites and cloned into vector pMK-RQ (GeneArt, Life Technologies, Carlsbad, CA, USA). Gene fragments were transferred to the binary vector pb7FWG2 (ref. 38) containing the CaMV 35S promoter and egfp at the C-termini using Gateway recombinational cloning.

**Figure 5 | Thylakoid ultrastructure.** Representative transmission electron micrographs are shown for leaf chloroplasts from 8-week-old wild-type (wt) plants and vccn1-1 mutant fixed after 16 h of darkness or 3 h after the onset of illumination (150 μmol photons per m\(^2\) per s). The mutant shows consistently longer thylakoid stacks (grana) than wt in the dark state. In light conditions, the grana of vccn1-1 are also longer, but often display a curved ‘banana-like’ shape (observed in 20–50% of the studied plastid sections). Scale bars, 1μm (upper panels) and 200 nm (lower panels).
(Invitrogen, Life Technologies). Constructs were transformed into the Agrobacterium tumefaciens strain GV3101 and subsequently in Arabidopsis vcn1-1 or vcn2-1 genetic backgrounds, respectively, using the floral dip method. To select for successfully transformed plants, seeds were sown on soil and sprayed for 3 days at the fourth leaf stage with 60 mg/mL glyphosate (Basta; Hoechst Schering AgroBio, Düsseldorf, Germany). GFP expression in transformed plants was verified using an Axio Scope A1 epifluorescence microscope (Carl Zeiss Microscopy, Göttingen, Germany). Presence of the VCCN transgene in transformants was confirmed by PCR and its expression verified by quantitative PCR.

Quantitative PCR. Total RNA was isolated from plant tissues of 7-week-old plants with an E.Z.N.A. R6827-01 Plant RNA kit (Omega Bio-Tech, Norcross, GA, USA) and residual DNA was removed with E1911 DNAse (Omega Bio-Tek). Total RNA was pooled from 30 plants was used for each analysis. Complementary DNA (cDNA) was prepared from 400 ng RNA with an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR analyses were conducted with a SoSAdvanced Universal SYBR Green Supermix on a C1000 Touch Thermal Cycler (Bio-Rad). PCR template cDNA corresponding to 40 ng total RNA was used in 15-μl reactions. Amplifications were undertaken in two-step PCR with the following cycling conditions: initial denaturation for 3 min at 95 °C, followed by 40 cycles of denaturation for 5 s at 95 °C and annealing/extension for 30 s at 60 °C. Melt-curve analyses were conducted for all primers after amplification. Expected product size were confirmed by agarose gel electrophoresis. Efficiency and efficiencies of PCR amplification of all target genes were established from serially diluted calibration curves. Melt-curve analyses were conducted for all primers after amplification. Expected product size were confirmed by agarose gel electrophoresis. Efficiency and efficiencies of PCR amplification of all target genes were established from serially diluted calibration curves. Efficiency and efficiencies of PCR amplification of all target genes were established from serially diluted calibration curves. Expected product size were confirmed by agarose gel electrophoresis. Efficiency and efficiencies of PCR amplification of all target genes were established from serially diluted calibration curves. Efficiency and efficiencies of PCR amplification of all target genes were established from serially diluted calibration curves. Efficiency and efficiencies of PCR amplification of all target genes were established from serially diluted calibration curves. Efficiency and efficiencies of PCR amplification of all target genes were established from serially diluted calibration curves. Efficiency and efficiencies of PCR amplification of all target genes were established from serially diluted calibration curves.
system (Carl Zeiss, Oberkochen, Germany) equipped with a LaB6 emitter at an accelerating voltage of 120 kV using a Veleta CCD camera and iTEM software (Olympus Soft Imaging Systems, Münster, Germany). ImageJ software was used to measure granum diameter (measured at the middle of perpendicular granum sections) on transmission electron micrographs. Calculations were done on randomly chosen grana (350–450) originating from 60–90 different plastids taken randomly from 60–90 different mesophyll cells from leaves of different genotypes per treatment. Statistical analyses were undertaken using Instat v.6.0 (GraphPad, La Jolla, CA, USA). Ultrastructural data did not follow a normal distribution, so Kruskal–Wallis non-parametric ANOVA was used followed by the Mann–Whitney rank-sum test. For all data, P < 0.05 was considered significant.

**SANS measurements.** SANS measurements were carried out on the SANS-1 (leaves) and SANS-2 instruments (isolated thylakoid membranes) at the Paul Scherrer Institute (Villigen, Switzerland). On SANS-1 and SANS-2, we used a wavelength of 6 Å, collimation of 11 and 6 m, and sample-detector distance of 11 and 6 m, respectively. Detached leaves were infiltrated in D₂O to enhance contrast. Owing to the relatively weak Bragg peak in Arabidopsis leaves compared with several other species 30, for each measurement 8–10 leaves were placed in a quartz cuvette (path length, 2 mm) filled with D₂O. To improve the signal-to-noise ratio, experiments were repeated three times for vcn1-1 and four times for wt, and the SANS curves were averaged. Hence, these SANS profiles represent the statistical average of ≈ 25–30 leaves. Thylakoid membranes, isolated as described by Posselt and colleagues 36, were suspended in a D₂O-containing reaction medium (20 mM Tricine, pH 7.6, 0.4 M sorbitol, 5 mM MgCl₂, 5 mM KCl) and measured at a chlorophyll concentration of ≈ 500 µg per ml in a quartz cuvette (optical path length 2 mm) placed in a magnetic field of 0.4 T.

Primary steps of data analyses and radial averaging in a 360° sector (leaves) and in two 75° sectors (isolated thylakoid membrane) of the two-dimensional scattering profiles were performed with the Graphical Reduction and Analysis SANS Program package (GRASP) developed by C. Dewhurst (Institut Laue-Langevin, Grenoble, France). RD of the thylakoid membrane was calculated from the position of the characteristic Bragg peak, which corresponded to the periodicity of grana thylakoid membranes. For this, SANS curves in 0.0151–0.0380 Å⁻¹ (leaves) and 0.0194–0.0310 Å⁻¹ (isolated thylakoids) regions were fitted with equation (1):

\[
I(q) = I_0 + A \left[ q^2 + \left( \frac{q}{q_0} \right)^2 \right]^{-\alpha},
\]

where \( I \) is the scattered intensity, \( I_0, A \) and \( \alpha \) are constants, \( q \) is scattering vector, \( q^* \) is position of the Bragg peak, \( p \) and \( c \) are parameters of the power function and Gaussian distribution, respectively. 71. The \( q^* \) parameter was used to calculate RD with equation (2):

\[
\text{RD} = \frac{2\pi q^*}{q^2}.
\]

For detached leaves, statistically averaged RD values for 25–30 leaves per treatment are given; the corresponding error values arise from uncertainty of the fitting. The variation of RD values among repeated measurements within 3 h was < 1 Å. Representative one-dimensional plots and RD values with the error of fitting are presented.

**CD spectroscopy.** CD measurements were made on a J-815 spectropolarimeter (JASCO, Tokyo, Japan). Detached, water-infiltrated leaves were placed between two glass slides in an optical cell. Spectra were recorded at room temperature between 400 and 800 nm at a scan speed of 100 nm per min, band-pass of 3 nm and step size of 1 nm. For each sample, 3–4 scans were averaged. Spectra were normalized to the absorption of the red-most peak of the spectra recorded at the same time as the CD spectra and were corrected for baseline distortions. Measurements were repeated on three different leaves for each genotype. Amplitudes of psi-type CD bands, at spectra and were corrected for baseline distortions. Measurements were repeated 8–10 times for each sample, 3–4 scans were averaged. Spectra were normalized to the glass slides in an optical cell. Spectra were recorded at room temperature between 400–900 nm. For the two-dimensional scattering profiles were performed with the Graphical Reduction and Analysis SANS Program package (GRASP) developed by C. Dewhurst (Institut Laue-Langevin, Grenoble, France). RD of the thylakoid membrane was calculated from the position of the characteristic Bragg peak, which corresponded to the periodicity of grana thylakoid membranes. For this, SANS curves in 0.0151–0.0380 Å⁻¹ (leaves) and 0.0194–0.0310 Å⁻¹ (isolated thylakoids) regions were fitted with equation (1):

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I(q) = I_0 + A \left[ q^2 + \left( \frac{q}{q_0} \right)^2 \right]^{-\alpha},
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where \( I \) is the scattered intensity, \( I_0, A \) and \( \alpha \) are constants, \( q \) is scattering vector, \( q^* \) is position of the Bragg peak, \( p \) and \( c \) are parameters of the power function and Gaussian distribution, respectively. 71. The \( q^* \) parameter was used to calculate RD with equation (2):

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\text{RD} = \frac{2\pi q^*}{q^2}.
\]

For detached leaves, statistically averaged RD values for 25–30 leaves per treatment are given; the corresponding error values arise from uncertainty of the fitting. The variation of RD values among repeated measurements within 3 h was < 1 Å. Representative one-dimensional plots and RD values with the error of fitting are presented.

**Phylogenetic analyses.** To identify candidates for the most closely related eucaytotic protein sequences to AtVCCN1, we used BlastP searches with ‘AtVCCN1’ as the query against the GenBank database 56. Initially, we selected best eukaryotic protein sequences to AtVCCN1, we used BlastP searches with the evolutionary relationships of the sequences were examined using MrBayes 3.2.2 (ref. 58); (a) the sampling scheme used MAFFT alignment as described previously 59. When including BEST1 from human we used the gene duplication that produced VCCN1 and VCCN2 occurred. MrBayes settings included reversible model jump MCMC over the substitution models, four chains, and paired runs for 5 million generations.

**Data availability.** The data that support the findings of this study are available from the corresponding authors on request.

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Author contributions

A.H., C.S. and B.L. conceived the study; A.H., G.G., I.S., C.S. and B.L. designed the analyses. O.Z. carried out CD analyses. S.D. conducted RT-PCR. B.L. carried out experiments. A.H., C.S. and B.L. wrote the manuscript. All authors helped to edit the manuscript.

Additional information

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