



# Impact of ion fluxes across thylakoid membranes on photosynthetic electron transport and photoprotection

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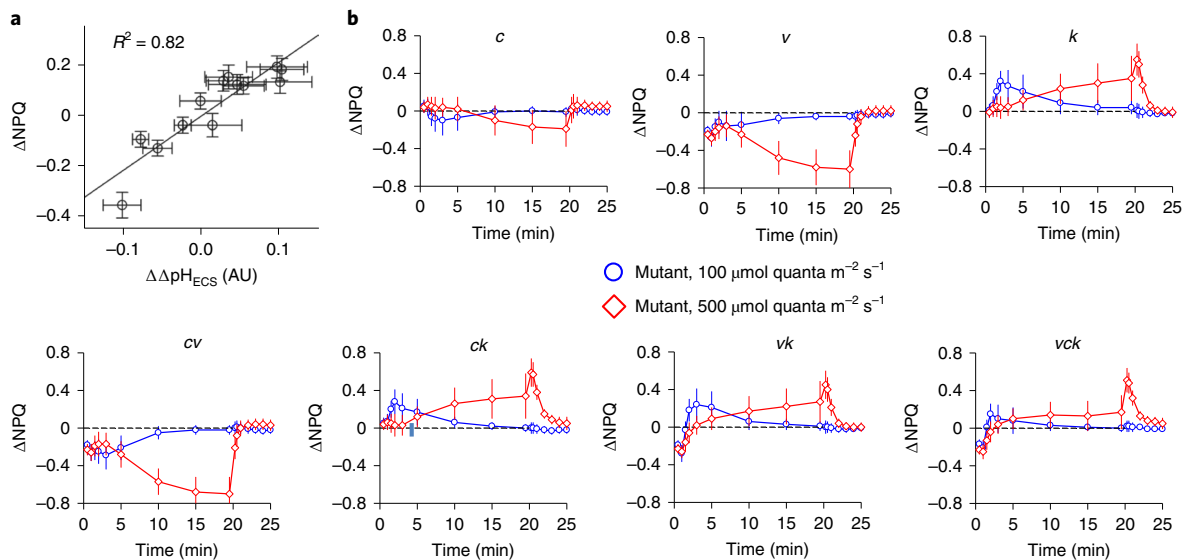
**In photosynthetic thylakoid membranes the proton motive force (pmf) not only drives ATP synthesis, in addition it is central to controlling and regulating energy conversion. As a consequence, dynamic fine-tuning of the two pmf components, electrical ( $\Delta\psi$ ) and chemical ( $\Delta\text{pH}$ ), is an essential element for adjusting photosynthetic light reactions to changing environmental conditions. Good evidence exists that the  $\Delta\psi/\Delta\text{pH}$  partitioning is controlled by thylakoid potassium and chloride ion transporters and channels. However, a detailed mechanistic understanding of how these thylakoid ion transporter/channels control pmf partitioning is lacking. Here, we combined functional measurements on potassium and chloride ion transporter and channel loss-of-function mutants with extended mathematical simulations of photosynthetic light reactions in thylakoid membranes to obtain detailed kinetic insights into the complex interrelationship between membrane energization and ion fluxes across thylakoid membranes. The data reveal that potassium and chloride fluxes in the thylakoid lumen determined by the  $\text{K}^+/\text{H}^+$  antiporter KEA3 and the voltage-gated  $\text{Cl}^-$  channel VCCN1/Best1 have distinct kinetic responses that lead to characteristic and light-intensity-dependent  $\Delta\psi/\Delta\text{pH}$  oscillations. These oscillations fine-tune photoprotective mechanisms and electron transport which are particularly important during the first minutes of illumination and under fluctuating light conditions. By employing the predictive power of the model, we unravelled the functional consequences of changes in KEA3 and VCCN1 abundance and regulatory/enzymatic parameters on membrane energization and photoprotection.**

A ubiquitous principal of biological energy conversion is transient energy storage as a proton motive force (pmf) across a proton-sealed membrane that is employed by ATP synthases for generation of the more stable and universal energy equivalent ATP from ADP and inorganic phosphate<sup>1–3</sup>. Because protons carry a charge, the pmf is the sum of two components: one electrical ( $\Delta\psi$ ) and one chemical ( $\Delta\text{pH}$ ). Both are equivalent in driving ATP synthesis at the ATPase enzyme<sup>4</sup>. Whereas in respiratory membranes the pmf is mainly stored as  $\Delta\psi$ , for a long time it was assumed that the  $\Delta\psi$  under steady-state illumination is small in photosynthetic thylakoid membranes and the pmf consists mainly of  $\Delta\text{pH}$ <sup>5</sup>. This view of thylakoid pmf storage has been challenged by improved spectroscopic analyses<sup>6–9</sup> that disentangle the contributions of  $\Delta\text{pH}$  and  $\Delta\psi$  to total pmf storage, combined with the identification and characterization of thylakoid ion channel/transporters mutants, implicating storage of a fraction of pmf as  $\Delta\psi$ . An explanation of the controversy about pmf partitioning in thylakoid membranes is that the  $\Delta\text{pH}/\Delta\psi$  ratio is not static but dynamic and could depend on environmental/metabolic conditions in vivo (for example ref. <sup>10</sup>), as well as the experimental conditions under which it is measured<sup>8</sup>. The attractiveness of dynamic pmf partitioning is that it enables fine-tuning of photosynthetic electron transport and light harvesting without compromising ATP synthesis. This is based on the fact that the main regulatory processes in thylakoid membranes are dependent on  $\Delta\text{pH}$  (more precisely on the acidity of the thylakoid lumen) but not  $\Delta\psi$ . In detail, proton-releasing reactions in

the thylakoid lumen, water splitting at photosystem II (PSII) and plastoquinol oxidation at the cytochrome  $b_6f$  (cyt  $b_6f$ ) complex, are slowed by lumen acidification in light (high  $\Delta\text{pH}$ )<sup>11,12</sup>, a phenomenon called photosynthetic control. Furthermore, the photoprotective non-photochemical quenching mechanism (NPQ, more precisely the dominating high-energy-quenching component (qE)) localized mainly in the thylakoid-hosted light-harvesting complexes II is activated and triggered at high  $\Delta\text{pH}$ <sup>13–15</sup>. Therefore, pmf redistribution from the  $\Delta\psi$  component to the  $\Delta\text{pH}$  component would downregulate light harvesting (NPQ) and electron transport (photosynthetic control) but leave ATPase activity unaffected<sup>9,16</sup>. How can pmf partitioning be adjusted? Here, thylakoid membrane ion channels and transporters come into play. The main ions in chloroplasts are potassium ( $\text{K}^+$ ) and chloride ( $\text{Cl}^-$ )<sup>17,18</sup>.  $\Delta\text{pH}/\Delta\psi$  partitioning can be tuned by the pmf-driven passive influx of  $\text{Cl}^-$  into the thylakoid lumen and/or by  $\text{K}^+/\text{H}^+$  antiport ( $\text{K}^+$  in,  $\text{H}^+$  out). The influx of  $\text{Cl}^-$  into the lumen is expected to decrease  $\Delta\psi$  (charge compensation in the lumen) allowing an increase in  $\Delta\text{pH}$  (at constant pmf), whereas a  $\text{K}^+/\text{H}^+$  antiport is expected to decrease  $\Delta\text{pH}$  allowing an increase in  $\Delta\psi$ . Early evidence from electrophysiological measurements suggesting the existence of  $\text{K}^+$  and  $\text{Cl}^-$  channels or transporters in thylakoid membranes<sup>19,20</sup> was recently validated by molecular biological studies (see reviews <sup>21,22</sup>). At present, bona fide thylakoid channels/transporters are the  $\text{K}^+/\text{H}^+$  antiporter KEA3 (ref. <sup>23</sup>), the voltage-gated  $\text{Cl}^-$  channel VCCN1/Best1 (refs. <sup>24,25</sup>) and the  $\text{Cl}^-$  channel ClCe<sup>26,27</sup>. A few years ago, the

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**Fig. 1 | NPQ characteristics in KEA3, VCCN1 and Clce mutants.** **a**, Correlation between NPQ changes in all mutants ( $\Delta\text{NPQ} = \text{NPQ}_{\text{mutant}} - \text{NPQ}_{\text{WT}}$ ) versus corresponding changes in the pH component of the ECS ( $\Delta\text{pH}_{\text{ECS}}$ ). Data are also represented as the difference of mutant minus WT ( $\Delta\Delta\text{pH}_{\text{ECS}} = \Delta\text{pH}_{\text{ECS, mutant}} - \Delta\text{pH}_{\text{ECS, WT}}$ ). Both parameters were measured on intact leaves. Data were taken after an average of 7.5 min illumination with 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . **b**, Difference in NPQ ( $\Delta\text{NPQ} = \text{NPQ}_{\text{mutant}} - \text{NPQ}_{\text{WT}}$ ) for two light intensities (100 and 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) measured on intact plants. Light was turned on at 0 min and off at 20 min. Data show the mean of  $n$  (WT, *c*, *v*, *k*, *cv*, *ck*, *vk*, *vck*) = (16, 26, 14, 14, 14, 13, 15, 13) plants with standard deviation. AU, arbitrary units.

two-pore  $\text{K}^+$  channel TPK3 was suggested as another thylakoid channel<sup>28</sup>. However, newer detailed studies make genuine chloroplast localization unlikely<sup>29–31</sup>. Although good characterizations of these thylakoid ion transporter/channels concerning sublocalization, molecular biological characterization and their implication in fine-tuning photosynthetic energy conversion exist<sup>21,22,32</sup> a mechanistic understanding of how they determine ion fluxes,  $\Delta\text{pH}$  and  $\Delta\psi$  across thylakoid membranes, both individually and in concert, is missing. This lack of a mechanistic understanding is, to some degree, due to the methodical challenge of measuring ion fluxes across thylakoid membranes *in vivo*. Here, we take an alternative approach to this challenge by combining measured photosynthetic parameters of thylakoid ion transporter/channel loss-of-function mutants with a computer model for photosynthetic light reactions that includes fluxes through thylakoid transporter/channels. The computer model is an extension of a recently published version<sup>9</sup>.

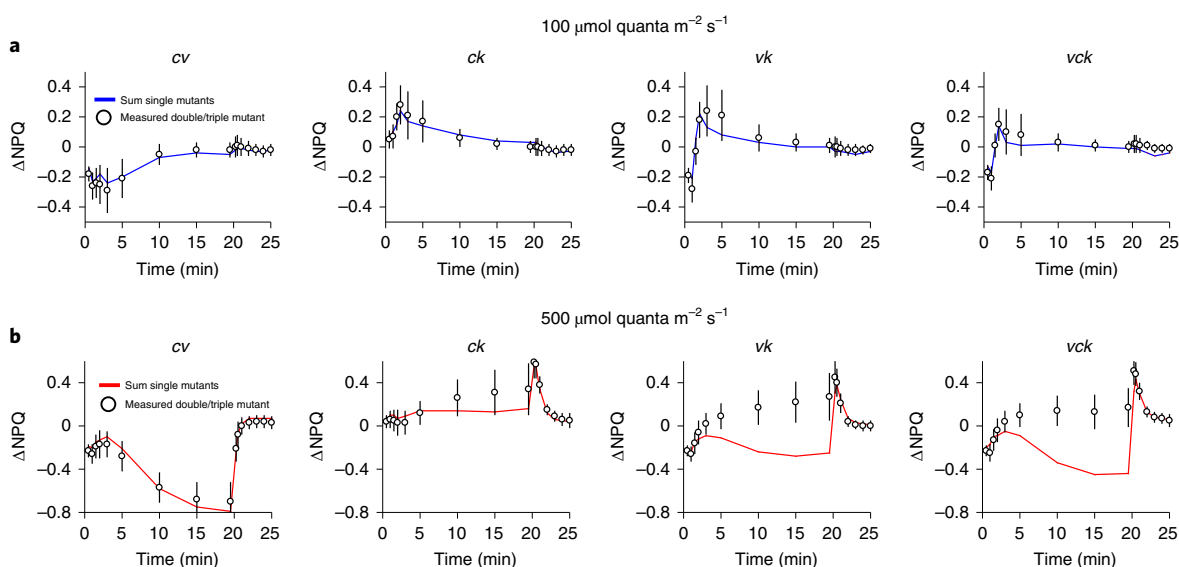
## Results

**Growth phenotype and characterization of thylakoid ion channel/transporter mutants.** For characterization of ion fluxes over thylakoid membranes by KEA3, VCCN1 and Clce transporter/channels, single and multiple loss-of-function mutants were generated. The single knockout mutants, *kea3-1* (ref. <sup>23</sup>), *vccn1-1* (SALK\_103612C)<sup>25</sup> and *clce-2* (Salk\_010237)<sup>26</sup>, were used to generate homozygous double mutants, *clce-2kea3-1* (*ck*), *clce-2vccn1-1* (*cv*) and *vccn1-1kea3-1* (*vk*), and the triple mutant, *vccn1-1clce-2kea3-1* (*vck*) (Supplementary Fig. 1). The composition of key thylakoid membrane components reveals that the abundance of the proteins light-harvesting complexes II, PSII, photosystem I, *cyt b<sub>6</sub>f* complex, ATPase and PsbS, as well as carotenoids, is very similar in all eight genotypes (Supplementary Fig. 2). It was reported that thylakoid ion transporter/channel mutants show functional phenotypes mainly under **fluctuating light conditions**<sup>25,33,34</sup>. To examine how fluctuating light intensities affect plant growth, total leaf areas were determined by plant imaging under constant and fluctuating light regimes (Supplementary Fig. 3). Only mutants that lack KEA3 antiport activity show statistically significant reduced growth and

only under fluctuating light conditions (Supplementary Fig. 3), in accordance with recently published data<sup>32</sup>.

Because it is expected that altered ion fluxes across the thylakoid membrane in ion transporter/channel mutants affect membrane energization,  $\Delta\text{pH}$  was examined *in vivo*. In the following, we express parameter changes in mutants as difference from wild-type (WT) plants (that is mutant minus WT) because this focuses directly on alterations caused by the mutation. A readily quantifiable proxy for  $\Delta\text{pH}$  across thylakoid membranes is NPQ<sup>35</sup>. However, NPQ also depends on the **PsbS protein and the xanthophyll zeaxanthin**<sup>14,15,36</sup>. As mentioned above, neither PsbS level nor xanthophyll pool size is different in the mutants compared with WT (Supplementary Fig. 2) providing justification for the use of NPQ as a qualitative measure of  $\Delta\text{pH}$ . To further validate the NPQ parameter as a proxy for  $\Delta\text{pH}$ , we measured the electrochromic shift signal (ECS) on dark-adapted intact leaves. From ECS signals, total pmf, as well as the  $\Delta\psi$  ( $\Delta\psi_{\text{ECS}}$ ) and  $\Delta\text{pH}$  ( $\Delta\text{pH}_{\text{ECS}}$ ) components can be extracted<sup>4,37,38</sup>. Experiments using the  $\Delta\text{pH}$  indicator 9-aminoacridine (9-AA<sup>39,40</sup>) on fresh isolated intact isolated thylakoid membranes confirm that  $\Delta\text{pH}_{\text{ECS}}$  measures  $\Delta\text{pH}$  (Supplementary Fig. 4). Figure 1a shows plots of  $\Delta\text{NPQ}$  versus  $\Delta\Delta\text{pH}_{\text{ECS}}$  (mutant minus WT) with 7.5 minutes of illumination. The good correlation supports that  $\Delta\text{NPQ}$  can indeed be used as a proxy for  $\Delta\text{pH}$  changes.

Figure 1b summarizes  $\Delta\text{NPQ}$  changes for growth (100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) and elevated (500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) light intensities (for original NPQ kinetics see Supplementary Fig. 5). The  $\Delta\text{NPQ}$  behaviours of the mutants are strikingly different. (1) A general trend is that NPQ was lower in mutants lacking either of the two functional chloride channels, whereas it was higher in the absence of the  $\text{K}^+/\text{H}^+$  antiporter KEA3, consistent with each proposed function in redistributing  $\Delta\text{pH}/\Delta\psi$ . (2) Clce had less impact on lowering NPQ than VCCN1. (3) During illumination, mutants without KEA3 (*k*, *ck*, *vk*, *vck*) showed a characteristic transient increase in NPQ at around 2 min at 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . (4) For the same light intensity, the *vccn1* mutant showed a fast (< 1 min) transient decline in  $\Delta\text{NPQ}$ . (5) For longer time points (>10 min), NPQ relaxed completely to WT levels at the lower light intensity in

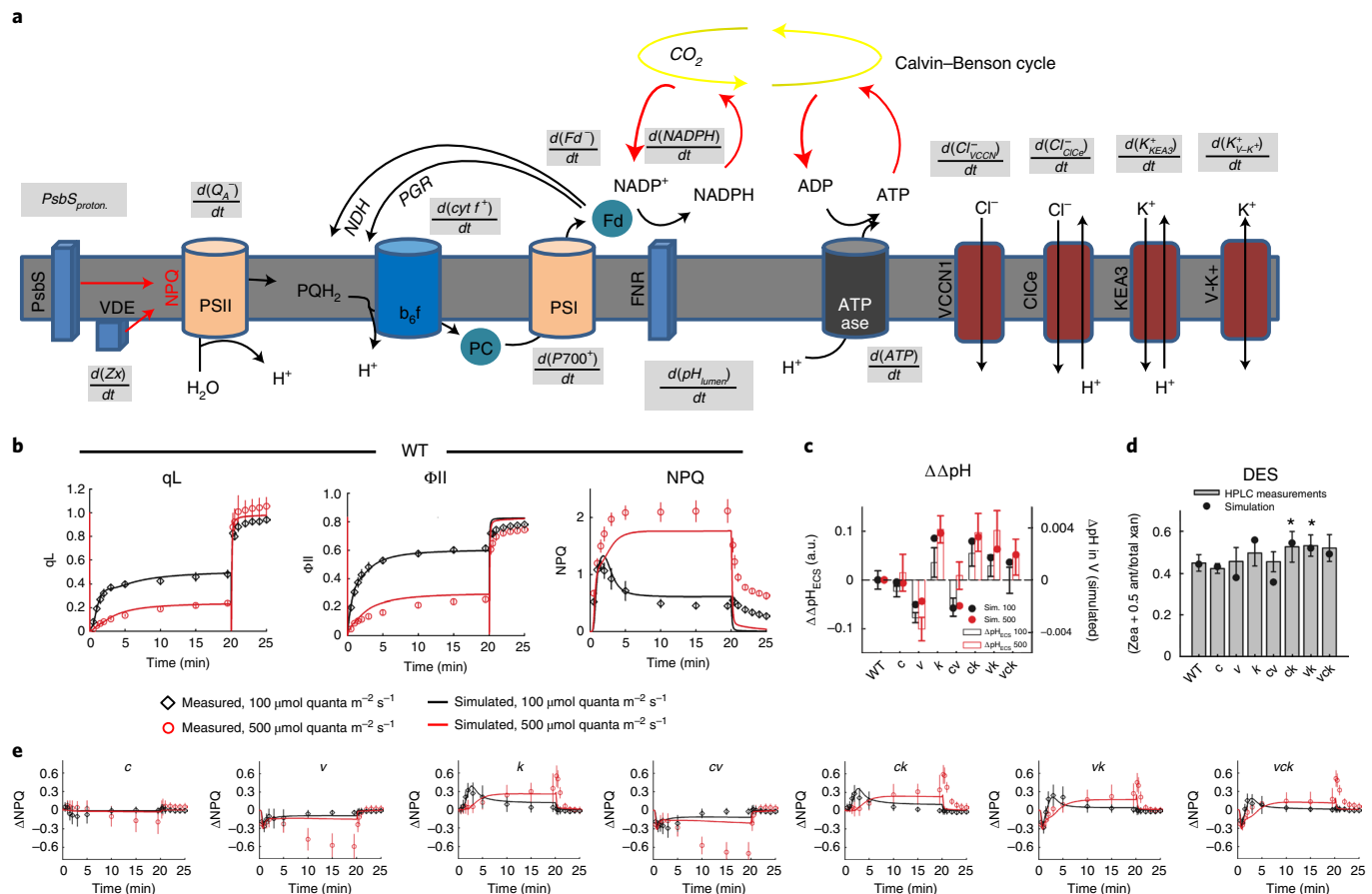


**Fig. 2 | Testing the independence of KEA3, VCCN1 and ClCe functionality.** The graphs show the measured  $\Delta\text{NPQ}$  for the double and triple mutants (circles) compared with their mathematical sum of the single mutants (lines). Independent functionality is indicated if the sum of single mutants equals the data for double and triple mutants. **a**, Results for  $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . **b**, Results for  $500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . Data show the mean of  $n$  (WT,  $c$ ,  $v$ ,  $k$ ,  $cv$ ,  $ck$ ,  $vk$ ,  $vck$ ) = (16, 26, 14, 14, 14, 13, 15, 13) plants with standard deviation.

all mutants, indicative of transient changes in  $\Delta\text{pH}$  and ion fluxes at  $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . (6) In contrast, at  $500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , a larger difference in  $\Delta\text{NPQ}$  appeared over the 20 min illumination period, with decreases in  $v$ -mutants and increases in  $k$ -mutants likely pointing to sustained changes in ion fluxes and  $\Delta\text{pH}$  at higher light or the involvement of other non-photochemical quenching types (see below). (7) All mutants without KEA3 showed slower NPQ relaxations at  $500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  visualized as a positive  $\Delta\text{NPQ}$  spike after light off (20 min). For shorter periods ( $<10$  min) the  $\Delta\text{NPQ}$  changes measured in Fig. 1 are in accordance with published data for VCCN1 (refs. <sup>25,32</sup>), KEA3 (refs. <sup>34,35</sup>) and the higher-order mutants<sup>29,32</sup>. To date, no results have been published for periods  $>10$  minutes.

**Do thylakoid ion transporter/channels operate independently from each other?** The availability of higher-order ion transporter/channel mutants allows the possibility of examining whether the transport proteins operate independently in thylakoid membranes. Independent operation of channels/transporters would be visible as a similar behaviour of measured higher-order mutants compared with the sum of their corresponding single mutants, whereas deviation between the two indicates that channels/transporters function non-independently. The latter could be explained by compensatory ion fluxes triggered by loss of function of a certain channel/transporter. In a recent study on thylakoid ion transporter/channels it was concluded that the three transporter/channels studied here work independently<sup>32</sup>. The  $\Delta\text{NPQ}$  results in Fig. 2 confirm this independency for  $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (Fig. 2, upper panel); that is, the  $\Delta\text{NPQ}$  change in higher-order mutants is the sum of respective single mutants. However, for  $500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  a different conclusion must be drawn. Although the chloride channel double-mutant  $cv$  shows clear indications of the independent operation of ClCe and VCCN1, the data for higher-order  $kea3$  mutants reveal that the behaviour in double and triple mutants deviates clearly from the sum of the single mutant for illumination times  $>3$  min (Fig. 2 bottom panel). The data indicate that at higher light intensities, the  $\Delta\text{NPQ}$  phenotype is dominated by the absence of KEA3 (measured data are more positive than the sum of single mutants).

**Validation of a computer model simulating photosynthetic light reactions.** Because ion fluxes, electron transport, proton pumping and thylakoid membrane energization form a complex, highly interwoven and dynamic functional network, it is difficult to comprehend the consequences of altered ion fluxes in thylakoid channel/transporter mutants on these parameters. To obtain mechanistic insights into the functional consequences of altered ion fluxes, we modified a recently published computer model<sup>9</sup> that describes light harvesting, electron transport, proton pumping, ATP synthesis, NPQ, ion fluxes and ATP/NADPH consumption in the Calvin–Benson cycle (Fig. 3a) by employing coupled differential equations. Rate constants, stoichiometries, pH dependencies and enzyme activities of individual reactions required for the modelling were taken from the literature. Details of the model are given in Supplementary Fig. 6 and Supplementary Methods. Owing to substantial progress over past decades, good quantitative values are available for many model parameters (Supplementary Methods). However, the best way to build up confidence in a model is to test its outcomes by experiments. As a first step, we compared measured kinetics of chlorophyll (Chl) fluorescence parameters  $q_L$  (fraction of open PSII centres),  $\Phi_{II}$  (operating efficiency of PSII) and NPQ for WT plants (Fig. 3b) with the outcome of the model. All three measuring parameters were well described by the model for  $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (black curves and symbols). The modelled NPQ amplitude for  $500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  was slightly lower and the relaxation was faster than measured. This may be explained by the fact that the model does not include slower NPQ components such as  $q_Z$  or  $q_I$  that can contribute notable at higher light intensities<sup>41,42</sup>; that is, the model assumes that NPQ is entirely the high-energy quenching part  $q_E$ . As a consequence of the  $\sim 15\%$  lower NPQ in the model for  $500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , the  $\Phi_{II}$  parameter, which is a measure of linear electron transport, is slightly higher compared with the measured data. Including  $q_Z$  and  $q_I$  in the model is not straightforward and the gain for the model is limited. Therefore, we decided not to model  $q_Z$  and  $q_I$ . The second comparison between the model and the experiment was the  $\Delta\Delta\text{pH}$  changes in the mutants relative to WT for the two light intensities examined (Fig. 3c). The model predicts the right direction of the changes and for many mutants is quantitatively correct (within the



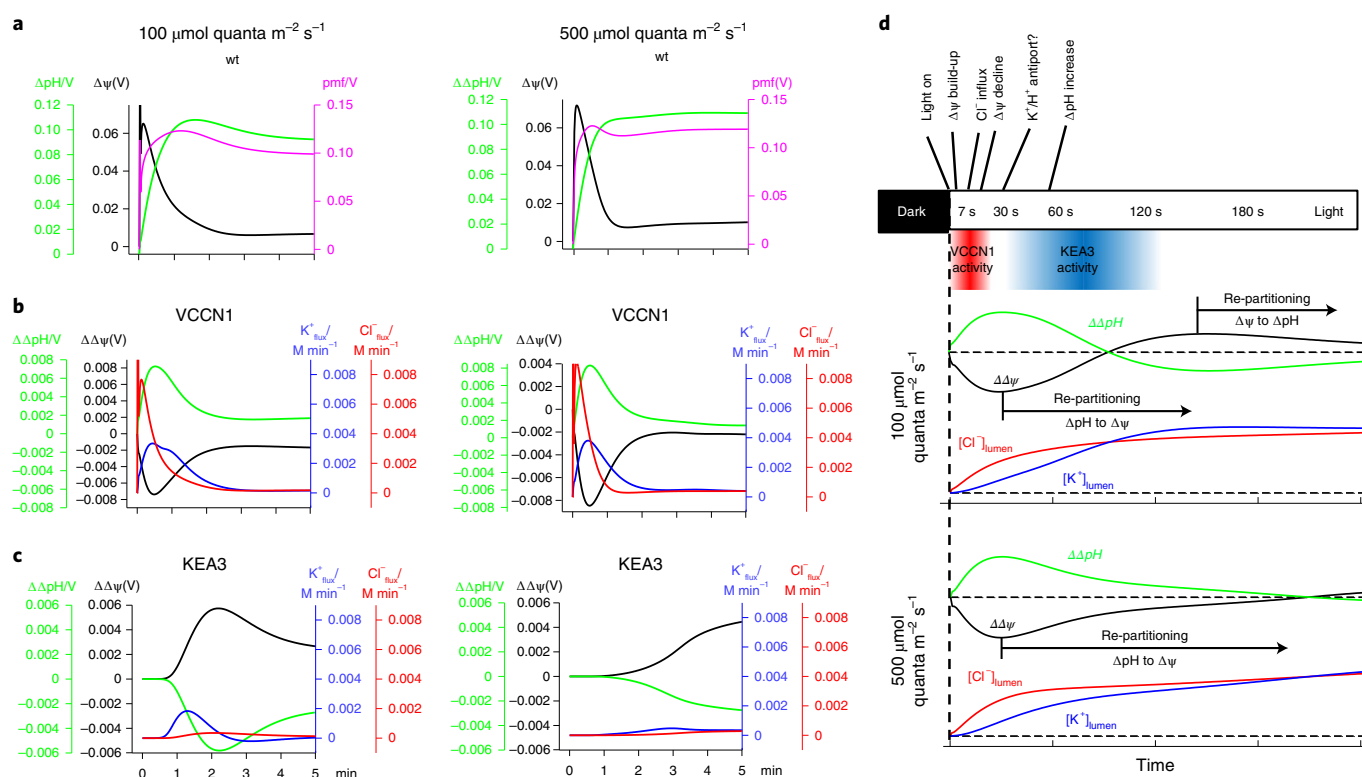
**Fig. 3 | Computer model of photosynthetic light harvesting coupled to  $CO_2$  fixation in the Calvin-Benson cycle.** **a**, Cartoon summarizing the model parameters. Electron, proton, ion and metabolite fluxes are expressed by differential equations that are defined in Supplementary Fig. 6. **b**, Comparison of modelled and measured  $qL$ ,  $\Phi II$  and  $NPQ$  data for WT for 100 and 500  $\mu mol quanta m^{-2} s^{-1}$ . **c**, Comparison of modelled and measured  $\Delta\Delta pH$  changes. **d**, Comparison of modelled and measured changes in the de-epoxidation status of the xanthophyll pool for 500  $\mu mol quanta m^{-2} s^{-1}$ . Asterisks indicate statistically significant changes (two-sided Student's  $t$ -test, for  $ck$ :  $P=0.0362$ ; for  $vk$ :  $P=0.0179$ ). **e**, Comparison of modelled and measured  $\Delta NPQ$  for all ion transporter and channel mutants. Data in **b** and **e** show the mean with standard deviation of  $n$  (WT, c, v, k, cv, ck, vk, vck) = (16, 26, 14, 14, 14, 13, 15, 13) biological independent samples. Data in **c** show the mean with standard deviation of  $n=16$  biological independent samples. Data in **d** show the mean with standard deviation of  $n=9$  biological independent samples. DES, de-epoxidation state; HPLC, high-performance liquid chromatography; PGR, proton gradient regulation 5; VDE, violaxanthin deepoxidase.

range of experimental deviation). Furthermore, the de-epoxidation state of the xanthophyll pool measured for leaves illuminated with 500  $\mu mol quanta m^{-2} s^{-1}$  light intensity is predicted quantitatively by our simulations (Fig. 3d). The 100  $\mu mol quanta m^{-2} s^{-1}$  light intensity was not examined because the de-epoxidation state is very low at this light intensity due to low violaxanthin-de-epoxidase activity. Finally, model performance was tested by its prediction of  $\Delta NPQ$  changes (Fig. 3e). Good agreement between simulated and measured  $\Delta NPQ$  kinetics is apparent with the exception that it fails to simulate the measured slow  $\Delta NPQ$  decline ( $>5$  min) in the  $v$  and  $cv$  mutants at 500  $\mu mol quanta m^{-2} s^{-1}$ . One possibility is that the slow  $\Delta NPQ$  decline reflects  $qZ$  or  $qL$ , which are not included in the simulations. Overall, in most cases, the model correctly predicts quantitatively different measured parameters, particularly for shorter ( $<5$  min) time scales, indicating that the set of constants and stoichiometries used in the model describe the system well. This result also supports the view that partitioning of  $\Delta\psi$  and  $\Delta pH$  by ion movements is important for controlling photosynthetic responses<sup>9,16,32,43</sup>. It is noteworthy that the model requires the involvement of cyclic electron transport for proper description of the measured data. Without implementation of cyclic electron transport the model failed to predict the experimental  $qL$ ,  $\Phi II$  and  $NPQ$  data

(Supplementary Fig. 7, compare with Fig. 3b that includes cyclic electron transport).

**Impact of KEA3, VCCN1 and ClCe activities on ion fluxes and thylakoid membrane energization.** Computer modelling allows deciphering of the individual contributions of ion transporter/channels on fluxes of  $Cl^-$  and  $K^+$  as well on membrane energization. Because the model predicts measured parameters more accurately over the first minutes of illumination, we decided to study ion fluxes,  $\Delta pH$  and  $\Delta\psi$  over the first 5 minutes only. This restriction to shorter periods is further justified by the fact that ion transporter/channel mutants show most pronounced phenotypes under fluctuating light conditions<sup>23,32,33</sup>; that is, when fast changes are required. Channel/transporter independence analysis in the higher-order mutants justifies analyses of KEA3, VCCN1 and ClCe individually over the first minutes of illumination to understand how they work in concert. In line with data from other groups<sup>26,27</sup>, the  $clce$  loss-of-function mutant phenotype is very weak for the conditions examined here (Supplementary Fig. 8b). Therefore, the following analyses focus on the impact of VCCN1 and KEA3 only, which reduces the complexity of the system without compromising essential aspects of ion fluxes across the thylakoid membrane. Figure 4b





**Fig. 4 | Specific role of KEA3 and VCCN1 for thylakoid energization and ion fluxes calculated from our mathematical model. a**, Light-induced changes (left, 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ; right, 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) in proton concentration in the lumen (green),  $\Delta\psi$  (black) and pmf (purple) in WT plants. The kinetics represent the first 5 minutes of illumination. **b**, Specific changes induced by the  $\text{Cl}^-$  channel VCCN1. Data show the simulated differences of WT minus *vccn1*, that is they express VCCN1-induced changes in WT plants. **c**, Specific changes induced by the  $\text{K}^+$  channel KEA3. **d**, The upper panel represents the time series of events after the light was turned on. The middle and lower panels show calculated differences in membrane energization and ion concentrations between WT and *vck* (WT minus *vck*), that is the kinetics reveal changes caused by the ion transporters/channels (VCCN1, KEA3 and  $\text{ClC}_e$ ) as they occur in WT plants for the two light intensities.  $\Delta\Delta\text{pH}$  are expressed in V units ( $\Delta\Delta\text{pH} \times 0.060$ ) to allow better comparison with  $\Delta\psi$ .

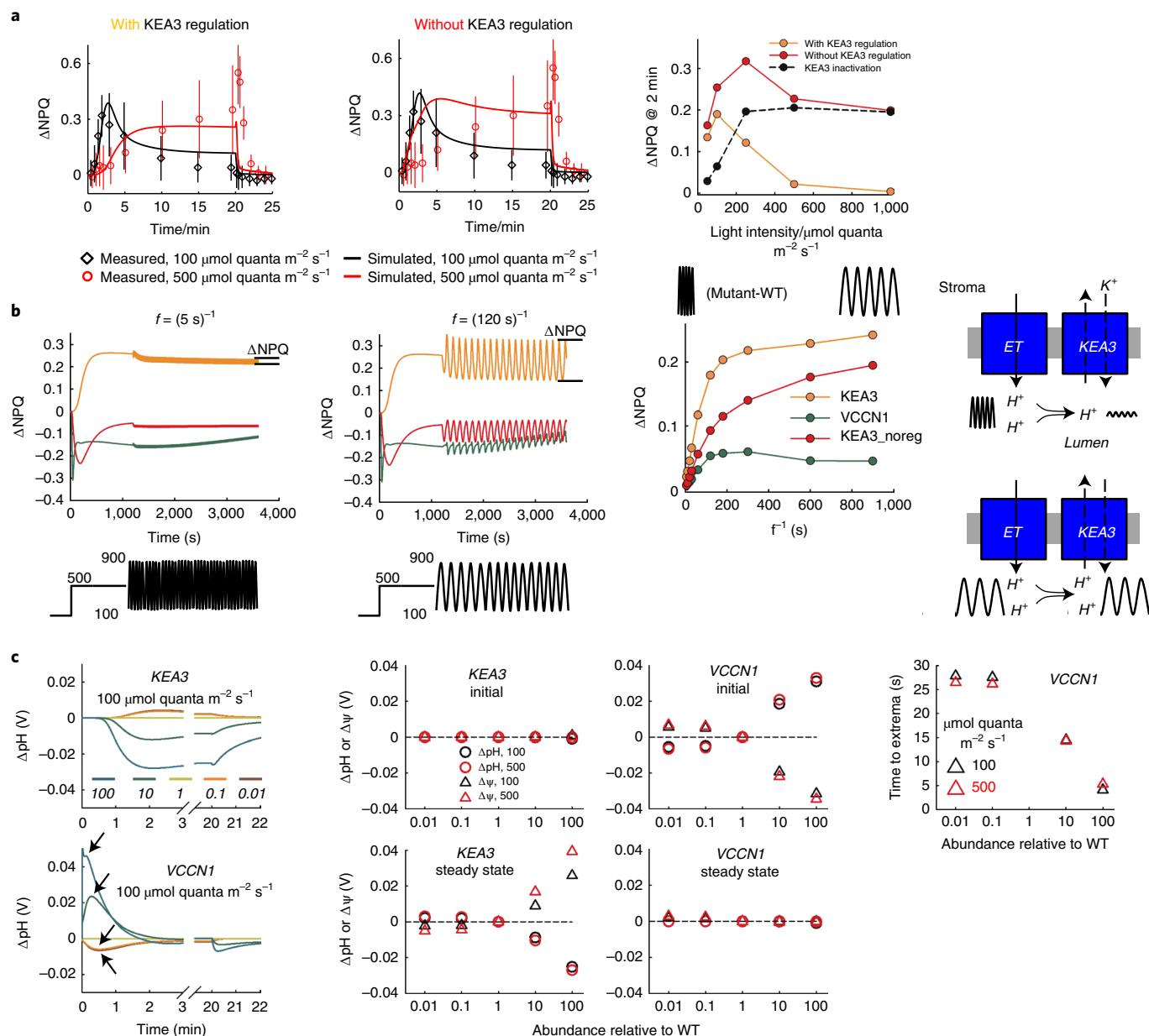
(VCCN1) and Fig. 4c (KEA3) show differences for WT minus mutant, that is it reports specifically on the function of the anion channel or  $\text{K}^+/\text{H}^+$  antiporter in WT plants.

**VCCN1.** The  $\text{Cl}^-$  channel VCCN1 was modelled as a resistor-like channel (Supplementary Methods), with its response to electrochemical potential ( $\text{Cl}^-$ ) fit to in vitro observations described in an earlier study<sup>25</sup>. During the first seconds of illumination with 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ,  $\Delta\psi$  rose steeply (peak  $\sim 7$  s, Fig. 4a left panel) leading to activation of the voltage-gated VCCN1 channel. The resulting  $\text{Cl}^-$  influx also peaks at  $\sim 7$  s (red curve in Fig. 4b) indicating the causal link between  $\Delta\psi$  and  $\text{Cl}^-$  fluxes, that is  $\Delta\psi$  dissipation involves mainly VCCN1 during the first minute of illumination. The VCCN1-specific  $\Delta\psi$  relaxation (Fig. 4b black) was accompanied by a build-up of the  $\Delta\text{pH}$  component seen as anti-parallel behaviour of  $\Delta\Delta\psi$  (black curve) and  $\Delta\Delta\text{pH}$  (green curve) in Fig. 4b peaking at  $\sim 30$  s. The kinetics of VCCN1-mediated ion fluxes are similar at 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (right panel in Fig. 4b) but the amplitudes of these fluxes were more intense at higher light intensities.

**KEA3.** KEA3 was modelled as a proton/potassium antiporter the activity of which is regulated by NADPH (we used qL as a proxy for NADPH level) and the  $\text{pH}_{\text{lumen}}$  in accordance with the literature (Supplementary Methods). In contrast to VCCN1, the simulated behaviour for KEA3 was strikingly different (Fig. 4c). No notable KEA3-specific changes were apparent over the first minute of illumination, in a time when most VCCN1-dependent changes

take place. Furthermore, in contrast to VCCN1, the kinetics of KEA3-induced changes are light-intensity dependent. At 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  alterations in ion fluxes in *kea3* were more rapid compared with 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (see also Fig. 1). We first focus on 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . KEA3-mediated  $\text{K}^+$  influx peaks at  $\sim 78$  s (blue curve Fig. 4c) in parallel to the  $\Delta\text{pH}$  build-up (Fig. 4a), which is expected for a proton-driven  $\text{K}^+$  import by KEA3. The  $\text{K}^+/\text{H}^+$  antiporter activity leads to a decline in  $\Delta\text{pH}$  (Fig. 4c green curve) paralleled by an increase in the  $\Delta\psi$  component (Fig. 4c black curve), that is a re-partitioning from  $\Delta\text{pH}$  to  $\Delta\psi$  occurring between 1 and 2 min. However, after  $\Delta\text{pH}$  and  $\Delta\psi$  reached their extrema ( $\sim 2$  min), the activity of KEA3 decreases, probably as a result of ATP synthase activation and its impact on  $\Delta\text{pH}$ <sup>43</sup>. This leads to a relaxation of  $\Delta\text{pH}$  and  $\Delta\psi$  at around 10 min (see  $\Delta\text{NPQ}$  for  $k$  in Fig. 1a). Similar to VCCN1, the antiparallel  $\Delta\text{pH}/\Delta\psi$  changes mediated by KEA3 are almost simultaneous. At 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  KEA3-mediated  $\text{K}^+$  influx into the lumen activates later and lower (Fig. 4c right, blue line) although the initial  $\Delta\text{pH}$  increase is similar to that for 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (compare green curves in Fig. 4a, right with left). The lack of activity of KEA3 at 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  indicates regulatory inactivation of this transporter at higher light intensity (see below). The slow and weak KEA3 activation leads to decreased and slower re-partitioning from  $\Delta\text{pH}$  to  $\Delta\psi$  (Fig. 4c right).

**Employing the predictive power of the computer model.** *KEA3 regulation.* Figure 4c reveals low KEA3 activity at 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , but not at 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . This is unexpected because



**Fig. 5 | Computer simulation for the functional characterization and regulation of KEA3 and VCCN1.** **a**, Comparison between measured (circles) and simulated (lines)  $\Delta NPQ$  (mutant minus WT) data for 20 min of illumination at two different light intensities. Redox-dependent downregulation of KEA3 activity is required for  $500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (left graph, red line and symbols). KEA3 inactivation (right graph, black curve) derived as the difference between  $\Delta NPQ$  with (orange) and without KEA3 (red) regulation. **b**, Dependence of VCCN1 and KEA3 ion fluxes expressed as  $\Delta NPQ$  on sinusoidal changes in light intensities. The two graphs on the left show examples for high (left) and low (right) frequencies responses for KEA3 (orange), KEA3 without redox-regulation (red) and VCCN1 (green). The plots at the bottom show the light protocol. Numbers indicate light intensities in  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . The amplitudes of the  $\Delta NPQ$  response for the two frequencies are indicated by black horizontal lines for KEA3. The graph on the right gives a detailed dependence of  $\Delta NPQ$  on the frequency of the fluctuating light. The panel on the far right illustrates how the antiporter activity of KEA3 responds to faster (top) and slower (bottom) changes in light intensities/proton pumping into the lumen. ET, electron transport chain. **c**, Response of  $\Delta pH$  and  $\Delta \psi$  to different abundances of VCCN1 and KEA3. The two graphs on the left show examples of  $\Delta pH$  responses if the transporter/channel concentration changes from 0.01- to 100-fold of the WT level. Black arrows for VCCN1 indicate a shift in the  $\Delta pH$  extrema. The middle plots show  $\Delta pH$  and  $\Delta \psi$  changes as function of KEA3 and VCCN1 abundances for 100 (black symbols) and 500 (red symbols)  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . The graphs on the upper panel illustrate initial  $pH/\Delta \psi$  changes (from 0 s to 50 s), the plots in the lower panel illustrate steady-state changes (20 min of illumination). The graph on the right shows how the time point of the early  $\Delta pH$  ( $\Delta \psi$ ) spike (black arrows in the graph to the bottom left) shifts as function of VCCN1 concentration in thylakoid membranes.

the driving force for  $H^+/K^+$  antiport at  $500 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  is even higher than at  $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (see  $\Delta pH$  in Fig. 4a). This observation points to a downregulation of KEA3 activity at higher light intensities. Recent studies provide evidence for KEA3 regulation via a so-called KTN domain located at the C-terminus

of the antiporter<sup>44,45</sup>. For the bacterial KEA3 homologue KefC it was shown that the regulatory KTN domain binds  $NAD(P)^+/NAD(P)H$ <sup>46</sup>. The reduced coenzyme has an inhibitory effect on the transport activity. Recently, independent studies in planta showed that the KTN domain of KEA3 extends into the stroma<sup>44,47</sup> where the

**Table 1 | Impact on enzymatic parameters of KEA3 on NPQ determined by the computer model**

pK <sub>a</sub> Hill	5.5	6.0	6.5
1	28 s 1.94	25 s 1.85	23 s 1.77
3	28 s 1.91	24 s 1.80	22 s 1.70
5	28 s 1.89	24 s 1.77	22 s 1.66

Conditions: Illumination for 60 min with 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  followed by change to 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . The upper number in each cell represents the time constant for NPQ relaxation after the light-intensity switch. The second number gives the steady-state NPQ value for 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  at the end of the 60-min period. The time constant was determined by bi-exponential fitting of the NPQ relaxation curve. The time represents the dominating fast relaxation component (amplitude 75–81%).

NADP(H) pools vary according to the photosynthetic status of the chloroplast. It is therefore likely that elevated NADPH/NADP ratios at higher light intensities downregulate KEA3 activity<sup>44,47</sup>. To test this, we ran simulations with or without NADPH-dependent downregulation of KEA3 activity at different light intensities. Comparison with measured  $\Delta\text{NPQ}$  data shows clearly that NADPH-triggered downregulation is required to explain experimental results at 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  but that this regulation is not required for 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (Fig. 5a, for other mutants see Supplementary Fig. 9). A detailed light-intensity dependency of the downregulation of KEA3 activity (black curve, Fig. 5a, right) derived from the difference of  $\Delta\text{NPQ}$  in the presence (orange) and absence (red) of KEA3 regulation reveals a steady inactivation of the antiporter with increasing light intensity reaching a plateau at 250  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ .

Furthermore, the model allows for the study of functional implications if regulatory parameters of KEA3 were altered. Table 1 summarizes changes in NPQ for different pK<sub>a</sub> values for the pH<sub>Lumen</sub>-dependent activation of the antiporter and for different Hill coefficients for the reductive inactivation of KEA3 by NADPH. Changing the pK<sub>a</sub> values for KEA3 from 5.5 to 6.5 leads to an ~20% acceleration in NPQ relaxation (from 28 s to 22 s), whereas changes in the Hill coefficient for NADPH-triggered inactivation has no apparent impact on the speed of NPQ kinetics. Alteration of the Hill coefficient has a small impact on the steady-state NPQ, whereas increasing the pK<sub>a</sub> value leads to a ~10% decrease in NPQ. The results presented in Table 1 demonstrate that modulation of the sensitivity of KEA3 to pH<sub>Lumen</sub> or to NADPH-dependent downregulation has only minor functional consequences.

**KEA3 and VCCN1 under fluctuating light.** A second application of the computer model is to determine how fast KEA3 and VCCN1 activities respond to fluctuating (sinusoidal) light intensities. To this end, the simulation started with a 20-min pre-illumination period to activate the Calvin–Benson cycle followed by sinusoidal changing light intensities alternating between 100 and 900  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  over a further 40-min period (Fig. 5b, bottom). Figure 5b gives the  $\Delta\text{NPQ}$  (mutant minus WT) for different frequencies of fluctuating light intensities. The two examples for high ( $f=(5 \text{ s})^{-1}$ , left) and low ( $f=(120 \text{ s})^{-1}$ , middle) frequencies demonstrate that the loss of KEA3 activity has a substantial impact on the fluctuating light response only for lower frequencies, but not if the light intensity changes faster. This is visualized by the changes in  $\Delta\text{NPQ}$  amplitudes in response to the sinusoidal light fluctuations for the two frequencies (indicated in Fig. 5b). The frequency dependency of  $\Delta\text{NPQ}$  in Fig. 5b (right) derived from this analysis monitors the frequency dependency of KEA3 and VCCN1 activities. It indicates that only if the fluctuation in light intensity and therefore

proton pumping into the lumen is slow enough does the lack of H<sup>+</sup>/K<sup>+</sup> antiport in the *kea3* mutant lead to a  $\Delta\text{NPQ}$  tuning phenotype (see panel to the far right in Fig. 5b). The change in VCCN1 amplitude is relatively small but that for KEA3 is notable ( $\Delta\text{NPQ} > 0.2$ ). Figure 5b (right) reveals that half of KEA3 antiport activity is reached at a frequency of  $\sim(60 \text{ s})^{-1}$ . Repeating this analysis with a KEA3 transport version without NADPH regulation shows that this leads to a less-efficient response over the entire frequency domain, that is this unregulated KEA3 version becomes a slow responding antiporter to fluctuating light.

**Impact of KEA3 and VCCN1 abundances on thylakoid energetics.** The mode of action of KEA3 and VCCN1 allows prediction of how changes in their relative abundance will impact  $\Delta\text{pH}$ ,  $\Delta\psi$ , NPQ and electron transport. In contrast to these more intuitive predictions, the magnitude of changes in these parameters in response to changes in antiporter and channel concentrations in the thylakoid membrane is not straightforward. Figure 5c shows how  $\Delta\text{pH}$  changes if KEA3 or VCCN1 abundances vary between 0.01 and 100 times WT levels (mutant minus WT) for a light intensity of 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . As expected (see above), an increase in KEA3 levels leads to a decrease in  $\Delta\text{pH}$  with a lag time of ~1 min, whereas an increase in VCCN1 abundance has the opposite effect, mainly at shorter times. These alterations in  $\Delta\text{pH}$  and  $\Delta\psi$  are analysed quantitatively in Fig. 5c (middle) for early time points ('initial' between 0 s and 50 s after light on) or for steady-state conditions (20 min light on). The analysis was performed for 100 (black symbols) and 500 (red symbols)  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . The reduction in KEA3 and VCCN1 abundances (0.1- and 0.01-fold) has a moderate effect on the pmf components. However, higher concentrations of KEA3 and VCCN1 lead to much larger changes in  $\Delta\text{pH}$  and  $\Delta\psi$  (in opposite directions). Note that  $\Delta\text{pH}$  changes are expressed in volts, for example a change in  $-0.03 \text{ V}$  as seen for 100-fold higher KEA3 abundance translates into a  $\Delta\text{pH}$  change of  $\sim 0.5 \text{ pH units}$  or an NPQ decrease of an impressive 1.5 units (Supplementary Fig. 10). A similar change in  $\Delta\text{pH}$ ,  $\Delta\psi$ , NPQ magnitudes are apparent for 10- and 100-fold increases in VCCN1 concentrations but in opposite directions and only for illumination times  $< 30 \text{ s}$ . An additional implication of changing the VCCN1 abundance in thylakoid membranes is that the position of the  $\Delta\text{pH}/\Delta\psi$  spike (black arrows in Fig. 5c, bottom left) shifts to shorter times if VCCN1 concentration increases (Fig. 5c, right). Changing VCCN1 from 0.1-fold to 100-fold accelerates the  $\Delta\text{pH}/\Delta\psi$  response (spike) from  $\sim 27 \text{ s}$  to  $\sim 5 \text{ s}$  independent of light intensity.

## Discussion

**Temporal sequence of events unravelled by mutant analysis and mathematical modelling.** In this study, we demonstrated that our extended mathematical model was able to describe a range of measured parameters (Fig. 3), as well as functional consequences for mutants that affect ion homeostasis around thylakoid membranes. The model allows deciphering of an order of events that is summarized in Fig. 4d for two light intensities. The data show differences between WT minus the *vck* triple mutant, which indicate how the concerted action of the three ion channels/transporters fine-tune the pmf through ion fluxes. At a growth light intensity of 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  the fast build-up of  $\Delta\psi$  triggers Cl<sup>−</sup> influx by the voltage-gated VCCN1 into the lumen leading to partitioning of the pmf from  $\Delta\psi$  to  $\Delta\text{pH}$ . This initial partition phase is completed after  $\sim 30 \text{ s}$ . At this point, the  $\Delta\text{pH}$  rise is half-maximal (Fig. 4a, green curve) leading to activation of KEA3 and re-partitioning from  $\Delta\psi$  to  $\Delta\text{pH}$  that is completed after  $\sim 120 \text{ s}$ . This is followed by a slower secondary re-partitioning phase from  $\Delta\text{pH}$  to  $\Delta\psi$ . Thus, the concerted actions of VCCN1 and KEA3 lead to oscillations in the  $\Delta\psi$  and  $\Delta\text{pH}$  components of the pmf. Physiologically these oscillations make sense because during the initial phase of illumination

the build-up of the  $\Delta pH$  provides well-needed photoprotection via NPQ and photosynthetic control. Additionally,  $\Delta pH$  build-up helps to activate ATP synthesis via ATP synthase by protonation of luminal amino acids<sup>48</sup>. After the initial period, a re-partition from  $\Delta pH$  into  $\Delta \psi$  allows faster electron transport by releasing the NPQ brake and photosynthetic control; that is, light is now converted more efficiently, which is beneficial under low light conditions. Figure 4d shows that an important contributor for these  $\Delta pH/\Delta \psi$  oscillations is the ratio of  $Cl^-$  to  $K^+$  concentrations in the thylakoid lumen controlled by VCCN1 and KEA3 activities. Inspection of Fig. 4d at both light intensities shows that  $\Delta pH$  increases and  $\Delta \psi$  decreases if the change in  $Cl^-$  concentration is higher than the change in  $K^+$  and vice versa. Consequently, at crossing points of ion concentrations the changes in both pmf components are also equal (compare cross points of red/blue and black/green lines in Fig. 4d). Overall Fig. 4d demonstrates relative fast changes in ion concentrations, and as a consequence pmf partitioning, that explain why phenotypes of thylakoid membrane ion transport/channel mutants are more apparent under fluctuating light (Supplementary Fig. 3, refs. <sup>25,33,49</sup>). In contrast to  $100 \mu mol \text{ quanta m}^{-2} \text{ s}^{-1}$ , re-partitioning from  $\Delta pH$  to  $\Delta \psi$  is much slower at  $500 \mu mol \text{ quanta m}^{-2} \text{ s}^{-1}$ ; that is, the  $\Delta \psi/\Delta pH$  crossing point is shifted from  $\sim 90$  s to  $\sim 210$  s (Fig. 4d). This delay in  $\Delta pH$  relaxation could help to photoprotect the system using NPQ/photosynthetic control at this higher light intensity. Mechanistically, the slower  $\Delta pH$  relaxation at  $500 \mu mol \text{ quanta m}^{-2} \text{ s}^{-1}$  can be explained by the low activity of KEA3 (see  $K^+$  flux in Fig. 4c right, blue line) caused by downregulation of the antiporter (see below).

**Insights in KEA3 and VCCN1 operation.** *Defining design strategies for thylakoid ion transporter/channels.* The power of our experientially validated computer model was explored for deeper physiological characterization of KEA3 and VCCN1 that is difficult to access experimentally. The analyses shown in Fig. 5a provide strong evidence for redox-dependent downregulation of KEA3, probably by NADPH, at higher light intensities in line with indications from the literature<sup>44,45</sup>. The light-intensity dependence of KEA3 inactivation reveals that 50% inactivation is reached at  $\sim 100 \mu mol \text{ quanta m}^{-2} \text{ s}^{-1}$ ; that is, the regulation of antiport activity is tuned around the growth light intensity. Further insights into KEA3 and VCCN1 operation are provided by their frequency dependency (Fig. 5b). For both VCCN1 and KEA3, a full response to sinusoidal modulated light intensities in light-adapted plants is only apparent at frequencies lower than  $\sim (100 \text{ s})^{-1}$ . This might reflect a limited capacity for ion transport, in particular for KEA3; that is, if the proton pumping into the lumen by electron transport fluctuates too fast a limiting capacity of KEA3 leads to a damping of the proton oscillation and the NPQ response. It is noteworthy that these results are exclusively caused by KEA3 and VCCN1 and not by pleiotropic effects that are possible in real mutant studies. This is a clear advantage of computer simulations. The analysis in Fig. 5b highlights the importance of redox-regulation of KEA3 activity under fluctuating light. A lack thereof would make the  $H^+/K^+$  antiporter slower and less responsive to fluctuating light intensities. Further analyses reveal that the biggest impact on thylakoid membrane energetics can be achieved by overexpressing VCCN1 and KEA3 but not by adjusting enzymatic parameters of KEA3 (Table 1). The latter has only a minor impact on NPQ. The overexpression simulation shows an intriguing high sensitivity of  $\Delta \psi$ ,  $\Delta pH$  and NPQ on higher concentrations of KEA3 and VCCN1. Furthermore, increasing VCCN1 abundance also accelerates the transient and early  $\Delta pH$  spike. The results of our analyses may guide genetic engineering strategies to adjust thylakoid energization to optimize and fine-tune energy transduction in plants. In this context, the extended computer program presented in this study can be employed to help develop custom-made plants with certain bioenergetic features by uncovering and exploring non-intuitive behaviours of thylakoid membrane components.

## Conclusion

Our computer simulations reveal that the different kinetic behaviours of VCCN1 and KEA3 and the accompanied ion fluxes determine mutual oscillatory increases and decreases in  $\Delta pH$  and  $\Delta \psi$  across thylakoid membranes required for the light-dependent control of electron transport and photoprotection at the onset of illumination. Furthermore, detailed characterization of KEA3 activity shows that the redox-dependent (NADPH) downregulation of KEA3 activity regulates NPQ and membrane energization around growth light intensities and enhances the response to fast changing fluctuating light intensities, a feature that is relevant for efficient plant growth in natural environments. In contrast, membrane energization and NPQ are relatively insensitive to the precise values of enzymatic KEA3 parameters that determine its regulatory power ( $pK_a$  of activation, Hill coefficient for downregulation). However, increasing VCCN1 and KEA3 abundance in thylakoid turned out to be a powerful way to tune photoprotection and electron transport.

## Methods

**Plant materials and growth conditions.** *Arabidopsis thaliana* WT (Col-0) and confirmed mutant seeds were sown on soil, and plants were maintained in a growth chamber at a constant temperature of  $21^\circ\text{C}$ , and 9 h illumination per day at  $120 \mu mol \text{ m}^{-2} \text{ s}^{-1}$ . Double mutants were acquired by crossing corresponding single mutants: *kea3-1* (ref. <sup>23</sup>), *vccn1-1* (SALK\_103612C) and *clce-2* (Salk\_010237). The triple mutant was obtained by crossing *kea3-1clce-2* with *vccn1*. Homozygous mutants were identified using a polymerase chain reaction with genomic DNA (Supplementary Fig. 1).

**Phenomics.** Plants that were 4 weeks old were moved to phenomic chambers with the same photoperiod and temperature settings as in the growth chamber. After a 2–7 day acclimation period, Chl fluorescence parameters were measured at night after  $>2$  h of dark adaptations. Data from the constant light phenomic chamber was acquired using the Fluorcam system (PSI Co.). Data from the fluctuating light phenomic chamber was acquired using PhenoCenter (LemnaTec). In the PhenoCenter during the day, after 20 min constant light at  $120 \mu mol \text{ m}^{-2} \text{ s}^{-1}$ , light intensity was set to fluctuate every 1 min, randomly among 50, 120, 250,  $400 \mu mol \text{ m}^{-2} \text{ s}^{-1}$  with an overall frequency of 0.6, 0.2, 0.1, 0.1 respectively, which ensured the average light intensity was close to  $120 \mu mol \text{ m}^{-2} \text{ s}^{-1}$ .

**Isolation of chloroplasts thylakoid membranes.** Chloroplast and thylakoid membrane isolations were done as described in a previous study<sup>50</sup>. Thylakoid membranes were used for gel electrophoresis and western blotting, whereas isolated chloroplasts were used for in vitro 9-AA fluorescence measurement.

**Gel electrophoresis and western blotting.** Isolated thylakoids were loaded on a 10% Tris–Tricine acrylamide gel ( $2 \mu g/\text{lane}$  for western blotting and  $4 \mu g/\text{lane}$  for Coomassie blue staining). For western blotting, gels were wet blotted onto a methanol-activated PVDF membrane ( $0.45 \mu m$ , Millipore). Blocking was done in 5% milk (Bio-Rad) in Tris-Buffered Saline with Tween20 buffer (pH 7.6) for 1 h at room temperature. Incubation with primary antibody raised against PsbS protein (kind gift from K.K. Niyogi, UC Berkeley, CA, USA) was done overnight in a cold room (dilution 1:2000). Secondary antibody anti-rabbit horseradish peroxidase-conjugated from donkey as a host animal (GE Healthcare) was incubated at room temperature for 2 h (dilution 1:50 000, Amersham, GE). Chemiluminescence (ECL) was recorded on Amersham Hyperfilm (GE) using Amersham ECL Western Blotting Detection Kit (GE).

**9-AA fluorescence.** 9-AA fluorescence kinetics was recorded with excitation at 405 nm (bandwidth 1 nm) and emission at 460 nm (bandwidth 7.5 nm). Isolated chloroplasts with  $40 \mu g$  Chl were added to 1 ml of shock buffer (25 mM HEPES, 7 mM  $MgCl_2$ , 40 mM KCl, pH 7.8 with KOH) in a reaction cuvette with constant stirring for  $\sim 1$  min followed by addition of 1 ml of double concentration buffer (25 mM HEPES, 7 mM  $MgCl_2$ , 260 mM KCl, 200 mM sorbitol, pH 7.8 with KOH). After  $\sim 1$  min,  $2 \mu l$  of 5 mM 9-AA were added before turning on the external light source following a stabilized fluorescence signal. Three minutes of illumination was followed by 2 min of dark relaxation.  $\Delta pH$  was calculated as described by Van et al.<sup>51</sup>.

**Pigments analysis.** Leaf samples illuminated under  $500 \mu mol \text{ m}^{-2} \text{ s}^{-1}$  light for 20 min were frozen in liquid  $N_2$  before carotenoid extraction in 100% ethanol on ice. Extracted pigments were stored at  $-20^\circ\text{C}$  and analysed using reverse-phase high-performance liquid chromatography within 24 hours<sup>52</sup>.

**Chl fluorescence measurements.** Before light adaptation, the minimum fluorescence ( $F_0$ ) and maximum fluorescence ( $F_m$ ) were determined. For



light-adapted leaves the steady-state fluorescence level ( $F_s$ ) and maximal fluorescence ( $F_m'$ ) were measured and the following parameters calculated:<sup>53</sup>  
 $F_v/F_m = (F_m - F_o)/F_m$ ;  $\Phi_{II} = (F_m' - F_s)/F_m'$ ;  $NPQ = (F_m - F_m')/F_m'$ ;  
 $q_L = ((F_m' - F_s)/(F_m' - F_o)) \times (F_o/F_s)$ .

**Difference absorption spectroscopy.** Cyt  $b_6f$  complex and PSII concentrations in thylakoid membranes were derived from chemical difference absorption spectra that quantify cyt  $b_6$  and cyt  $f$  (for cyt  $b_6f$  complex) and cyt  $b_{559}$  (for PSII). Signals were recorded using a Hitachi U3900 spectrometer (2-nm slit width, 530–580 nm). For details see ref.<sup>54</sup>.

**ECS.** The ECS signals at 520 and 545 nm were measured on dark-adapted plants. The dark interval relaxation kinetics was recorded after illuminating a leaf at 500  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  light for a period (1, 2, 3, 5, 10, 19.5 min). Eight plants were used for each genotype. The  $\Delta\text{pH}$  and  $\Delta\psi$  components were calculated as described in ref.<sup>29</sup>.

**Modelling.** The photosynthetic light reaction was modelled by modifying a previously described model<sup>9</sup>. Key improvements involve a close match between the simulated data and measured data from leaf samples. Detailed updates and explanations are available in Supplementary Methods.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Data availability

The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Code availability

The computer code for our model is available online. A link is provided in Supplementary Information.

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## Author contributions

M.L., V.S., G.D. and H.-H.K. performed experiments and analysed data. M.L., G.D., D.K. H.-H.K. and H.K. designed the study. H.K. wrote the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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## Antibodies

Antibodies used	Secondary antibody from GE Healthcare Anti-Rabbit HRP conjugated from host donkey as a host animal (NIF824, lot: 9794849). The PsbS antibody was a kind gift of Dr. Krishna K. Niyogi (UC Berkeley, CA, USA).
Validation	Li, X.-P., Phippard, A., Pasari, J., and Niyogi, K. K. (2002) Structure-function analysis of photosystem II subunit S (PsbS) in vivo. <i>Funct. Plant Biol.</i> 29, 1131–1139