Cryo-EM structures of thylakoid-located voltage-dependent chloride channel VCCN1

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In the light reaction of plant photosynthesis, modulation of electron transport chain reactions is important to maintain the efficiency of photosynthesis under a broad range of light intensities. VCCN1 was recently identified as a voltage-gated chloride channel residing in the thylakoid membrane, where it plays a key role in photoreaction tuning to avoid the generation of reactive oxygen species (ROS). Here, we present the cryo-EM structures of Malus domestica VCCN1 (MdVCCN1) in nanodiscs and detergent at 2.7 Å and 3.0 Å resolutions, respectively, and the structure-based electrophysiological analyses. VCCN1 structurally resembles its animal homolog, bestrophin, a Ca²⁺-gated anion channel. However, unlike bestrophin channels, VCCN1 lacks the Ca²⁺-binding motif but instead contains an N-terminal charged helix that is anchored to the lipid membrane through an additional amphipathic helix. Electrophysiological experiments demonstrate that these structural elements are essential for the channel activity, thus revealing the distinct activation mechanism of VCCN1.
photosynthesis by plant cells is a crucial metabolic reaction that generates oxygen and carbohydrates. In this reaction, light energy is absorbed and converted into chemical molecules, including ATP, at the thylakoid membrane of chloroplasts. Absorbed light energy drives water oxidation and H⁺ transport by the photosystem II (PSII) and cytochrome b6f complex, to generate the proton motive force (PMF) across the membrane. The PMF, which is widely conserved among plants, dissipates excessive light photochemical quenching (NPQ) at the thylakoid membrane, located two-pore K⁺ channels, and activates ATP synthesis.

Although light-induced PMF generation is necessary, excessive photoreaction induces the generation of reactive oxygen species (ROS), which cause serious damage to chloroplasts. Therefore, plants have adopted various regulating systems to control excess photon production. For example, non-photochemical quenching (NPQ) at the thylakoid membrane, which is widely conserved among plants, dissipates excessive light energy as heat. The acidic luminal environment formed by the H⁺ uptake enhances NPQ, and therefore the PMF is mainly stored as ΔΨ. The thylakoid membrane is dissipated by ion counterbalancing across the thylakoid membrane, via cation efflux and anion influx by various membrane transporters and channels. The thylakoid-localized two-pore K⁺ channel, TPK3, functions in cation efflux. For NPQ downregulation, the K⁺/H⁺ antiporter KEA3 reportedly effluxes H⁺ to adjust photosynthesis for the transition from high to low light. As for anion influx, patch-clamp studies suggested the existence of a voltage-gated anion channel in the thylakoid membrane, which could play a key role in rapid Cl⁻ influx in response to the ΔΨ change caused by H⁺ uptake. VCCN1, the voltage-gated Cl⁻ channel localized in the thylakoid membrane, was identified by mutant screening using a mutagenic alkylating agent (Ethyl Methanesulfonate; EMS) according to the diminished NPQ. Further mutant analyses revealed that VCCN1 plays a key role in partitioning the PMF and contributes to the fine-tuning of photosynthesis in the transition from low to high light (Supplementary Fig. 1).

VCCN1 belongs to the bestrophin family, which is widely conserved among kingdoms. In animal cells, bestrophin channels function as Ca²⁺-activated Cl⁻ channels, and mutations of BEST1, the most functionally characterized protein among the bestrophin channels, are associated with retinal diseases, including vitelliform macular dystrophy. The structures of bestrophin channels and their bacterial homologs are well characterized; both form a homopentamer with each subunit containing four transmembrane helices. Furthermore, bestrophin channels are gated by Ca²⁺-binding to the conserved motif termed the “Ca²⁺ clasp” and by a long C-terminal inactivation peptide. A previous electrophysiological experiment using cell-free expressed AtVCCN1 proteins reconstituted in planar lipid bilayers revealed voltage-sensitive anion channel activity, suggesting that VCCN1 is predominantly regulated by the membrane voltage. Accordingly, VCCN1 has low amino acid sequence similarity with bestrophin channels — 9% sequence identity between Arabidopsis thaliana VCCN1 (AtVCCN1) and human Bestrophin 1 (HsBEST1) — and is predicted to lack both the Ca²⁺ clasp and long C-terminal petide. Instead, VCCN1 has a unique N-terminal sequence that is not present in bestrophin channels. These differences between the animal and plant homologs suggest that the regulatory mechanism of VCCN1 is distinct from those of bestrophin channels.

In this work, we present the cryo-EM structures of the apple VCCN1 in lipid nanodiscs and in detergent micelles. Our structures, together with electrophysiological experiments, provide insights into the channel properties and gating mechanism of VCCN1.

**Results**

**Functional characterization and structure determination of MdVCCN1.** We first created various constructs of plant VCCN1 homologs with truncations of the N-terminal chloroplast targeting peptides, and screened them by fluorescence detection size-exclusion chromatography (FSEC). One of the truncated constructs from the apple VCCN1 protein (MdVCCN1; “Md” refers to Malus domestica, and hereafter referred to as the “wild-type” construct), which shares 73% sequence identity with AtVCCN1 (Supplementary Fig. 2), showed a monodisperse FSEC profile with high expression. We next assessed the functional properties of MdVCCN1 by whole-cell patch-clamp recordings. HEK293 cells expressing MdVCCN1 showed outwardly rectifying currents (Fig. 1a), and the channel activity was diminished by the addition of a Cl⁻ channel inhibitor, 4,4‘-disothiocyanato-2,2‘-stilbenedisulfonic acid; DIDS (Fig. 1b, c), consistent with the previous experiment using the AtVCCN1 proteins reconstituted in a lipid environment.

**Fig. 1 Functional characterization of MdVCCN1.** a Whole-cell current-voltage relationships in HEK293 cells expressing MdVCCN1 (n = 7), expressing GFP by transfection of the empty GFP co-expression vector (n = 10), and non-transfected HEK293 cells (n = 9). Error bars represent s.e.m. Source data are provided as a Source Data file. b Whole-cell current-voltage relationships in HEK293 cells expressing MdVCCN1, before (black) and after (red) the addition of 0.5 mM DIDS (n = 8). Error bars represent s.e.m. Source data are provided as a Source Data file. c Representative traces of whole-cell currents in HEK cells expressing MdVCCN1, before and after the addition of 0.5 mM DIDS to the bath solution. Source data are provided as a Source Data file.
To further investigate the physiological properties of VCCN1, we evaluated its topology in the thylakoid membrane. The ‘positive-inside rule’ is also applicable to thylakoid membrane proteins: positively charged residues are more likely to be located within the stromal side of thylakoids (corresponding to the cytoplasmic side in cellular membranes)\(^2\). Based on this rule, the C-terminus of VCCN1 is predicted to be in the stroma. To experimentally prove this prediction, we conducted a protein digestion assay with C-terminally monomeric GFP-fused AtVCCN1 (AtVCCN1-mGFP), using chloroplasts isolated from Arabidopsis thaliana. The fluorescence of the AtVCCN1-mGFP was quenched upon the treatment with endoproteinase Arg-C, whereas such quenching was not observed without the proteinase treatment (Fig. 2a, b). Western blotting analyses confirmed that this quench is due to mGFP digestion, whereas a thylakoid protein, PsbO, one of the major components in the PSI core complex, was not digested by this treatment (Fig. 2c). These results demonstrated that the C-terminus of VCCN1 is exposed to the stroma, thus supporting our predicted topology in chloroplasts.

We then performed single-particle cryo-EM analyses of MdVCCN1 in detergent micelles (glyco-diosgenin; GDN), and in nanodiscs to investigate the structure in a native-like membrane environment (Supplementary Figs. 3–5). The structures were determined at 3.0 Å and 2.7 Å, respectively, with root mean square deviation (RMSD) values below 0.52 Å for 329 residues. The overall structures were determined bestrophin channel structures from chicken Bestrophin 1 (GgBEST1; PDB: 4WD8), with amino acids 84 to 419 in GDN and 78 to 418 in nanodiscs (Fig. 3b and Supplementary Fig. 6a). Both structures are basically similar, with an N-terminal amphipathic helix (NAH), an N-terminal acidic residue (NCH), and two short stromal helices (S1 and S2). The NAH and S2 helices are amphipathic and run parallel to the membrane, while the NCH and S1 helices are located on the outer and inner sides of the transmembrane region, respectively, and TM3 and TM4 are sandwiched by these helices (Fig. 3b, e). TM2 constitutes the pentameric interface, and thus forms a central ion permeation pore. The NTD is composed of an N-terminal amphipathic helix (NAH), an N-terminal charged helix (NCH), and two short stromal helices (S1 and S2). The NAH and S2 helices are amphipathic and run parallel to the membrane, while the NCH and S1 helices are located on the stromal side (Fig. 3c, d). The NAH and NCH helices are only observed in VCCN1, whereas the S1 and S2 helices are also conserved in bestrophin channels. The SMD is composed of the C-terminal half of the long TM2 helix and six stromal helices (S3–S8). The previously determined GgBEST1 structures, the long loop between the TM4 and S8 helices contains a cluster of acidic residues and constitutes the Ca\(^{2+}\) clasp, which plays an essential role in the Ca\(^{2+}\)-dependent activation of bestrophin channels\(^3\) (Supplementary Figs. 7 and 8). By contrast, the corresponding loop of MdVCCN1 lacks these acidic residues (Supplementary Fig. 7). The C-terminus of the bestrophin channel forms a long loop (> 200 amino acids) that plays an essential role in channel inactivation, by allosterically interacting with the cytosolic surface\(^25\). By contrast, the C-terminus of VCCN1 is not involved in the channel regulation observed in bestrophin channels.

### Ion permeation pathway

The ion permeation pathway of MdVCCN1 is formed by the TM2 and S7 helices from each subunit and located along the central axis, perpendicular to the membrane plane (Fig. 4a). The present structure revealed two constrictions along the pathway: one is located at the membrane region and the other is at the stromal end (Fig. 4a and Supplementary Fig. 6c). These constrictions at the membrane and the stromal end are conserved in other homolog structures and called

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**Fig. 2 Topology evaluation of VCCN1 in chloroplasts.** a Representative images of mGFP digestion by Arg-C on isolated chloroplasts with transient expression of AtVCCN1-mGFP. The scale bar represents 50 μm. b Quenching level of GFP fluorescence with or without Arg-C treatment for 60 min. GFP signals were detected with a fluorescent microscope. The GFP signal was normalized with the chlorophyll autofluorescence signal. Error bars represent mean ± S.D. from independent experiments (n = 3). **P < 0.01, ***P < 0.001, two-sided student’s t test. Exact P values are as follows, **P = 0.00329, ***P = 0.00099. Source data are provided as a Source Data file. c Western blotting analyses of AtVCCN1-mGFP and PsbO, with or without Arg-C treatment. AtVCCN1-mGFP and PsbO were immunoblotted by an anti-GFP antibody and an anti-PsbO antibody, respectively. The experiment was repeated three times independently, with similar results.

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**Overall structure.** The folding and subunit assembly of MdVCCN1 are consistent with those of the two previously determined bestrophin channel structures from chicken Bestrophin 1 (GgBEST1; the “Gg” refers to Gallus gallus) and bovine Bestrophin 2 (BbBEST2; the “Bb” refers to Bos taurus), as well as the bacterial homolog structure\(^18\)–\(^20\) (Fig. 3b–e). Since GgBEST1 is the best characterized channel, in terms of the structural and functional aspects among the previously studied species, we compared MdVCCN1 with GgBEST1. MdVCCN1 forms a symmetric homopentamer, with each subunit consisting of three domains: transmembrane domain (TMD), N-terminal domain (NTD), and stromal domain (SMD) (Fig. 3b). The TMD is composed of four transmembrane (TM) helices. TM1 and TM2 are located on the outer and inner sides of the transmembrane region, respectively, and TM3 and TM4 are sandwiched by these helices (Fig. 3b, e). TM2 constitutes the pentameric interface, and thus forms a central ion permeation pore. The NTD is composed of an N-terminal amphipathic helix (NAH), an N-terminal charged helix (NCH), and two short stromal helices (S1 and S2). The NAH and S2 helices are amphipathic and run parallel to the membrane, while the NCH and S1 helices are located on the stromal side (Fig. 3c, d). The NAH and NCH helices are only observed in VCCN1, whereas the S1 and S2 helices are also conserved in bestrophin channels. The SMD is composed of the C-terminal half of the long TM2 helix and six stromal helices (S3–S8). The previously determined GgBEST1 structures, the long loop between the TM4 and S8 helices contains a cluster of acidic residues and constitutes the Ca\(^{2+}\) clasp, which plays an essential role in the Ca\(^{2+}\)-dependent activation of bestrophin channels\(^3\) (Supplementary Figs. 7 and 8). By contrast, the corresponding loop of MdVCCN1 lacks these acidic residues (Supplementary Fig. 7). The C-terminus of the bestrophin channel forms a long loop (> 200 amino acids) that plays an essential role in channel inactivation, by allosterically interacting with the cytosolic surface\(^25\). By contrast, the C-terminus of VCCN1 is not involved in the channel regulation observed in bestrophin channels.
A comparison between the structures of the closed and Ca^{2+}-bound open GgBEST1 states revealed that the neck widens upon channel activation, while the aperture lacks significant conformational changes, demonstrating that the neck is mainly responsible for channel gating. In the GgBEST1 structure, the neck is formed by a set of three hydrophobic residues (Ile76, Phe80, and Phe84) (Fig. 4b). Similarly, in the MdVCCN1 structure, the neck is composed of a set of three hydrophobic residues (Pro184, Leu188, and Phe192) (Fig. 4a, b and Supplementary Fig. 2) and constitutes the narrowest constriction along the pore, with a 1.40 Å radius, which is too narrow to allow the permeation of dehydrated Cl^{-} (1.81 Å) (Fig. 4a). Therefore, the present MdVCCN1 structure represents the closed state.

The aperture of MdVCCN1 is formed by Ser301 from each subunit (Fig. 4c and Supplementary Fig. 2). In GgBEST1, Val205 constitutes the aperture and its side chain blocks hydrated Cl^{-} permeation. By contrast, MdVCCN1 has a wider aperture (3.30 Å), which is too narrow to allow the permeation of hydrated Cl^{-} (1.81 Å) (Fig. 4a). Therefore, the present MdVCCN1 structure represents the closed state.

Overall, the aperture of MdVCCN1 does not seem to function as the selective filter, in contrast to GgBEST1. It should be noted that the GgBEST1 mutant with a wider aperture shows less selective permeability even between SCN^{-} and other anions, suggesting the distinct mechanisms of ion selectivity between VCCN1 and BEST1.

Subunit interface. While the subunit interface is mainly formed by hydrophobic interactions through TM2, TM3, and S7, a pair of non-hydrophobic residues, Glu195 in TM2 and Tyr332 in TM3, forms a direct hydrogen bond in the transmembrane region between the adjacent subunits. These two residues are highly conserved among the VCCN1 proteins, suggesting the importance of this hydrogen bond (Supplementary Fig. 2). Accordingly, we hypothesized that this hydrogen bond contributes to maintaining the structural integrity of the neck region and thereby the proper channel gating.
or more (Fig. 5b and Supplementary Fig. 9a–e). However, the Y332F mutant exhibited greatly reduced whole-cell currents, whereas the Y332A mutant showed only slightly reduced currents (Fig. 5b and Supplementary Fig. 9f, g). We also performed an FSEC analysis for all mutants to check their pentameric assembly (Supplementary Fig. 10a, b). A cell-surface biotinylation assay of E195A and Y332F, representative mutants with greatly reduced currents, was also performed to check plasma-membrane targeting of the mutants (Supplementary Fig. 10d), and suggested that the decrease in the whole-cell current is due to their reduction in each channel activity. Taken together, while all of these mutations designed to disrupt the hydrogen bond between Glu195 and Tyr332 diminished the channel activity, the Y332A mutation exceptionally retains the comparable whole-cell current. The bulky sidechain of Tyr332, located at the interface of the two TM2 helices, is reminiscent of Trp287 of GgBEST1, which is known to be critical for the channel gating in GgBEST1. By the structural analogy, we assumed that the conformational change of the Tyr332 residue triggers MdVCCN1 gating via the hydrogen bond interaction, and that the Y332A mutation may mimic the state after the conformational change. Accordingly, to test our hypothesis, we determined the structure of the MdVCCN1 Y332A mutant reconstituted in nanodiscs by cryo-EM (Supplementary Figs. 11 and 12). The overall assembly and subunit folding of the Y332A mutant are quite similar to those of the WT structure in nanodiscs, with RMSD values below 0.36 Å for 335 Cα atoms (Fig. 5c), demonstrating that the Y332A mutation does not affect the overall structural integrity. In the mutant structure, however, the Glu195 residue flips toward the central axis, and the Phe192 residue in the neck flips away from the central axis and resides in the space occupied by Tyr332 in the WT structure (Fig. 5d, e and Supplementary Fig. 13). Consequently, Glu195 is directly exposed to the ion permeation pathway, and thus may affect the anion permeability. Notably, this local conformational change of the Phe residue was similarly observed in the open structure of GgBEST1, while the other neck residues remained in the closed conformation (Fig. 5d). Consistently, the pore radius of the Y332A mutant was widened only at the position of Phe192 (Fig. 5e). Thus, the Y332A mutant seems to mimic the ‘partially opened’ state of VCCN1, whereas the whole neck opening requires larger movements, triggered by the conformational change of Tyr332. Taken together, these electrophysiological and structural analyses demonstrated that the inter-helical interactions near the neck region are important for channel activity in the VCCN1 family.

**N-terminal extension.** On the luminal side of the MdVCCN1 structure, the N-terminal 40 residues are folded into two short α-helices, an N-terminal amphipathic helix (NAH) and an N-terminal charged helix (NCH), which is a unique feature in VCCN1 and not conserved in the previously determined homolog structures (Supplementary Fig. 7). The NAH runs parallel to the membrane,
with its hydrophobic side directly anchored to the membrane plane (Figs. 3c, d and 6a; Supplementary Fig. 14a). The NCH forms an intra-subunit interaction with the N-terminal tip of the TM1 helix and an inter-subunit interaction with the S1 helix of the adjacent subunit (Figs. 3c, d and 6a). Notably, the NAH is only visualized in the nanodisc structure and is disordered in the detergent micelles (Supplementary Fig. 14b), indicating that the lipid environment is essential to stabilize this helix.

To investigate the functional roles of NAH and NCH, we performed whole-cell patch-clamp recordings using various truncated or mutated constructs of the two helices (Supplementary Fig. 14b), indicating that the lipid environment is essential to stabilize this helix.

Discussion

In this study, we determined the cryo-EM structures of MdVCCN1, a plant homolog of bestrophin channels, in detergent micelles and in nanodiscs. As in other bestrophin family proteins, the overall structure exhibits a pentameric assembly with the ion permeation pathway along the central axis, which contains two constriction sites: neck and aperture. The neck of MdVCCN1 consists of three hydrophobic residues and is likely to restrict the permeation pathway along the central axis, which contains two constriction sites: neck and aperture. The neck of MdVCCN1 consists of three hydrophobic residues and is likely to restrict the ion permeation of MdVCCN1 in the closed state, similar to bestrophin channels. The other constriction site, the aperture, has properties and functions of the apertures may vary among the species.

or less than that of ΔN (Fig. 6b), indicating the essential role of the charged residues for the channel activity. Taken together, these results suggest that the electric charge distribution in NCH and its interaction with the lipid membrane through NAH contribute to the channel function of MdVCCN1.

Fig. 5 The hydrogen bond between Glu195 and Tyr332. a Close-up view of the neck and the hydrogen bond between Glu195 and Tyr332. b Whole-cell current densities at +80 mV in HEK293 cells expressing MdVCCN1 wild-type, Glu195 mutant, or Tyr332 mutant (wild-type (n = 16), E195A (n = 11)), E195D (n = 7), E195L (n = 8), E195Q (n = 9), E195R (n = 8), Y332A (n = 10), Y332F (n = 7)). Error bars represent s.e.m. Source data are provided as a Source data file. c Comparison of Cα traces of the MdVCCN1 Y332A mutant (violet) and wild-type (green). d Neck structures of the MdVCCN1 Y332A mutant (upper panels) and wild-type (lower panels), in which the left panels are views from the side and the right panels are from the top. e Pore radii of the MdVCCN1 Y332A mutant (violet) and wild-type (green) along the center axis of the channels. Black and red dashed lines represent the radii of dehydrated and hydrated Cl−, respectively.
lumen and that the NCH of VCCN1 is located in the stroma, VCCN1 might not be activated by the change of AP', but by other secondary signals on the stromal side derived from the light-induced PMF generation. While previous studies focused only on the physiological aspects of VCCN1, the current study for the first time has revealed the existence of the N-terminal extension, which is unique in VCCN1 and essential for channel function.

The structural comparison between MdVCCN1 and GgBEST1 revealed that the NCH and the following loop of MdVCCN1 are located at positions structurally similar to those of the Ca<sup>2+</sup>-clasp and the C-terminal inactivation peptide in GgBEST1, on the stromal/cytosolic surface of the channels (Supplementary Figs. 8 and 16). These structural analogies suggest that the N-terminal extension of MdVCCN1 is involved in the channel regulation in VCCN1, which is consistent with our patch-clamp experiments on the mutants lacking these regulatory elements. As a result, the N-terminal extension enables VCCN1 to be activated by a distinct stimulus from that of bestrophin, but in a similar manner. In bestrophin channels, the structural changes in the Ca<sup>2+</sup>-clasp are propagated to the neck region to open the channel pore. Similarly, in VCCN1, the membrane voltage probably induces conformational changes in the NTD, which may be propagated to the neck region through inter-helical interactions, including the hydrogren bond between Glu on TM2 and Tyr on TM3. Although further functional and structural studies are required to clarify this gating mechanism, the present study provides the structural framework for understanding the molecular mechanisms of VCCN1, which plays a key role in photoreaction tuning. Recently, the detailed kinetics of VCCN1 in the photoreaction was investigated by using computer simulation. This work suggested that further understanding of the NPQ regulation mechanisms and underlying kinetic features of ion channels and transporters in the thylakoid membrane can be applied to genetic engineering in plants, to optimize energy transduction in the photoreaction. Therefore, our findings help pave the way for such applications in bioenergetics.

**Methods**

**Protein expression, purification, and sample preparation for cryo-EM.** The region encoding the N-terminally truncated MdVCCN1 protein (accession number XP_028961536.1), which lacks the N-terminal 61 amino acids (Leu2–Asp68) and contains one natural mutation (S207A), was amplified from a cDNA library prepared from Malus domestica leaves (ZYAGEN) and inserted into the pEG BacMam vector<sup>28</sup>, with a C-terminal enhanced green fluorescent protein (eGFP)-His<sub>8</sub> tag and a tobacco etch virus (TEV) protease cleavage site. Baculoviruses were generated in Spodoptera frugiperda Sf9 cells (American Type Culture Collection, catalog no. CRL-1711), using the Bac-to-Bac system (Invitrogen). HEK293s GnTI<sup>−</sup> (N-acetylglucosaminyl-transferase I-negative) cells (American Type Culture Collection, catalog no. CRL-3022) were cultured in Freestyle 293 medium (Invitrogen) supplemented with 2% FBS (Sigma-Aldrich) in the presence of 8% CO<sub>2</sub>, and infected by a P2 baculovirus at a density of approximately 3 × 10<sup>6</sup> cells ml<sup>−1</sup>. After a 24 h incubation at 37 °C, the culture was supplemented with 5 mM sodium butyrate and incubated at 30 °C for 10 h. The cells were collected by centrifugation (6,000 × g, 20 min, 4 °C), and solubilized for 1 h at 4 °C in solubilization buffer (300 nM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 1% n-dodecyl-β-D-maltoside (DDM; Calbiochem)). Insoluble materials were removed by ultracentrifugation (186,000 × g, 1 h, 4 °C). The detergent-soluble fraction was incubated with CNBr-Activated Sepharose 4 Fast Flow beads (GE Healthcare) coupled with an anti-GFP nanobody (GFP enhancer) for 2.5 h at 4 °C.

While the bestrophin channel is activated by Ca<sup>2+</sup>, VCCN1 reportedly serves as a voltage-gated Cl<sup>−</sup> channel<sup>14,15</sup>. Consistently, our MdVCCN1 structure lacks the cluster of acidic amino acids that constitutes the Ca<sup>2+</sup>-binding motif of bestrophin channels<sup>24</sup>. Instead, VCCN1 contains two N-terminal helices (NAH and NCH) that play crucial roles in the channel gating. Typical voltage-gated ion channels (VGIC) contain a cluster of positively charged residues on the transmembrane helix, known as the S4 segment, which serves as the voltage sensor<sup>26,27</sup>. The current structure revealed that VCCN1 has a similar cluster of charged residues on the NCH in the N-terminal extension, but it is located on the stromal side and not embedded in the membrane, unlike the S4 segment. This structural difference may explain the different voltage dependency profiles between VCCN1 and VGIC. VCCN1 was first identified as a voltage-gated anion channel in thylakoid membranes, based on the phenotypes of the mutants<sup>14,13</sup>, but our electrophysiological experiments, as well as the previous research on AtVCCN1<sup>15</sup>, revealed the much lower voltage-dependency of VCCN1, as compared to those of typical VGICs. Although this weak voltage-dependence might be partly due to our experimental environments, which may differ from thylakoid membranes in many aspects, it is conceivable that the voltage-gating profile of VCCN1 may be distinct from those of canonical VGICs. Furthermore, considering that the activation of VCCN1 is associated with the pH change in the chloroplast...
Nanodisc reconstitution and sample preparation for cryo-EM. Membrane scaffold protein (MSP13D1) purification and protein reconstitution into nanodiscs were performed as previously described, with some modifications. L-α-phosphatidylcholine (Soy) (SoyPC) dissolved in chloroform (Avanti) was dried to a powder, and the resulting films were reconstituted into liposomes with the van der Waals radii of the ion pathway were calculated using the HOLE program. The figures depicting the molecular structures were prepared with CootEM (http://www.cuemol.org/).

Patch-clamp. For patch-clamp experiments, HEK293T cells (American Type Culture Collection, catalog no. CRL-11268) were cultured on polyleucine treated coverslips for 24 h, and then transfected with the receptor constructs, using Lipofectamine 2000 (Invitrogen), and the mdVCCN1 constructs were over-expressed by the pIREs2-AcGFP1 vector. Whole-cell patch-clamp recordings were then performed 36 h after transfection. The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose and 10 mM HEPES, pH 7.4. Borosilicate pipettes (Harvard Apparatus), with a resistance of 5–8 MΩ, were filled with the pipette solution (148 mM tGCl, 0.5 mM CaCl2, 2 mM MgCl2, 2 mM EGTA, 10 mM NaCl and 10 mM HEPES, pH 7.2). For selectivity experiments, the NaCl was replaced by NaBr, NaI, NaNO3, and NaSCN. The final concentration of DIDS was 0.5 mM in the bath solution. Currents were measured before and after the addition of DIDS. The voltage was clamped and currents were recorded using an Axopatch 700B amplifier (Axon) and filtered at 1 kHz and sampled at 5 kHz.

Transient expression of AtVCCN1-mGFP using the protoplast transfection assay. Total RNA was extracted from Arabidopsis thaliana rosette leaves using Isogen II (Nippon Gene, Japan) and reverse transcribed to cDNA using a PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa, Bio). The AtVCCN1 gene was then amplified and fused with the mGFP gene. The AtVCCN1-mGFP genes were inserted between SacI and Sali sites of the pDH301 plasmid. Arabidopsis thaliana wild type plants were grown in soil for 8 weeks in a growth chamber with an 8/16 h day and night cycle, at 23 °C and 60% relative humidity. Protoplasts derived from Arabidopsis thaliana mesophyll cells were generated by using the Tape-Arabidopsis Sandwich method. The protoplasts were then transfected by the TEAP method with slight modifications. Approximately 2 × 10^5 protoplasts in 0.5 ml MesMg solution (4 mM MES-KOH, pH 5.7, 15 mM MgCl2, 400 mM mannitol) were mixed with 100 µg of plasmid DNA at room temperature. An equal volume of PEG8000 with transfection solution (35% PEG8000, 100 mM CaCl2, 200 mM mannitol) was added gently and incubated at room temperature for 15 min. Afterwards, 1 ml of W5 solution (2 mM MesMg-KOH, pH 5.7, 154 mM NaCl, 125 mM CaCl2, 5 mM KCl) was added gently and mixed. Protoplasts were centrifuged at 200 × g for 30 s and the supernatant was removed. Protoplasts were resuspended gently in WI solution (4 mM MES-KOH, pH 5.7, 20 mM KCl, 500 mM mannitol). A 500 µl aliquot of the protoplast suspension was dispensed into each well of a 12 well plate and incubated at 23 °C for 16 to 20 h.

Protease protection assay. Protoplasts were osmotically disrupted by suspension in low osmotic buffer (50 mM HEPES pH 7.6, 7 mM MgCl2, 1 mM MnCl2, 2 mM EDTA, 30 mM KCl, 0.25 mM KHPO4, 20 mM DTT, 0.2% BSA) to isolate chloroplasts. The protease protection assay was performed by treating the chloroplast suspension with proteinase K (20 µg/ml) for 30 min. To terminate the reaction, 20 mM PMFS was added. The GFP signals of the treated chloroplasts were observed with a fluorescence microscope BZ-X810 (Keyence, Japan). Image analysis was performed using the ImageJ software (https://imagej.nih.gov/ij/). Samples were denatured by adding Laemmli’s sample buffer and 2 µg of chloroplast protein was subjected to 13% SDS-PAGE followed by a western blot analysis. The anti-GFP antibody (MBL, Japan), anti-P60 antibody (Agriera, Sweden) and anti-Rabbit IgG antibody (MBL, Japan) were used at dilutions of 1/3,000, 1/3,000, and 1/5,000 respectively.

Cell surface biotinylation assay. The cell surface biotinylation assay was performed as previously described, with slight modifications as follows. For transient transfections, HEK293T cells were grown in poly-L-lysine-coated 6-well plates to ~50% confluence, and then transfected with 1 µg plasmid and 3 µl Mirus TransIT-LT1 in 100 µl Opti-MEM. Two days after transfection, the cells were washed twice with cold PBS and incubated with 1 ml PBS supplemented with 5 µg/ml DIDS for 15 min. The cells were washed twice with ice-cold PBS and quenched by 50 mM NH4Cl in PBS for 10 min at 4 °C. After another wash, the cells were lysed by 1 ml ice-cold lysis buffer (0.5% Triton X-100 and 1 mM EDTA in PBS) containing cOmplete protease inhibitor cocktail (Roche) and incubated on ice for 20 min. After centrifugation at 20,000 g for 15 min at 4 °C, ten percent of the cleared lysate was saved for immunoblotting, and the rest was incubated with 30 µl of Pierce High Capacity Streptavidin Agarose beads for 1 h at 4 °C. The beads were washed five times with lysis buffer, incubated in SDS-PAGE sample buffer containing 10% β-mercaptoethanol at 95 °C for 5 min to dissociate the biotinylated cell surface proteins from the beads, and analyzed by immunoblotting using an anti-GFP antibody (Roche) at dilution of 1/2,000.
Received: 6 April 2021; Accepted: 22 April 2022;
Published online: 06 May 2022

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Author contributions

T.H. purified VCC1 and performed the cryo-EM trial, determined the structure, and
planned the mutational analyses, under the supervision of T. Kato, T.N., and O.N. K.K.
T. Kusakizako, K.Y. and T.N. assisted with the EM image data collection and the data analyses. S.H. and H.K. performed the protease protection assays. T.S. performed the cell surface biotinylation assays. Y.F. assisted with the functional characterization. A.D.M. performed patch-clamp analyses. T.H., T. Kato, G.K., T.N., and O.N. wrote the manuscript, with feedback from all of the authors. T.N. and O.N. supervised the research.

Competing interests
O.N. is a co-founder and scientific advisor for Curreio. The other authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-30292-w.

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Peer review information Nature Communications thanks So Iwata and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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