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Journal

Proceedings of the National Academy of Sciences of the United States of America, 111(20)

ISSN

0027-8424

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Publication Date

2014-05-20

DOI

10.1073/pnas.1323899111

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Plastidial transporters KEA1, -2, and -3 are essential for chloroplast osmoregulation, integrity, and pH regulation in *Arabidopsis*

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Edited by Bob B. Buchanan, University of California, Berkeley, CA, and approved April 8, 2014 (received for review January 4, 2014)

Multiple K^+ transporters and channels and the corresponding mutants have been described and studied in the plasma membrane and organelle membranes of plant cells. However, knowledge about the molecular identity of chloroplast K^+ transporters is limited. Potassium transport and a well-balanced K^+ homeostasis were suggested to play important roles in chloroplast function. Because no loss-of-function mutants have been identified, the importance of K^+ transporters for chloroplast function and photosynthesis remains to be determined. Here, we report single and higher-order loss-of-function mutants in members of the cation/proton antiporters-2 antiporter superfamily *KEA1*, *KEA2*, and *KEA3*. KEA1 and KEA2 proteins are targeted to the inner envelope membrane of chloroplasts, whereas KEA3 is targeted to the thylakoid membrane. Higher-order but not single mutants showed increasingly impaired photosynthesis along with pale green leaves and severely stunted growth. The pH component of the proton motive force across the thylakoid membrane was significantly decreased in the *kea1kea2* mutants, but increased in the *kea3* mutant, indicating an altered chloroplast pH homeostasis. Electron microscopy of *kea1kea2* leaf cells revealed dramatically swollen chloroplasts with disrupted envelope membranes and reduced thylakoid membrane density. Unexpectedly, exogenous NaCl application reversed the observed phenotypes. Furthermore, the *kea1kea2* background enables genetic analyses of the functional significance of other chloroplast transporters as exemplified here in *kea1kea2*Na^+/H^+ *antiporter1* (*nhd1*) triple mutants. Taken together, the presented data demonstrate a fundamental role of inner envelope KEA1 and KEA2 and thylakoid KEA3 transporters in chloroplast osmoregulation, integrity, and ion and pH homeostasis.

The regulation of ion and pH homeostasis is a vitally important feature of all living organisms. The existence of organelles in eukaryotic cells has added complexity to this circumstance. Proper function of chloroplasts in plant cells is not only crucial for the organism's survival but affects all life forms as chloroplasts convert light into chemical energy and fix carbon from the atmosphere. With up to 10% of the dry weight, K^+ is the most abundant cation found in plants; it fulfills numerous essential roles: for example, in osmoregulation, as a pH regulator, in motor cell movements, and in membrane polarization (1).

Early studies on isolated chloroplasts suggested that K^+ transport occurs in exchange for H^+ (2, 3). Later studies on reconstituted envelope membranes supported the notion that K^+ and H^+ transport are functionally connected (4, 5). A K^+ transport system across envelope membranes has been proposed to be crucial for chloroplast function because even small changes in the osmotic pressure or electrochemical potential led to a dramatic decrease in photosynthesis (6, 7). However, the molecular mechanisms that mediate K^+ transport across chloroplast membranes and that regulate the osmotic pressure and pH and ion homeostasis of the chloroplast are poorly understood. Two possible mechanisms have been proposed for K^+ transport across the inner envelope membrane: K^+ channels (4, 5) and K^+/H^+ antiporters (7, 8). Furthermore, it remains unknown whether K^+ transport across chloroplast membranes is rate-limiting for chloroplast function. These gaps in knowledge could be closed by genetic analyses to assess the proposed roles of K^+ in chloroplast function and could also lead to a mechanistic understanding for transport models. In this study we sought to characterize K^+ transporters of the chloroplast and to identify their biological functions in plants.

A variety of different ion/H^+ transporters, K^+ channels, and H^+ pumps are located in the plasma and endomembranes of plants. These transporters maintain organelle-specific ion contents and pH, which create gradients over membranes that not only energize secondary transport processes but also result in unique biochemical reaction compartments. The electro neutral cation/H^+ antiporters in *Arabidopsis thaliana* build the superfamily of monovalent cation/proton antiporters (CPA) (44 predicted genes), which further subdivides into the CPA1 and CPA2 families (9, 10). CPA1 consists of the $Na^+(K^+)(Li^+)/H^+$ exchangers NHX1–8. Although NHX1–6 were identified in endomembranes (11–13), NHX7 and -8 localize to the plasma membrane (14, 15) and are more distantly related to the first six members, thus forming a subfamily (10, 16). SOS1/NHX7 has been studied in detail because loss-of-function of the gene leads to salt sensitivity (14).

The second family CPA2 covers two larger subfamilies, which include Cation/H^+ exchangers (CHX) and putative K^+-efflux antiporters (KEA) (10). Twenty-eight different CHXs exist in *A. thaliana*, with members targeted to the plasma membrane, prevacuolar membrane, or the endoplasmic reticulum, where

Significance

Photosynthesis is the key biochemical reaction in plants. The molecular mechanisms of potassium (K^+) transport across chloroplast membranes and their relevance for chloroplast function and photosynthesis remain unknown. In our report, we identify and characterize the molecular basis of K^+ (KEA1, KEA2, KEA3) and sodium (NHD1) transporters in chloroplast membranes. We demonstrate that these inner envelope and thylakoid-targeted transporters are essential for chloroplast osmoregulation and thylakoid density. In addition, we discover an unexpected high Na^+ restoration of photosynthetic activity in the mutants.

Author contributions: H.-H.K., M.G., A.H., M.S.-C., D.M.K., C.S., and J.I.S. designed research; H.-H.K., M.G., A.H., and M.S.-C. performed research; H.-H.K., M.G., A.H., M.S.-C., D.M.K., C.S., and J.I.S. analyzed data; and H.-H.K. and J.I.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1323899111/-/DCSupplemental.

they exchange K^+ against H^+ (17–19). The significance of this transporter class was shown in *chx20* loss-of-function mutants, in which endomembrane dynamics and osmoregulation needed for stomatal opening is affected (20). Therefore, K^+/H^+ antiporters represent major osmo- and pH-regulators for organelles (9). A CPA2 family member, CHX23, long thought to be a chloroplast K^+/H^+ antiporter, was recently found not to target to chloroplasts but to the endoplasmic reticulum (19, 21). In addition, *CHX23* was found to be preferentially expressed in pollen (9), and the described phenotypes in *A. thaliana* CHX23 RNAi and *chx23-1* tilling mutants (22) could not be confirmed in T-DNA mutants (19, 21); thus, the molecular nature and biological function of chloroplast K^+ transporters remain unknown.

Recently, strong evidence was presented that the KEA2 protein could fulfill the role of plastidial K^+/H^+ antiport. A half-sized C-terminal transmembrane domain containing an AtKEA2 fragment was shown to complement a yeast mutant deficient in the endosomal $Na^+(K^+)/H^+$ exchanger NHX1p (23). In addition, in vitro measurements showed K^+/H^+ transport capacity for the half-sized protein fragment. A 100-aa N-terminal protein fragment of AtKEA2 suggested that the full-length AtKEA2 protein may be targeted to chloroplasts (23). However, no mutant phenotypes or chloroplast functions have yet been ascribed for KEA2 and investigation of the full-length gene was unsuccessful because of gene toxicity in *Escherichia coli* (23). Here, we have identified three members of the CPA2 family, KEA1, KEA2, and KEA3, as chloroplast K^+/H^+ antiporters that have critical function in the inner envelope (KEA1, KEA2) and in the thylakoid membrane (KEA3). Our findings reveal their essential role in plant chloroplast function, osmoregulation, and pH regulation.

Results

Candidate Identification and Phenotypic Analyses. Protein alignments of plant, algae, and cyanobacteria CPA2 members and in silico prediction identified KEA1 and KEA2 as putative candidates for plastidial localized K^+ transport family members (10). More evidence came from an N-terminal peptide of KEA2 that targeted to the chloroplast in a transient assay (23). To overcome potential overlapping functions of two homologous putative K^+ transporters, a gene-family–based artificial micro RNA (amiRNA) was designed that targeted *KEA1* and *KEA2* with similar efficiency (24). Isolated T1 generation amiRNA*kea1/2* plants were comparable in size and development to Col-0 wild-type control plants (Fig. 1*A*). Early studies on isolated chloroplasts indicated that an imbalance of chloroplast osmoregulation and pH results in decreased rates of photosynthesis (6, 7). Therefore, T1 individuals were screened for changes in the chlorophyll *a* fluorescence yield in pulse amplitude modulated (PAM) measurements, providing information about the quantum yield of photosystem II (F_v/F_m). Although Col-0 control plants had uniform value of 0.77 for this parameter, independent T1 amiRNA*kea1/2* plants revealed decreased F_v/F_m ratios down to 0.57 in the center of the rosette (Fig. 1*A*). The young leaves of independent amiRNA*kea1/2* mutant lines also displayed discreet pale green regions (Fig. 1*A*).

To verify these results, gene-disruption T-DNA insertion mutants of *KEA1* and *KEA2* were isolated (Fig. 1*B* and Fig. S1 *A* and *B*). Neither *kea1-1* nor *kea2-1* single mutants had a visible phenotype (Fig. 1*B*). However, *kea1-1kea2-1* mutants displayed strong growth retardation along with pale green leaves (Fig. 1*B*). The leaf paleness was most strongly pronounced in younger leaves. PAM recordings revealed a strong decrease of F_v/F_m ratio in the pale green areas of *kea1-1kea2-1* mutants (Fig. 1*C*). Interestingly, older leaves were found to be greener and reached almost Col-0 F_v/F_m levels (Fig. 1*C*). Subsequently the phenotype was confirmed by analyzing an independent double-mutant line *kea1-2kea2-2* with similar results (Fig. S2*A*). The observed *kea1kea2* mutant phenotype implies an important role of KEA1 and KEA2 in chloroplast photosynthetic function.

KEA1 and KEA2 Localize to the Chloroplast. To test the targeting of functional full-length KEA proteins, C-terminal fusions of *KEA1* and *KEA2* genomic DNA loci with Venus fluorophores were cloned and transformed into *kea1-1kea2-1* mutants. Lines that complemented the *kea1kea2* mutant phenotype were analyzed for Venus fluorescence. Both KEA1 and KEA2 clearly localized to the chloroplast (Fig. 1*D*). The ring-shaped Venus signal surrounding the central red chlorophyll fluorescence is characteristic for envelope membrane localization (Fig. 1*D*).

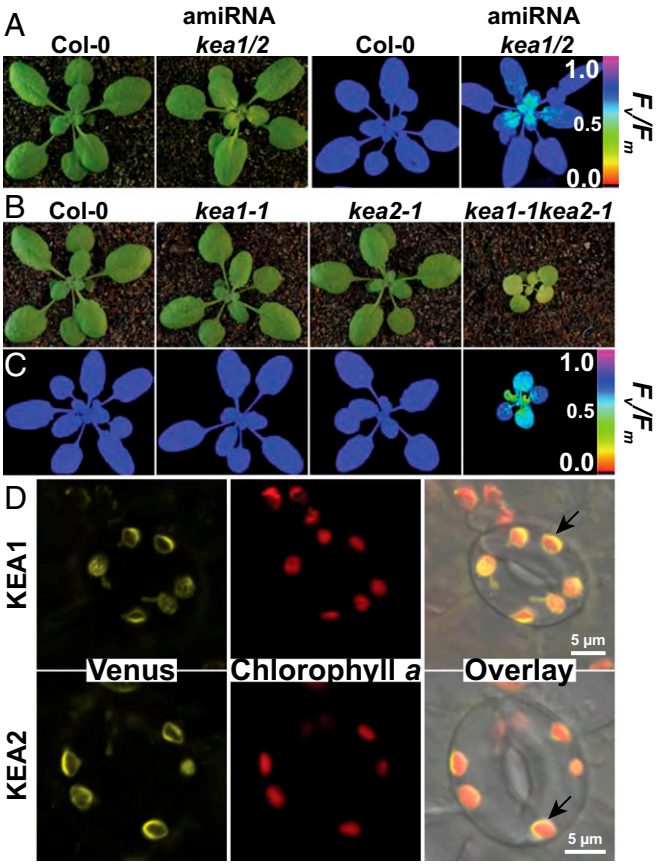

Fig. 1. Reduced F_v/F_m ratio and growth in amiRNA*kea1/2* and *kea1-1kea2-1* T-DNA mutant plants and subcellular localization of KEA1 and KEA2. (*A*) PAM F_v/F_m images in false colors (scale bar: wild-type F_v/F_m in dark blue). Three-week-old long-day grown amiRNA*kea1/2* plants showed decreased F_v/F_m ratio compared with Col-0. (*B*) *kea1-1* and *kea2-1* single mutants were indistinguishable from Col-0 controls whereas *kea1-1kea2-1* were smaller with pale green young leaves. (*C*) F_v/F_m ratio was strongly decreased in *kea1-1kea2-1* plants but not in single mutants or Col-0. (*D*) AtKEA1 and AtKEA2 localized in chloroplast envelope membranes.

KEA3, a Shorter Homolog, also Encodes for a Plastidial Transporter. A protein alignment and cladogram of all six KEA proteins from *A. thaliana* indicated that KEA3 is more closely related to the plastidial envelope KEA1 and KEA2 transporters than to the remaining family members KEA4, KEA5, and KEA6 (Fig. S2*B*). *KEA3* encodes only for a 637-aa protein of approximately half the size of KEA1 and KEA2 and lacks the long N-terminal hydrophilic loop of KEA1 and KEA2 (Fig. S2*C*). Despite this, all three members share two conserved PFAM domains frequently found in Na^+/H^+ antiporters (PF00999) and K^+ transporters (PF02254) (Fig. S2*C*). A genomic DNA KEA3 Venus fusion was transformed into Col-0 plants and a defined localization within the chloroplast was detected (Fig. 2*A*). Compared with the larger KEA1 and KEA2 proteins, the signal did not appear in the ring-shaped structure of envelope membranes but matched the red chlorophyll fluorescence signal in overlay image analyses (Fig. 2*A*). This finding indicates that KEA3 might be part of a different plastidial membrane (i.e., the thylakoid). For an independent

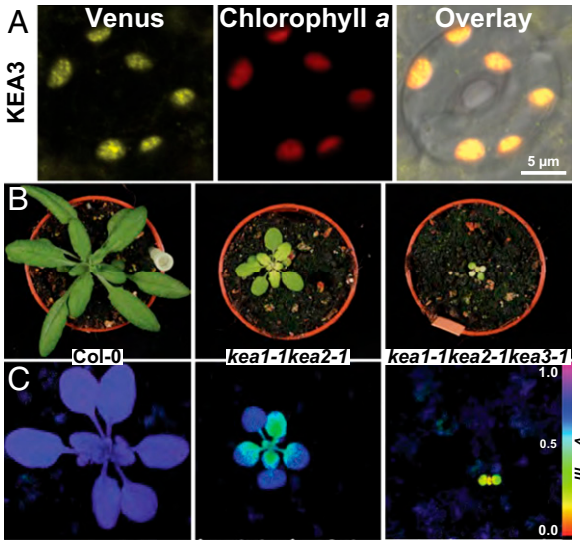

Fig. 2. *kea1-1kea2-1kea3-1* triple mutants show increased defects in photosynthesis and growth. (*A*) KEA3-Venus localized to the chloroplast but not to the envelope. (*B*) *kea1-1kea2-1kea3-1* mutants were extremely small and died before producing seeds on soil. (*C*) F_v/F_m ratio was more dramatically decreased in *kea1-1kea2-1kea3-1* compared with *kea1-1kea2-1* mutants.

analysis of KEA1, -2, and -3 membrane targeting, intact chloroplasts were isolated from all lines and the plastidial membranes fractioned. Immunodetection verified the protein localization for KEA1 and KEA2 in the chloroplast envelope membrane and KEA3 in the thylakoid membrane (Fig. S2*D*).

***kea1-1kea2-1kea3-1* Triple Mutants Are Barely Viable.** To investigate the functional relationship between the three plastidial KEA transporters, a homozygous T-DNA insertion line *kea3-1* was isolated and crossed with *kea1-1kea2-1*. Under normal long-day growth conditions, isolated *kea3-1* single-mutant plants did not display strong visible phenotypes and F_v/F_m was comparable to Col-0 (Fig. S2*E*). In contrast, plants with simultaneous loss of all three plastidial KEAs (*kea1-1kea2-1kea3-1*) were extremely stunted in size with entirely pale leaves and died before setting seeds (Fig. 2*B*). The same trend was observed in PAM measurements. Although *kea1-1kea2-1* mutant plants showed a recovery of the F_v/F_m ratio in older leaves, this effect was absent in *kea1-1kea2-1kea3-1* mutant, which had uniformly dramatically low F_v/F_m in all of the leaves (Fig. 2*C*).

Drastic Morphological Defects in *kea1-1kea2-1* and *kea1-1kea2-1kea3-1* Mutant Chloroplasts. The subcellular morphological defects resulting from loss-of-function of the KEA1, KEA2, and KEA3 plastidial transporters were investigated in leaf tissue of *kea1-1kea2-1* mutants, *kea1-1kea2-1kea3-1* mutants, and corresponding Col-0 controls by transmission electron microscopy (TEM). In Col-0, the chloroplasts were found to be intact, with a large number of grana and stroma thylakoid membranes (Fig. 3 *A* and *D*). Clearly, starch grains were visible in Col-0 chloroplasts (asterisks in Fig. 3*D*), which contributed to their characteristic half-moon shape. In contrast, the *kea1-1kea2-1* mutant chloroplasts were rounded or ball-shaped and larger in size (Fig. 3 *B*, *E*, and *J*). In many cases the chloroplast intactness was compromised and envelope membranes were found to be disrupted with stroma content apparently leaking out of the plastid (Fig. 3*E*, arrow, and Fig. S3*B*). Additionally, the thylakoid membranes were reduced in abundance and in grana number and size (Fig. 3*G*). Furthermore, starch granules were barely present or smaller in size (Fig. 3 *H* and *I*). The chloroplasts in fixed *kea1-1kea2-1kea3-1* mutant leaf tissue exhibited a greatly reduced number (Fig. 3*C*) and had a similarly swollen appearance, as described for *kea1-1kea2-1*, and again disruption of the envelope could be documented (Fig. 3*F*, arrow). In addition, the number of thylakoid membranes was even further reduced (Fig. 3*F*). The remaining thylakoid membranes appeared as dark spots with short, disintegrated membrane fragments. Starch grains were completely absent in *kea1-1kea2-1kea3-1* mutants.

Consistent with the low number of starch granules detected in TEM, the faint iodine staining confirmed strongly decreased starch content in *kea1-1kea2-1* mutant leaf tissue (Fig. S3*C*). However, no obvious differences were observed in the case of statolith starch in heterotrophic tissue (i.e., root tips) (Fig. S3*D*).

The *kea1-1kea2-1* and *kea1-1kea2-1kea3-1* Mutant Phenotypes Are Rescued by External NaCl. The transport substrates of bacterial homologs KefB/C and recent transport assays on a purified truncated KEA2 fragment provided evidence that the KEA-type transporters facilitate transport of K^+ and, to a lesser degree, Na^+ ions in counter exchange with H^+ (23, 25). Furthermore, excessive Na^+ concentration in leaves can cause chlorosis and K^+ ions can counteract Na^+ toxicity. To study the impact of increased ion levels in the absence of plastidial KEA transporters *kea1-1kea2-1* and *kea1-1kea2-1kea3-1*, mutant fitness and phenotype was investigated in the presence of elevated exogenous KCl and NaCl concentrations. Three-day-old seedlings were transferred to either control or supplemented media and grown for additional 17 d. Unexpectedly, a beneficial effect of growing *kea1-1kea2-1* mutants on elevated salt levels of 75 mM NaCl was discovered (Fig. 4 *A* and *B*). The characteristic pale green color of younger *kea1-1kea2-1* leaves was reduced, double mutants gained

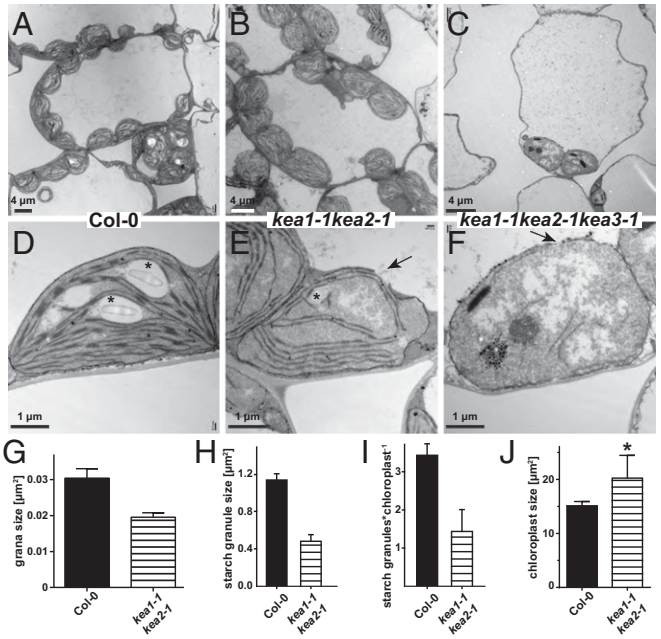

Fig. 3. Micrographs of Col-0, *kea1-1kea2-1* double mutants, and *kea1-1kea2-1kea3-1* triple mutants reveal chloroplast disintegrations. (*A*–*C*) Col-0 chloroplasts showed the characteristic half-moon shape with high starch accumulation. *kea1-1kea2-1* mutant plastids appeared ball-shaped with disorganized thylakoid membranes. In *kea1-1kea2-1kea3-1* mutants, the total number of chloroplasts was decreased and thylakoid membranes if present reduced to single disconnected stacks. (*D*–*F*) Detail chloroplast pictures reveal malfunction in higher order *kea* mutants. Although small starch granules (asterisk) were still detectable in double mutants, they were entirely absent in *kea1-1kea2-1kea3-1* mutants. Numerous disruptions of the envelope membrane were discovered in both mutant lines (arrows). (*G*) The thylakoid grana size was lower in *kea1-1kea2-1*. (*H* and *I*) The number and size of starch granules found in *kea1-1kea2-1* was decreased. (*J*) The average size of *kea1-1kea2-1* chloroplasts was larger compared with Col-0 (± SEM, $*P < 0.05$, $n = 3$ Col-0, $n = 4$ *kea1-1kea2-1*).

chlorophyll *a* and chlorophyll *b* (Fig. S3*E*), and fresh weight compared with *kea1-1kea2-1* mutants grown in the absence of NaCl (Fig. 4*C*). Elevated salt levels are toxic to plants and thus Col-0 had decreased fresh weight if exposed to 75 mM NaCl. However, Col-0 total fresh weight was still higher than in *kea1kea2* mutants (Fig. 4 *A* and *C*).

To study this observation in vivo and more objectively, agar plates were used in PAM measurements. When 75 mM NaCl was present, the F_v/F_m ratio of *kea1-1kea2-1* showed a strong recovery increasing from 0.15 up to 0.52 (Fig. 4 *B* and *D*). To a lesser extent, KCl had a beneficial effect with an F_v/F_m increase to 0.4 (Fig. 4*D*). Intriguingly, 75 mM KCl lowered F_v/F_m of all lines indicating an unbeneficial effect of increased K^+ levels on photosynthesis in general. This phenomenon could be a result of depolarization of the membrane potential caused by high K^+ (26). To rule out that a general osmotic effect is responsible for the salt complementation of *kea1-1kea2-1* mutants, iso-osmotic 150 mM sorbitol treatments were carried out. However, no F_v/F_m recovery was measured in the 150 mM sorbitol treatment (Fig. 4*D*).

We also examined the salt complementation effect on chloroplast morphology of *kea1-1kea2-1* by TEM. As documented before in the control, *kea1-1kea2-1* plastids appeared to be swollen with disorganized thylakoid membranes and low abundance of starch granules (Fig. 4*E*). However, in *kea1-1kea2-1* plants grown in the presence of 75 mM NaCl, the chloroplast morphology was indistinguishable from Col-0 controls; that is, the plastids appeared in half-moon shape, with well-organized, stacked thylakoid membranes and well-defined starch granules (Fig. 4 *E* and *F*). To test whether NaCl also has a beneficial effect on *kea1-1kea2-1kea3-1* mutants, seedlings were transferred to media supplemented with or without 75 mM NaCl. Astonishingly, even in the absence of all three plastidial KEA transporters the pale and retarded phenotype was strongly suppressed by the availability of 75 mM NaCl in the medium (Fig. 4*G*). The *kea1-1kea2-1kea3-1* mutant plants gained green color with only small pale regions close to the leaf veins remaining and produced seeds.

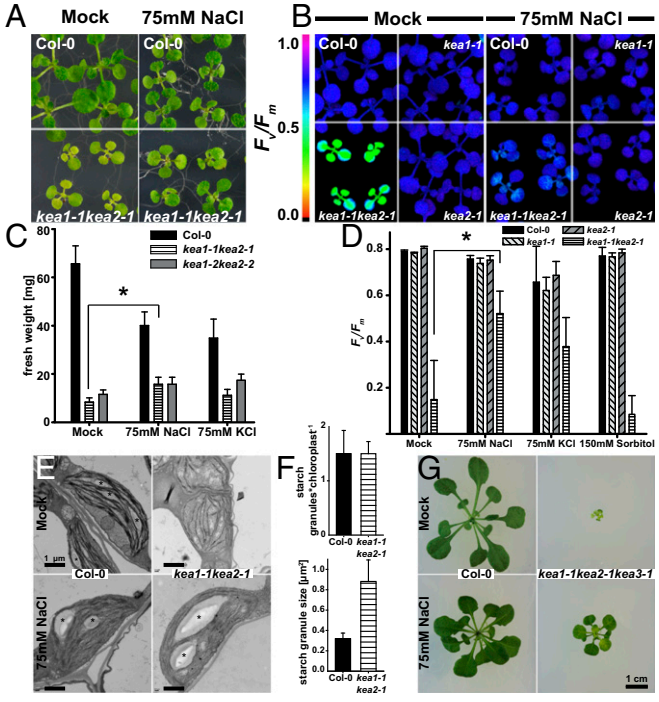

Fig. 4. Exogenous salt rescues *kea1-1kea2-1* and *kea1-1kea2-1kea3-1* mutant phenotypes. (*A*) *kea1-1kea2-1* mutants grown in the presence of 75 mM NaCl were slightly larger and less pale. (*B*) *kea1-1kea2-1* showed the characteristic low F_v/F_m ratio under control conditions. In the presence of 75 mM NaCl F_v/F_m ratio in *kea1-1kea2-1* increased to almost Col-0 levels. (*C*) Col-0 lost fresh weight in the presence of salt, whereas *kea1-1kea2-1* showed a significant increase. Col-0 was still larger than *kea1kea2* mutants (± SEM, $*P < 0.01$ $n = 3$, 12–15 plants). (*D*) Sorbitol (150 mM) did not increase F_v/F_m ratio in *kea1-1kea2-1* as found for NaCl and KCl. (± SD, $*P < 0.01$ $n = 10$–20). (*E*) In the absence of salt, *kea1-1kea2-1* mutant chloroplasts were swollen with disorganized thylakoid membranes and low starch. In the presence of 75 mM NaCl, *kea1-1kea2-1* mutant chloroplasts were indistinguishable from Col-0 plastids and starch granules (asterisk) were clearly visible. (*F*) NaCl (75 mM) in the growth media restored starch accumulation in *kea1-1kea2-1* chloroplasts (± SEM $n = 3$). (*G*) Five-week-old *kea1-1kea2-1kea3-1* mutants on 75 mM NaCl had green leaves, were larger, and produced seeds.

The *kea1-1kea2-1* Mutant Enables Functional Analysis of Other Plastidial Ion Transporters. Recently, a new type of plastidial Na^+/H^+ antiporter NHD1 was identified and biochemically characterized from *Flaveria bidentis* (27). NHD1 was reported to release Na^+ ions from chloroplasts in exchange for H^+ to balance the Na^+ uptake caused by members of the bile acid:Na^+ symporter family (27). However, the physiological function of NHD1 has not been studied in a mutant thus far. Homozygous individuals of the T-DNA line *nhd1-1* were isolated (Fig. S1 *A* and *D*) but did not show phenotypic differences from Col-0 under normal long-day conditions (Fig. S4*A*). To investigate a possible connection between the plastidial KEA transporters and NHD1, homozygous *kea1-1kea2-1nhd1-1* mutant plants were isolated. The plants showed a more dramatic phenotype compared with *kea1-1kea2-1* mutants (Fig. S4*A*); they were extremely stunted in growth with small uniformly pale green leaves but eventually produced a small number of homozygous seeds. Moreover, the growth rate of *kea1-1kea2-1nhd1-1* mutants could also be improved by 75 mM NaCl treatment (Fig. S4*B*).

***kea1kea2* and *kea3* Mutants Reveal Altered pH Gradient (ΔpH) Across the Thylakoid Membrane Compared with Col-0.** KEA1 and KEA2 are predicted to exchange K^+ ions with H^+ electroneutrally across the inner envelope membrane (23), whereas KEA3 acts in the thylakoid membrane. To determine if a lack of transport activity by the plastidial KEAs affects chloroplast pH homeostasis and thus the proton motive force (pmf) across the thylakoid membrane in vivo, electrochromic shift measurements were carried out (28). The ΔpH component is the major contributor to the pmf across the thylakoid membrane (29). The two independent *kea1kea2* mutant alleles displayed a significant decrease in total pmf by ∼20% compared with Col-0 plants (Fig. 5*A*). Although only minor changes in the electric field component (ΔΨ) of the pmf were found, a significant loss in ΔpH (24–30%) was detected in the mutants (Fig. 5*A* and Fig. S5*A*). Two independent *kea3* single mutant lines displayed approximate Col-0 levels of pmf but possibly a slightly altered partitioning between the ΔΨ and the ΔpH, namely higher ΔpH and lower ΔΨ than in Col-0 (Fig. S5*B*). In line with these findings, mutant plants displayed altered ability to acclimate to excess light, as indicated by measurements of nonphotochemical quenching (NPQ) (Fig. 5*B* and Fig. S5*C*). Interestingly, in salt treatments energy-dependent quenching (qE) recovered only to a moderate extent in *kea1kea2* and strongly increased in the wild-type control plants (Fig. S3*F*).

Discussion

The molecular identity of chloroplast K^+ transporters has remained unknown and direct genetic studies analyzing the physiological roles of K^+ transporters in chloroplast function are needed. Here we present findings of three members of the KEA-family, KEA1, KEA2, and KEA3. All three transporters target to the chloroplast, although to different membranes. Their loss-of-function has dramatic impact on photosynthetic performance, chloroplast structure, and plant growth.

The characteristic ring-shaped Venus fluorescence signal of KEA1 and KEA2 surrounding the chlorophyll fluorescence of chloroplasts (Fig. 1*D*), and the concomitant phenotype complementation, show that both Venus-tagged transporters are targeted to the chloroplast envelope membrane in vivo. Consistent with

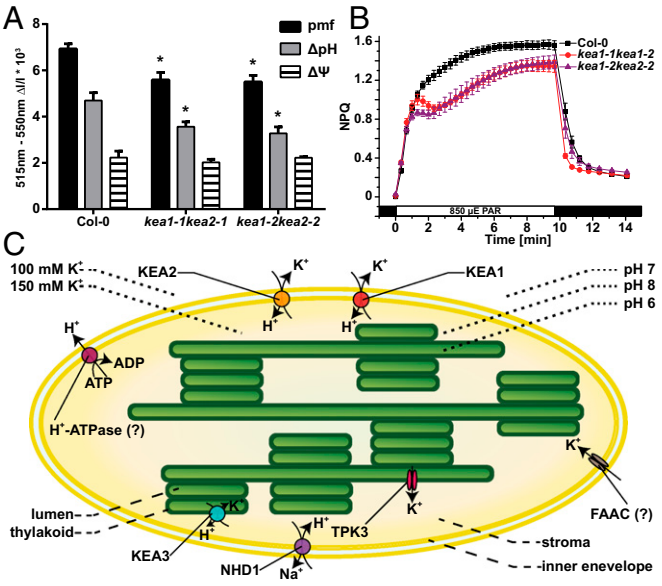

Fig. 5. (*A*) The proton motive force across the thylakoid membrane is compromised in both *kea1kea2* alleles because of decreased ΔpH ($n = 5 \pm$ SEM, $*P < 0.02$). ΔΨ was not significantly changed. (*B*) NPQ was consistently decreased in *kea1kea2* mutant lines. (*C*) Model of cation/H^+ exchange in the chloroplast. KEA1 and KEA2 are localized in the inner envelope membrane. Driven by the H^+ gradient generated by an envelope H^+-ATPase, both transporters are modeled to counter-exchange K^+ ions. NHD1 was proposed to transport Na^+ ions in the same manner and direction. KEA3 targets to the thylakoid membrane. The low pH of the thylakoid lumen drives lumenal K^+ uptake by KEA3 and release by TPK3.

this finding, a recent proteomic study on outer and inner envelope fractions detected KEA1 and KEA2 peptides in the inner envelope membrane (30). Additional evidence comes from an N-terminal fragment of KEA2 which targets to chloroplasts of *A. thaliana* cotyledons (23). Although KEA3 was also found in the chloroplast (Fig. 2*A*), the signal was distinct from KEA1 and KEA2 (Fig. 1*D*). An overlay with the chlorophyll fluorescence indicated that KEA3 could be targeted to the thylakoids. These results were confirmed by immunoblot analyses of chloroplast membrane fractions (Fig. S2*D*).

The characterization of isolated amiRNA *kea1/2* and T-DNA *kea1-1kea2-1* mutants revealed strong phenotypic defects that led to decreased photosynthetic activity, pale leaves, and stunted plant growth (Fig. 1 *A–C* and Fig. S2*A*). Observed phenotypes were only present if *KEA1* and *KEA2* transporter genes were disrupted or decreased in transcription simultaneously. The *kea1-1kea2-1kea3-1* mutant, which in addition lacks putative thylakoid K^+/H^+ transport, was barely viable on soil and died before producing seeds (Fig. 2*B*). TEM of *kea* double and triple mutants showed progressive morphological damage of chloroplasts most likely caused by osmotic swelling (Fig. 3). Disturbance of the chloroplast osmotic potential was reported before with similar damage: that is, chlorotic leaves, stunted growth, and decreased photosynthesis (31). Because of the lower grana size a role of plastidial KEAs in chloroplast development cannot be ruled out. KEA1 was also found in proplastid envelope membranes and might have an important function early on (32).

Known plasma membrane high- and low-affinity K^+ transporters contribute to plant growth and development (33, 34), but to date no single or higher-order loss-of-function plant mutant has been found that has a detrimental phenotype as strong as *kea1-1kea2-1* double or the *kea1-1kea2-1kea3-1* triple mutants (1, 26, 35, 36). This finding highlights the biological relevance of chloroplast K^+ transporters identified here and in an earlier biochemical study (23).

Based on the findings, we propose a model that integrates our results with present data (Fig. 5*C*). Although the molecular identity has not been solved yet, transport activity of an outward pumping H^+-ATPase and an inward directed fast-activated chloroplast channel (FACC) have been measured at the envelope membrane (37, 38). FACC seems to have little preference and showed conductivity for all physiologically essential inorganic cations (K^+, Na^+, Ca^{2+}, Mg^{2+}) (38). To avoid bursting of the organelle, electro-neutral K^+ transporters have been postulated for chloroplasts and mitochondria that mediate organelle K^+ efflux (8).

Other related secondary active ion/H^+ exchangers are known to fulfill a dual role (i.e., osmotic potential and pH regulation of the vacuole and other intracellular compartments) (16). Throughout the day, a pH gradient over the envelope membrane is built up by H^+-ATPases and H^+ pumping into the thylakoid lumen to ensure pH 8 in the stroma. A stromal pH 8 is crucial for photosynthesis because Calvin-Benson cycle enzyme activities are pH-dependent (7). The pH gradient represents a strong driving force for secondary active H^+-coupled transport at the inner envelope and is used for Fe^{2+} uptake into chloroplasts by an unknown transporter or Na^+ release by NHD1 in counter exchange with H^+ (27, 39). The prevailing pH gradient across the inner envelope membrane throughout the day calls for a KEA1 and KEA2 function in which they release K^+ from the chloroplast in exchange for H^+ influx to avoid osmotic swelling and bursting of the organelle. Uptake of H^+ is balanced by either the envelope H^+-ATPases or H^+ uptake into the thylakoid lumen coupled to photosynthetic electron transport (Fig. 5*C*). The size of pmf across the thylakoid membrane not only depends on light but also on the stromal pH of the chloroplast (40). Intriguingly, the lack of envelope K^+/H^+ exchange in *kea1kea2* mutants caused downstream effects leading to a decreased ΔpH across the thylakoid membrane. This effect leads to reduced photoprotection against high light, as indicated by lower levels of NPQ in the double mutants compared with Col-0 (Fig. 5 *A* and *B*).

Recently a K^+ efflux channel in the thylakoid lumen has been identified as TPK3 (41). This channel seems to play an important role in the ΔΨ component of the pmf (41). The existence of a K^+ channel calls for a K^+ loading mechanism into the thylakoid lumen. KEA3 is a likely candidate because the steep pmf across the thylakoid membrane of up to 2.5 units in the light could drive K^+/H^+ antiport mediating K^+ uptake into the thylakoid lumen (Fig. 5*C*). NhaS3 from *Synechocystis* is the closest CPA2 relative to KEA3 with a proposed function. This thylakoid Na^+/H^+ antiporter uses the H^+ gradient to sequester Na^+ into the thylakoid lumen (10). The lack of thylakoid KEA3-mediated K^+/H^+ exchange in *kea3* mutants resulted in slightly increased ΔpH and compensatory decreased ΔΨ across the thylakoid membrane (Fig. S5*B*). These are opposite effects to those recently described for mutants lacking TPK3 activity (41), suggesting that both the K^+ channel and KEA3 modulate the partitioning of pmf through K^+ ion counter-exchange across the thylakoid membrane. The function of plastidial thylakoid and envelope membrane KEAs are linked. K^+ released from the thylakoids during proton pumping via the channel can leave the chloroplast via KEA1 and KEA2. In exchange, H^+ is resupplied to the stroma, thereby enabling continued operation of the electron transport chain across the thylakoid membrane.

The envelope Na^+/H^+ antiporter NHD1 has been suggested to release Na^+ from the chloroplast taken up by Na^+-dependent transporters at the inner envelope (27), but no *nhd1* plant mutants have been studied thus far. Under normal growth conditions no phenotype was visible in *nhd1-1* (Fig. S4*A*). However, if NHD1 function was impaired in the *kea1-1kea2-1* background, the phenotype was aggravated with plants growing extremely slow and only low amounts of seeds (Fig. S4*A*). This finding indicates requirement for a well-balanced set of cation/H^+ transporters in the chloroplast inner envelope membrane of *A. thaliana*, which might have overlapping substrate specificity and therefore functionally overlap to some extent. Thus, in the future *kea1-1kea2-1* can be used to identify new ion transporters and channels in chloroplasts.

Previously, in vitro assays revealed that the half-length KEA2 fragment was also capable to exchange Na^+ ions for H^+ with lower affinity (23). Surprisingly, in *kea1-1kea2-1* mutant plants that lack KEA1 and KEA2 transport activity, exogenous NaCl in the growth media clearly alleviated the phenotypic defects (Fig. 4 *A* and *E*). Additionally, mutant plant chlorophyll content and photosynthetic activity recovered almost to wild-type level (Fig. S3*E* and Fig. 4*B*). This phenomenon cannot be explained in a simple manner. Isolation of intact chloroplasts for organelle-specific inductively coupled plasma measurements were unsuccessful because *kea1-1kea2-1* control plastids constantly burst in isolation buffers. However, as reported, elevated NaCl concentration in the media leads to an increase of ions in the cytosol followed by sequestration into the vacuole (11). The ion accumulation would decrease the water potential relative to the chloroplasts. Thus, less water would diffuse into *kea1-1kea2-1* mutant chloroplasts, preventing them from swelling and alleviating damage. Plants do not take up sorbitol, which explains the absence of beneficial effects as found in the case of salt (Fig. 4*D*).

In summary, the findings presented here show that KEA1 and KEA2 in the inner envelope and KEA3 in the thylakoid membrane are essential for chloroplast stability, osmoregulation, and maintenance of thylakoid membrane density.

Materials and Methods

A MAXI-IMAGING-PAM fluorometer (Walz Instruments) was used for in vivo chlorophyll *a* fluorescence assays on intact dark-adapted plants. For phenotype quantification, a light induction curve using standard settings was recorded. Procedures and analyses are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank E. Boutet for the template http://commons.wikimedia.org/wiki/File:Chloroplast.svg. KEA functional research was funded by the Division of Chemical, Geo, and Biosciences, Office of Basic Energy Sciences, US Department of Energy Grant DE-FG02-03ER15449 and in part by National Science Foundation Grant MCB0918220 (*Fv/Fm* experiments); National Institute on Environmental Health Sciences Grant P42ES010337 (to J.I.S.); the Swedish Research Council (experiments in Fig. 5 *A* and *B* and Fig. S5) (C.S.); and a Human Frontier Science Program Long-Term fellowship and an Alexander von Humboldt Feodor Lynen fellowship (to H.-H.K.).

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