



Conserved and diversified gene families of monovalent cation/H⁺ antiporters from algae to flowering plants

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All organisms have evolved strategies to regulate ion and pH homeostasis in response to developmental and environmental cues. One strategy is mediated by monovalent cation–proton antiporters (CPA) that are classified in two superfamilies. Many CPA1 genes from bacteria, fungi, metazoa, and plants have been functionally characterized; though roles of plant CPA2 genes encoding K⁺-efflux antiporter (KEA) and cation/H⁺ exchanger (CHX) families are largely unknown. Phylogenetic analysis showed that three clades of the CPA1 Na⁺–H⁺ exchanger (NHX) family have been conserved from single-celled algae to *Arabidopsis*. These are (i) plasma membrane-bound SOS1/AtNHX7 that share ancestry with prokaryote NhaP, (ii) endosomal AtNHX5/6 that is part of the eukaryote Intracellular-NHE clade, and (iii) a vacuolar NHX clade (AtNHX1–4) specific to plants. Early diversification of KEA genes possibly from an ancestral cyanobacterium gene is suggested by three types seen in all plants. Intriguingly, CHX genes diversified from three to four members in one subclade of early land plants to 28 genes in eight subclades of *Arabidopsis*. Homologs from *Spirogyra* or *Physcomitrella* share high similarity with AtCHX20, suggesting that guard cell-specific AtCHX20 and its closest relatives are founders of the family, and pollen-expressed CHX genes appeared later in monocots and early eudicots. AtCHX proteins mediate K⁺ transport and pH homeostasis, and have been localized to intracellular and plasma membrane. Thus KEA genes are conserved from green algae to angiosperms, and their presence in red algae and secondary endosymbionts suggest a role in plastids. In contrast, AtNHX1–4 subtype evolved in plant cells to handle ion homeostasis of vacuoles. The great diversity of CHX genes in land plants compared to metazoa, fungi, or algae would imply a significant role of ion and pH homeostasis at dynamic endomembranes in the vegetative and reproductive success of flowering plants.

Keywords: cation homeostasis, pH homeostasis, dynamic endomembrane, secretory system, protein, cargo sorting

INTRODUCTION

Cells have evolved mechanisms to regulate ion and pH homeostasis in order to respond to developmental cues and to adapt to a constantly changing environment. In prokaryotes, this feat is accomplished with a diverse array of transporters at the plasma membrane. In eukaryotes, cells have increased in size and contain more intracellular membranes, including organelles and various compartments of the secretory and endocytic pathways. Consequently, the number of ion transporters localized to endomembranes has increased considerably. As plant ancestors initially living in an aquatic or marine environment colonized land, we wondered how transport systems required for growth, reproduction, and adaptation evolved in their structure and function. The genomes of diverse plants have been sequenced in recent years offering the first opportunity to understand how plant transporters evolved over one billion years. Here we focus on the monovalent cation/proton antiporter (CPA) superfamily whose members are thought to regulate cation and pH homeostasis (Brett et al., 2005a) by exchanging Na⁺, Li⁺, or K⁺ for H⁺. Prokaryote

and eukaryote CPA members fall into two major families designated as CPA1 (2.A.36) and CPA2 (2.A.37; Saier, 2000; Brett et al., 2005a). In plants, CPA1 includes the well-studied Na⁺–H⁺ exchanger (NHX) family, and the relatively obscure CPA2 family that includes K⁺ efflux Antiporter (KEA) and Cation–H⁺ exchanger (CHX) families. The purpose of this study is to understand the evolutionary relationship of NHX, KEA, and CHX homologs from algae to flowering plants, to use comparative biology to infer functions of uncharacterized plant genes (Chang et al., 2004), and hopefully reveal new model systems to determine functions of KEA and CHX genes which remain largely uncharacterized.

MATERIALS AND METHODS

ORGANISMS

Plant genomes that have been completely sequenced and available in Phytozome were used. The list of plant species and their abbreviated names in parenthesis include *Manihot esculenta* (*Mes*), *Ricinus communis* (*Rco*), *Populus trichocarpa* (*Ptr*), *Medicago truncatula*

(*Mtr*), *Glycine max* (*Gma*), *Cucumis sativus* (*Csa*), *Arabidopsis thaliana* (*Ath*), *Arabidopsis lyrata* (*Aly*), *Carica papaya* (*Cpa*), *Eucalyptus grandis* (*Egr*), *Vitis vinifera* (*Vvi*), *Mimulus guttatus* (*Mgu*), *Sorghum bicolor* (*Sbi*), *Zea mays* (*Zma*), *Oryza sativa* (*Osa*), *Brachypodium distachyon* (*Bdi*), *Selaginella moellendorffii* (*Smo* or *club moss*), *Physcomitrella patens* (*Ppa* or *moss*), *Volvox carteri* (*Vca*), and *Chlamydomonas reinhardtii* (*Cre*).

COLLECTING SEQUENCES FROM PLANTS

Deduced protein sequences from diverse plants were initially collected from Phytozome¹ by Blast analyses using NHX1, NHX5, NHX7, KEA1, KEA3, KEA4, or CHX17 from *Arabidopsis thaliana*. The sequences of each plant species were then compared with *Arabidopsis* protein by alignment with Clustal W or MUSCLE (Edgar, 2004) and analyzed with T-coffee (Notredame et al., 2000) or MEGA5 (Tamura et al., 2011). Abbreviated names were given to each gene according to the nomenclature used for *A. thaliana* (Maser et al., 2001). Blast analyses with *Arabidopsis* genes also recovered predicted proteins from an EST sequencing project of Charophyceae (Timme et al., 2012); though many transcripts were truncated and were not used in phylogenetic analysis. Other

sequences from bacteria, cyanobacteria, fungi, protist, or metazoa were obtained by BLAST from JGI² or NCBI³.

ALIGNMENT AND CONSTRUCTING TREES

RAxML

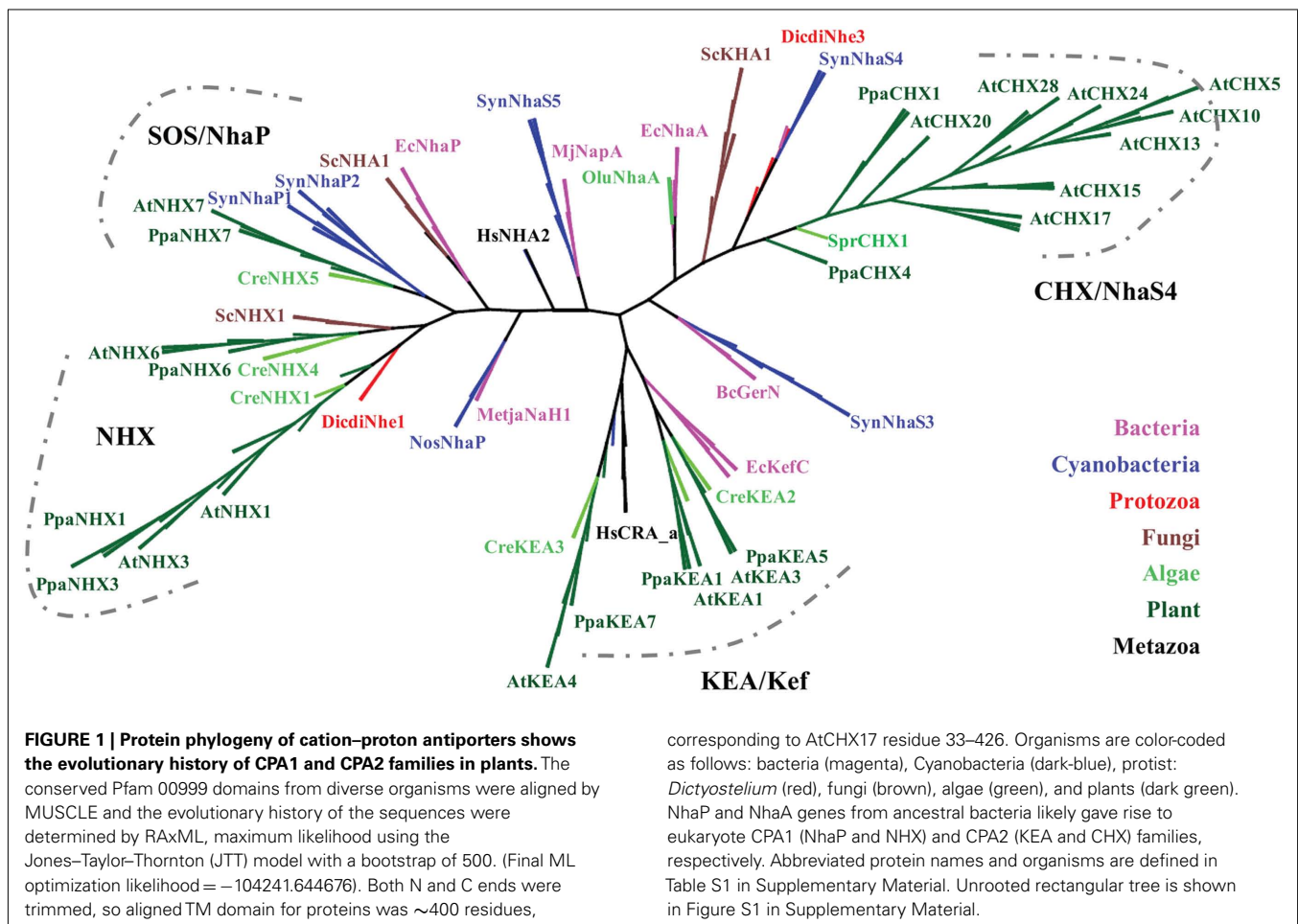
Sequences were assembled using Clustal X2 program (Larkin et al., 2007) to an original fasta file (fa.), and then aligned by Muscle software (Edgar, 2004). Alignment was exported and saved as fasta (afa), phylip (phy), or MSF format. RAxML was used for phylogenetic analysis and MSF was read by Genedoc (Nicholas et al., 1997⁴) for editing. After alignment with Clustal X2 or MUSCLE, sequences that included the Pfam domain (00999) Na/H exchanger domain were retained for further analysis. Short sequences or those lacking >30% of the transmembrane domain of ~400 residues were dropped, though they resemble members of the family. When sequences outside the conserved domain diverged significantly, the N-terminus and C-tail were trimmed to maximize alignment (e.g., Figure 1). We used maximum-likelihood and tested phylogeny with a bootstrap of 100–500, JTT model (Jones et al., 1992), to construct trees with either

²http://www.genome.jp/sit-bin/search_sequence?prog=blast&db=gvi&seqid=aa

³<http://www.ncbi.nlm.nih.gov/protein>

⁴<http://www.nrbsc.org/gfx/genedoc/>

¹Phytozome.net



RAXML (Stamatakis, 2006) or MEGA5 (Tamura et al., 2011). The bestTree file generated from the program gives the evolutionary value whereas the bipartition file informs the statistical value.

RESULTS

We first examined the evolutionary origin of plant cation–proton antiporters (CPA1 and CPA2) using *Arabidopsis* and rice as representatives of flowering plants. All these sequences had a conserved Na/H exchanger domain (Pfam00999) of about 400 residues. **Figure 1** shows three distinct branches which are labeled as CPA1 (NHX and NHAP), CPA2 with KEA, and with CHX families.

MONOVALENT CATION PROTON ANTIPORTER-1

Several prokaryotic and archaea NhaP genes are located at the base of the CPA1 branch supporting the idea that an ancestral NhaP gene gave rise to eukaryote CPA1 genes (**Figures 1** and **2**). This family of transporters is predicted to have 10–12 membrane-spanning domains, and bacterial NhaP mediates electroneutral Na⁺/H⁺ exchange (see **Table 1**). CPA1-type transporters are found in all kingdoms, including archaea, bacteria, fungi, plants, and metazoa (Brett et al., 2005a). Our analysis shows that ancestral NhaP evolved and diverged to give two distinct types: NHX and NHAP (**Figure 2**).

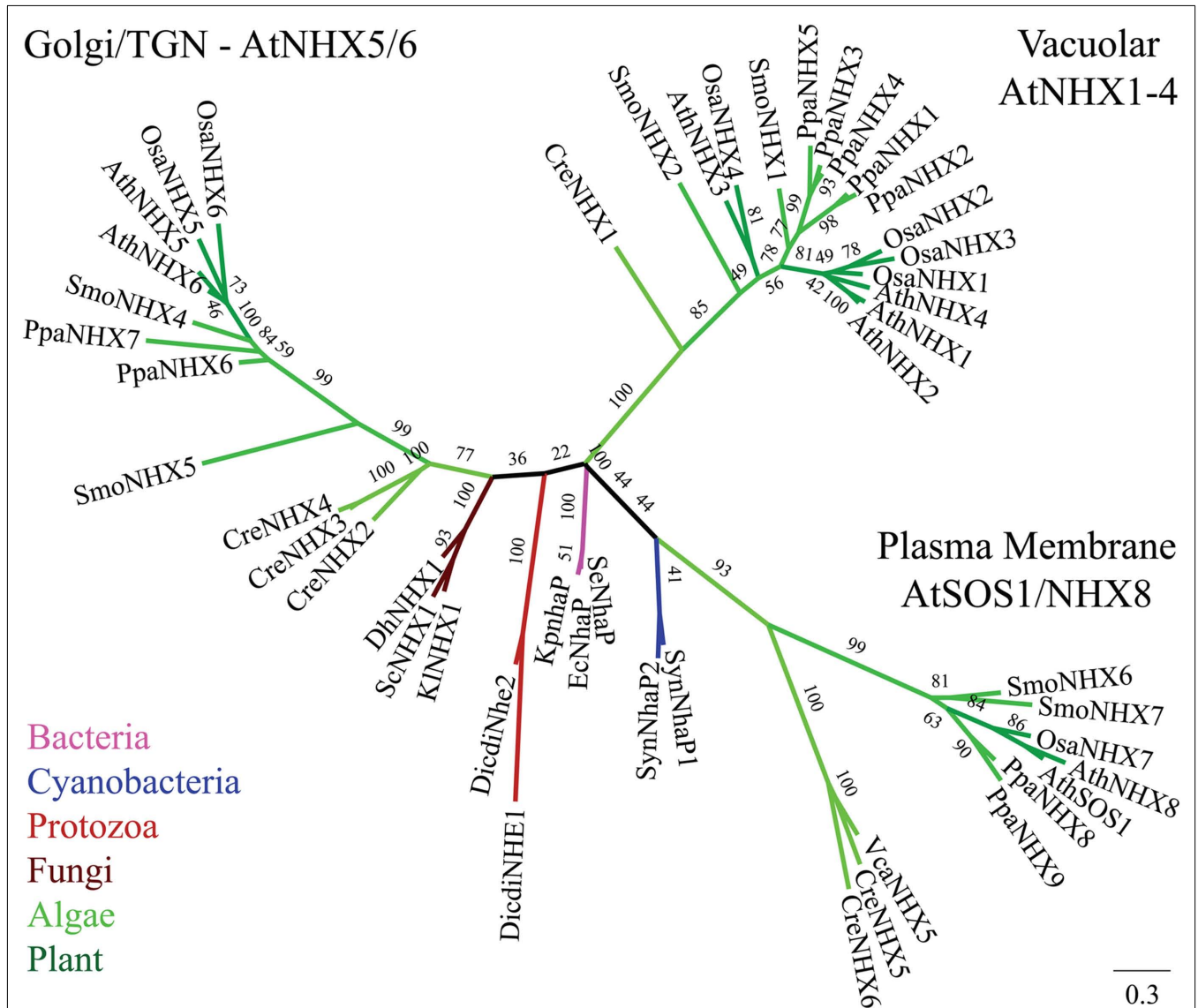


FIGURE 2 | Diversification of CPA1 genes into three clades preceded the evolution of early land plants. The C-tails were trimmed to maximize alignment, so AtNHX1 sequence included residues 1–448. Tree was generated with maximum likelihood of RAXML using the Jones–Taylor–Thornton (JTT) model and tested by bootstrap of 500. (Final ML optimization likelihood = –30244.238457). Single-celled alga (Cre), moss

(Ppa), and club moss (Smo) NHX members were present in each of three clades, NhaP/SOS1, endosomal AtNHX5/6, and vacuolar AtNHX1–4. Organisms and proteins are defined in Table S2 in Supplementary Material, and color-coded as bacteria (magenta), Cyanobacteria (dark-blue), fungi (brown), protist (red), green algae (green), early land plants, and angiosperms (dark green). The “vacuolar” AtNHX1–4 clade is specific to plants.

Table 1 | Functions of prokaryote cation/proton antiporters (CPA).

Prokaryote isoform	Transport mode	Membrane location (aa)	Main functions	Plant homolog (CPA)	Reference
EcNhaA	(Na ⁺ , Li ⁺)/2H ⁺ electrogenic	Plasma membrane (388)	Regulation of cellular acidity and salinity. Activated by external pH above 7	None (CPA2)	Arkin et al. (2007), Hunte et al. (2005)
SynNhaS1, S2 (SynNhaP, P2)	(Na ⁺ , Li ⁺)/H ⁺ electroneutral	Plasma membrane (527, 540)	Salt tolerance	SOS1/AtNHX7/8 (CPA1)	Hamada et al. (2001), Inaba et al. (2001)
MjNhaP	(Na ⁺ , Li ⁺)/H ⁺	Plasma membrane (426)	Cytoplasmic pH control using Na ⁺ gradient. Active between pH 6 and 7	SOS1/AtNHX7/8	Hellmer et al. (2002), Vinothkumar et al. (2005), Goswami et al. (2011)
SynNhaS3	(Na ⁺ , Li ⁺)/H ⁺	Thylakoid membrane (461)	Salt tolerance. Ion homeostasis K ⁺ , Na ⁺ , H ⁺ in cytoplasm, and thylakoid lumen	KEA (CPA2)	Tsunekawa et al. (2009)
SynNhaS4	(K ⁺ /H ⁺) suggested	? (410)	Growth in K ⁺ depleted conditions	CHX (CPA2)	Inaba et al. (2001)
SynNhaS5	?	? (698)	?	? (CPA2)	
EhNapA	(Na ⁺ , Li ⁺)/H ⁺	Plasma membrane (383)	Salt tolerance at neutral pH	(CPA2)	Waser et al. (1992), Strausak et al. (1993)
BcGerN/GerT	(Na ⁺ , Li ⁺)/(H ⁺ -K ⁺)	Plasma membrane (387)	Spore germination/Spore outgrowth in alkaline or saline conditions	KEA (CPA2)	Thackray et al. (2001), Southworth et al. (2001), Senior and Moir (2008)
EcKefC	K ⁺ uniport or K ⁺ /H ⁺ exchange (Rb ⁺ , Li ⁺ , Na ⁺)	Plasma membrane (620)	Cytoplasmic acidification coupled to K ⁺ efflux, survival exposure to electrophiles	KEA (CPA2)	Fujisawa et al. (2007), Ferguson et al. (2000)

"aa" Refers to the number of residues in the protein. Ec, *Escherichia coli*; Eh, *Enterococcus hirae*; Bc, *Bacillus cereus*; Syn, *Synechocystis sp. PCC 6803*; Mj, *Methanococcus jannaschii*. (See Table S1 in Supplementary Material for protein accession no.)

NHAP/SOS1 clade

Phylogenetic analysis showed that plant members of Na⁺/H⁺ antiporter (NHAP) clade arose from ancestral NhaP1 and NhaP2 genes of a cyanobacterium (Figures 1 and 2). The best characterized plant NHAP is SOS1/AtNHX7 which mediates electroneutral Na⁺/H⁺ exchange (Qiu et al., 2003). Although AtSOS1 and AtNHX8 were annotated previously as NHX, our analysis places this group within NHAP clade consistent with results of Brett et al. (2005a). AtSOS1 and its homolog, AtNHX8, are localized to the plasma membrane (Qiu et al., 2003; An et al., 2007). Intriguingly, plant NHAP, such as SOS1/NHX7, has a particularly long C-terminal tail compared to other plant NHX. Recent studies show that this long C-tail interacts with other regulatory proteins (Katiyar-Agarwal et al., 2006; Quintero et al., 2011).

Arabidopsis NHAP or AtSOS1 has been extensively studied since the discovery that *sos1* mutant is salt-overly sensitive (Wu et al., 1996; Quintero et al., 2002; Zhou et al., 2006; Fraile-Escanciano et al., 2010; see Table 2). SOS1 was shown to be associated with salt stress response (Shi et al., 2000, 2002) by mediating extrusion of Na⁺ (Qiu et al., 2003; Olias et al., 2009). It was shown that SOS1 had a physiological role in protecting plasma membrane K⁺ transporters from Na⁺ inhibition (Qi and Spalding, 2004). Furthermore, interaction of AtSOS1 with radical-induced cell death (RCD1) prevented RCD1, which is important for oxidative stress response, from nuclear localization (Katiyar-Agarwal et al., 2006). This relationship suggested a role of SOS1 in signal transduction. Analysis of *sos1* mutant revealed the consequence of Na⁺ accumulation which affected membrane trafficking, vacuolar biogenesis, and pH homeostasis in roots (Oh et al., 2010). Interestingly, cation

specificity for AtSOS1 and AtNHX8 is limited to Na⁺ and Li⁺ (Qiu et al., 2003; An et al., 2007) consistent with the substrate specificity of prokaryotic NhaP (Table 1). Results indicate that the major role of plant SOS1 transporters, like other NHAP members, is to extrude Na⁺ and confer tolerance to salt stress.

Two NHX clades

The diversification of NHAP to eukaryote intracellular-NHE (IC-NHE) and PM-NHE occurred early in evolution most likely when primitive eukaryote cells appeared (Brett et al., 2005a; Figure 1). Plant NHX members (excluding SOS1) belong to the IC-NHE family. NHX genes are present in single-celled algae like *Chlamydomonas*, early land plants, like *Physcomitrella patens*, as well as *Arabidopsis thaliana* (Figure 2). Furthermore, plant NHX has diversified into two clades that cluster either with *Arabidopsis* NHX5/6 or with AtNHX1–4. Consistent with the phylogenetic analysis, NHX proteins have been localized to intracellular membranes. In flowering plants, AtNHX5/6 is localized to Golgi or TGN, and AtNHX1 is at the vacuolar membrane (see Table 2 for references). The functions and localization of plant NHX and NhaP/SOS1 proteins are summarized in Table 2. These findings support the idea that NHX genes evolved as endomembranes appeared in early eukaryote and plant cells.

Initial studies showed that vacuolar NHX1 mediated electroneutral Na⁺/H⁺ exchange (Darley et al., 2000), though later studies broadened cation specificity to K⁺ in plants (Table 2). Vacuolar NHX was associated with salt stress responses and pH regulation (reviewed by Rodriguez-Rosales et al., 2009). InNHX1/2 of Japanese morning glory was shown to alkalize vacuolar pH

Table 2 | Functions of CPA1 members (NHXs and SOS1) from *Arabidopsis* and other plants.

Plant isoform	Expression	Membrane location (aa)	Main functions	Reference
AtNHX1	Ubiquitous: epidermis of siliques, inflorescence, stems, leaves, petals induced by salt, osmolarity, ABA	Tonoplast (538)	Na ⁺ (K ⁺)/H ⁺ antiport salt tolerance K ⁺ sequestered in vacuole vacuolar pH alkalization cell expansion, interact with calmodulin (AtCAM15) regulating Na ⁺ /K ⁺ selectivity	Apse et al. (1999, 2003), Gaxiola et al. (1999), Quintero et al. (2000), Shi et al. (2002), Venema et al. (2002), Yokoi et al. (2002), Yamaguchi et al. (2003, 2005), Leidi et al. (2010), Bassil et al. (2011b)
AtNHX2	Ubiquitous, induced by salt, osmolyte, ABA	Tonoplast (546)	Na ⁺ (K ⁺)/H ⁺ antiport, K ⁺ sequestered in vacuole, vacuolar pH alkalization, cell expansion, salt tolerance	Yokoi et al. (2002), Bassil et al. (2011b)
AtNHX3	Roots, germinating seeds, flowers, and siliques	Tonoplast (529)	K ⁺ /H ⁺ Low K ⁺ tolerance	Yokoi et al. (2002), Liu et al. (2010)
AtNHX4	Ubiquitous: stem, induced by high Li ⁺ or K ⁺ , ABA	Tonoplast (503)	Increase sensitivity to salt	Yokoi et al. (2002), Li et al. (2009)
AtNHX5/LeNHX2	Ubiquitous, induced by salt	Golgi/TGN (521)	Vesicular trafficking, salt tolerance	Yokoi et al. (2002), Venema et al. (2003), Bassil et al. (2011a)
AtNHX6	Ubiquitous	Golgi/TGN (535)	Vesicular trafficking, salt tolerance	Yokoi et al. (2002), Bassil et al. (2011a)
AtSOS1 (AtNHX7)	Root-, stem- and leaf-xylem parenchyma, and epidermal cells of the root tip, induced by NaCl	Plasma membrane (1146)	Na ⁺ /H ⁺ , salt tolerance, Na ⁺ transport from root to shoot, protect K ⁺ permeability, regulated by protein kinase SOS2 and Ca ²⁺ sensor SOS3 at C terminus, interact with RCD1 regulating oxidative stress response	Shi et al. (2000, 2002), Qiu et al. (2002), Quintero et al. (2002, 2011), Qi and Spalding (2004), Katiyar-Agarwal et al. (2006)
AtNHX8	Ubiquitous	Plasma membrane (756)	Li ⁺ tolerance	An et al. (2007)

"aa" Refers to number of residues in protein. At, *Arabidopsis thaliana*; Le, tomato.

causing purple buds to turn into blue flowers (Yamaguchi et al., 2001; Ohnishi et al., 2005). The increase in vacuolar pH was accompanied by K⁺ accumulation indicating that NHX1 mediated K⁺/H⁺ exchange in *Ipomea tricolor* (Yoshida et al., 2009). Cation specificity was directly shown using proteoliposome where AtNHX1 catalyzed Na⁺/H⁺ and K⁺/H⁺ exchange with similar affinity (Venema et al., 2002). Moreover, silencing *LeNHX2* resulted in tomato plants that accumulated less K⁺ and were susceptible to salt stress (Rodriguez-Rosales et al., 2008). In contrast, over-expression of AtNHX1 in tomato resulted in enhanced K⁺ accumulation and resistance to salt (Leidi et al., 2010). Finally, *nhx1/nhx2* double mutants displayed reduced K⁺ concentration and pH in vacuoles, supporting a role of AtNHX1 and AtNHX2 in mediating H⁺ efflux coupled to K⁺ uptake. Thus regulating intravacuolar (K⁺) and pH are essential to cell expansion and flower development (Bassil et al., 2011b). There is increasing acceptance that plant NHX has a major role in pH and K⁺ homeostasis in plant cells where salt tolerance is conferred by enrichment of intracellular K⁺ pool and sequestration of Na⁺ in vacuole.

Interestingly, endosomal NHXs, such as LeNHX2, AtNHX5, and AtNHX6 were able to confer salt tolerance (Yokoi et al., 2002; Rodriguez-Rosales et al., 2008). Double mutants of *nhx5/nhx6* were hypersensitive to salt (Bassil et al., 2011a). In previous studies of yeast, evidence emerged that ScNHX1 affected endosomal

pH and K⁺ homeostasis that influenced protein sorting and membrane trafficking (Bowers et al., 2000; Ali et al., 2004; Brett et al., 2005b; Wagner et al., 2006). A recent study with plants suggested that regulation of endosomal pH and K⁺ homeostasis by AtNHX5 and AtNHX6 had a role in sorting of vacuolar protein and cellular stress responses (Bassil et al., 2011a), consistent with the phylogenetic analysis showing ScNHX1 and AtNHX5/6 are in the same clade (Figures 1 and 2).

Curiously, budding yeast (and probably most fungi) has only one endosomal NHX1 localized to endosome/prevacuolar compartment (PVC), and does not have genes related to "plant vacuolar-type" AtNHX1–4. However, *Dictyostelium* Nhe1 and Nhe2 are at the base of the plant vacuolar NHX clade (Figure 2), suggesting plant vacuolar NHX arose from a gene in an ancestral protozoa possibly with contractile vacuoles for osmoregulation (Allen and Naitoh, 2002; Gerisch et al., 2002; Uchikawa et al., 2011). The plant-specific AtNHX1–4 type has apparently undergone gene duplications (Figure 2) underscoring the significance and diversity of vacuole functions in multicellular land plants.

MONOVALENT CATION PROTON ANTIPTER-2

Members of CPA2 superfamily are found in bacteria, fungi, and plants; however, they are rare in metazoa (Brett et al., 2005a). They are predicted to have 8–14 membrane-spanning domains with a Pfam00999 domain for Na⁺(K⁺)/H⁺ exchange. Interestingly,

substrate and transport mode of CPA2 from bacteria vary among family members. The general transport mode of CPA2 is M^+ (in) + nH^+ (out) $\rightleftharpoons M^+$ (out) + (in) for the carrier-mediated mode, whereas some members may catalyze M^+ (in) $\rightleftharpoons M^+$ (out) in the channel-mediated mode (Saier, 2000). The CPA2 superfamily consists of three families named here as NHA/NhaA, KEA/Kef, and CHX/NhaS4 based on phylogenetic analyses and prokaryote ancestry.

NHA clade

A eukaryote NHA clade was previously classified within CPA2 (Brett et al., 2005a), though they seem related to the NHAP clade in CPA1 (Figure 1). Eukaryote NHA, like NhaP, is localized at the plasma membrane (Kinclova et al., 2001). Functional studies of yeast show that plasma membrane ScNHA1 mediates both Na^+/H^+ and K^+/H^+ exchange (Banuelos et al., 1998; Sychrova et al., 1999; Ohgaki et al., 2005). Human NHA2 is localized to the PM and its expression in yeast conferred tolerance to Na^+ and Li^+ , but not to K^+ (Xiang et al., 2007), suggesting it mediates Na^+ transport similar to NhaP or NHX of CPA1. Our phylogenetic analysis suggests that eukaryote NHA is likely evolved from an *NhaP* gene of an ancestral bacterium (Figure 1). Unlike NhaP, *E. coli* NhaA is active at pH 8 and inactive at pH 6 (Taglicht et al., 1991; see Table 1). EcNhaA mediated exchange of $2H^+$ for $1Na^+$ or $1Li^+$ (Padan, 2008). Intriguingly, only tiny marine green alga, such as *Ostreococcus lucimarinus* and *Micromonas pusilla* (Chlorophyta) have a protein homologous to bacterial NhaA (Figure 1; Table S1 in Supplementary Material). NHA members are thus found in bacteria, animals, and fungi (Brett et al., 2005a), but not in early land plants or flowering plants (Figure 1).

KEA family

Genes encoding putative KEA from higher plants were first classified in *Arabidopsis thaliana* (Maser et al., 2001); thus AtKEA1–AtKEA6 members are used as a reference. KEA-like transporters from bacteria have been extensively studied, and are characterized by an N-terminal Na_H exchanger domain (Pfam PF00999), and a C-terminal KTN NAD(H)-binding domain, also called TrkA_N domain (Pfam PF02254; Figure 4). For example, Kch, TrkA, Ybal, and KefB or KefC are K^+ channels or transporters with a C-terminal KTN domain (Choe, 2002). The minimal functional unit of the KTN domain is a dimeric molecule connected by a flexible hinge. Hinge motion can be physically coupled to transmembrane loops to control K^+ flux, providing a mechanism of gating control (Choe, 2002). The domain contains the typical Rossman fold GXGXXG...D Glycine motif involved in NAD binding, indicating metabolic control of transporter function (Jiang et al., 2001; Roosild et al., 2002).

Divergence of two KEA clades. To understand the evolutionary origin of higher plant KEA, we searched and analyzed homologs in prokaryotes, protists, metazoa, and plants. AtKEA genes diverged into two main branches with AtKEA1–3 in clade I and AtKEA4–6 in clade II. Clade I consists of the only eukaryote-encoded proteins with a complete C-terminal KTN domain (Figures 3 and 4), and are closely related to EcKefB and EcKefC-like proteins from bacteria, including cyanobacterium *Gloeobacter violaceus* (Figure 3).

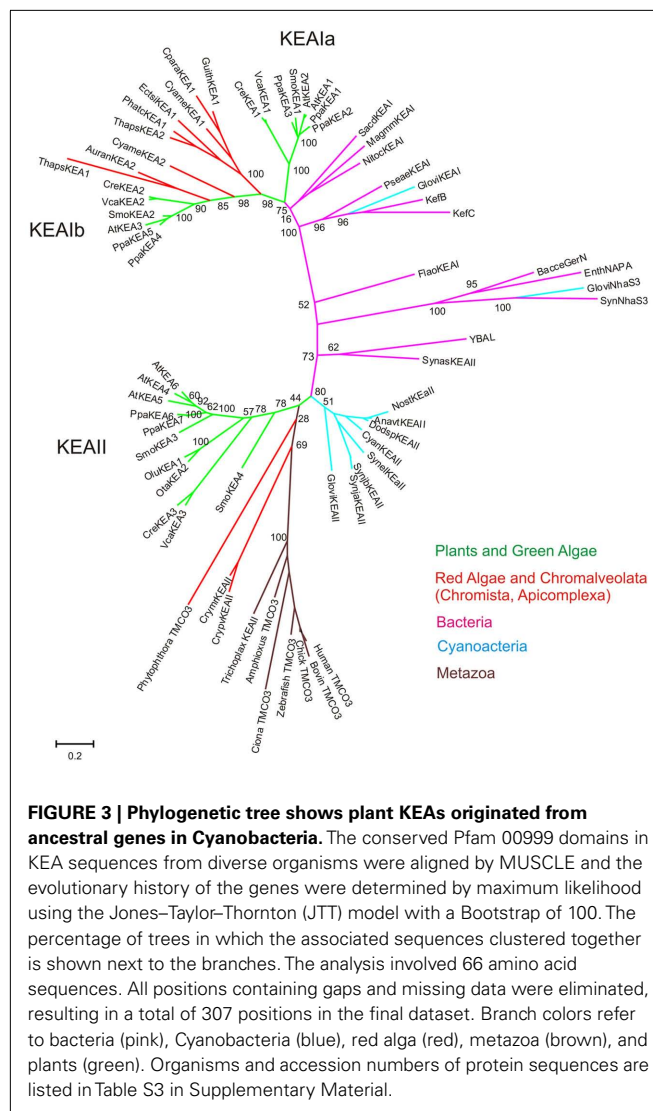


FIGURE 3 | Phylogenetic tree shows plant KEAs originated from ancestral genes in Cyanobacteria. The conserved Pfam 00999 domains in KEA sequences from diverse organisms were aligned by MUSCLE and the evolutionary history of the genes were determined by maximum likelihood using the Jones–Taylor–Thornton (JTT) model with a Bootstrap of 100. The percentage of trees in which the associated sequences clustered together is shown next to the branches. The analysis involved 66 amino acid sequences. All positions containing gaps and missing data were eliminated, resulting in a total of 307 positions in the final dataset. Branch colors refer to bacteria (pink), Cyanobacteria (blue), red alga (red), metazoa (brown), and plants (green). Organisms and accession numbers of protein sequences are listed in Table S3 in Supplementary Material.

Red algae as well as secondary photosynthetic organisms of the Kingdom Chromista contain sequences from clade I exclusively (Figure 3; Table S3 in Supplementary Material). In contrast, proteins of clade II lack a KTN domain (Figures 3, 4, and 6; Table S3 in Supplementary Material). Phylogenetic analysis suggests AtKEA4–6 arose from an ancestral cyanobacterium judging by the presence of related proteins in unicellular and filamentous cyanobacteria (Figure 3). Clade II genes are also present in parasitic organisms of the phylum Apicomplexa. These organisms contain a high number of plant and bacterial-like proteins (Abrahamsen et al., 2004) possibly due to apicoplast (plastid) resulting from secondary endosymbiont of an ancestral green or red alga (Keeling, 2004). A clade II protein is found in the oomycete, *Phytophthora infestans*, possibly also related to the presence of a plastid in a common photosynthetic ancestor of the chromista (Tyler et al., 2006). Oddly, AtKEA4–6 like proteins are also found in *Trichoplax adhaerens* (a Placozoa), *Ciona intestinalis*, a basal Chordata, and in higher Chordata, such as mammals, amphibians, and fish (Figure 3, Table S3 in Supplementary Material). These AtKEA4–6 like proteins contain



FIGURE 4 | Alignment of AtKEA sequences with their bacterial homologs – AtKEA sequences were aligned using MUSCLE (Edgar, 2004) with their closest prokaryotic homologs: *E. coli* KefC and Cyanobacterial sequence SynjbKEAII (Q2J145 Table S3 in Supplementary Material). The soluble N-terminal domain of AtKEA1 was removed. Conserved residues are shown in orange. Approximate positions of possible transmembrane helices are shown above the alignment. Secondary structure of the KTN domain is

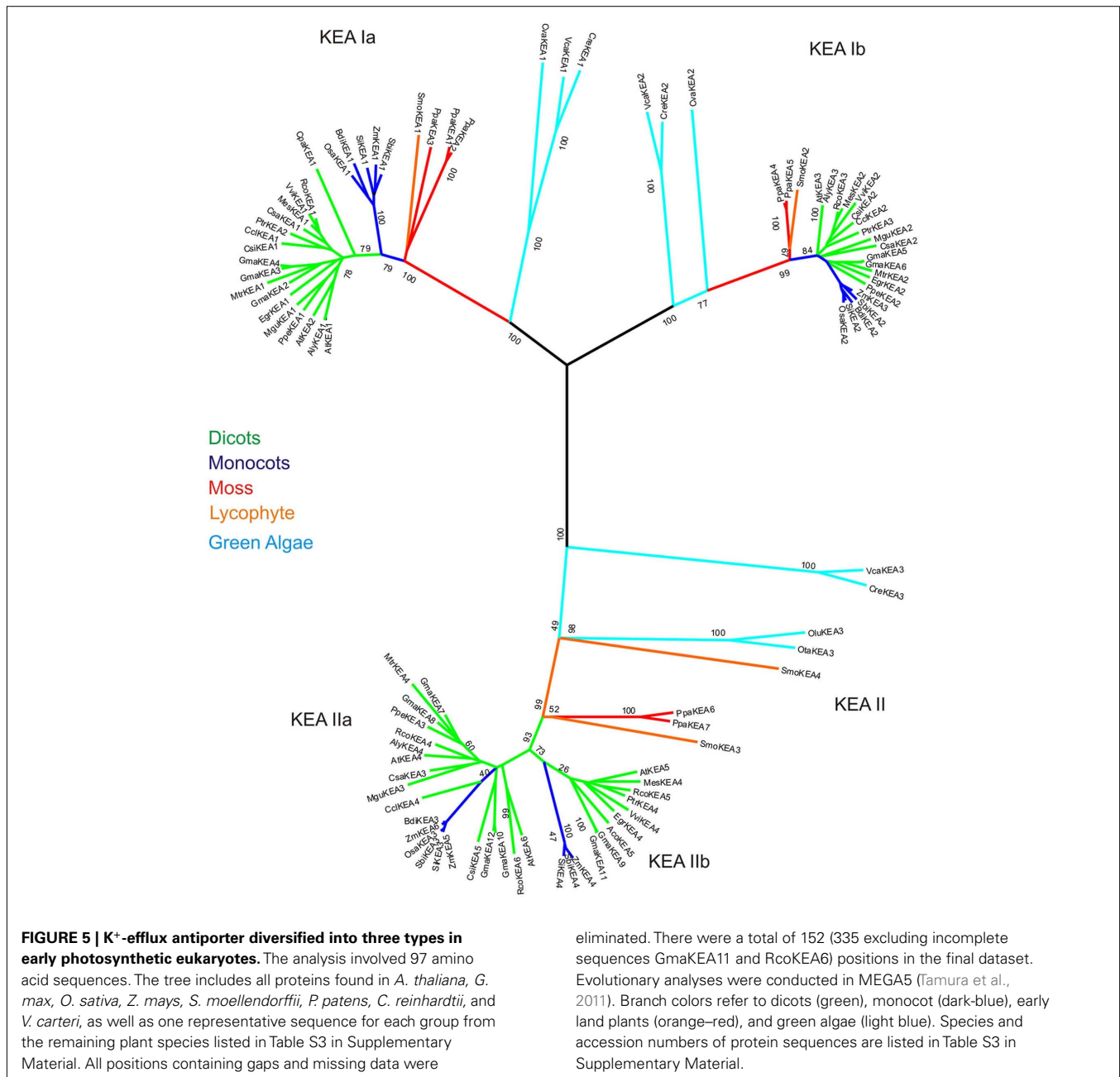
indicated as well as residues that were shown to be involved in Glutathione-binding in the KefC KTN structure. A short cytoplasmic regulatory loop in the *E. coli* KefC sequence as well as the conserved residues in the *Arabidopsis* sequences are shown in magenta. The residues corresponding to the proton binding residues in the structure of the bacterial NhaA sequence are shown above the alignment. The Rossman fold glycine motif is shown in green. Below the alignment the two specific clade Ib inserts in different plants are shown.

a trans membrane coiled coil protein 3 (TMCO3) and also lack the KTN domain.

These results would suggest that KEA genes were acquired by primary endosymbiosis of a cyanobacterium, and by secondary endosymbiosis in the Archaeplastida ancestor of red and green algae (Adl et al., 2005; Bowman et al., 2007). Furthermore, it appears the divergence of clade I and clade II KEAs occurred early as prokaryotes diversified perhaps before eukaryote cells evolved.

Intriguingly, KEA genes from clade I are found in a red alga (*C. merolae*) and in chromista (Figure 3). Chromista possibly

became photosynthetic by endosymbiosis with a unicellular red alga, as genes related to KEA sequences in *C. merolae* are also present in these species. In chromista of the cryptomad subgroup, a KEA gene constitutes one of three transport genes that are maintained in the highly reduced nucleomorph genome (Douglas et al., 2001). Nucleomorphs are the remnant nuclei of algal endosymbionts that were engulfed by non-photosynthetic eukaryotes (Tanifuji et al., 2011). This observation suggests that KEAs play important roles in these organisms, such as ion transport across one of four chloroplast membranes. Its role is apparently not in photosynthesis, as KEA-like gene is conserved in the

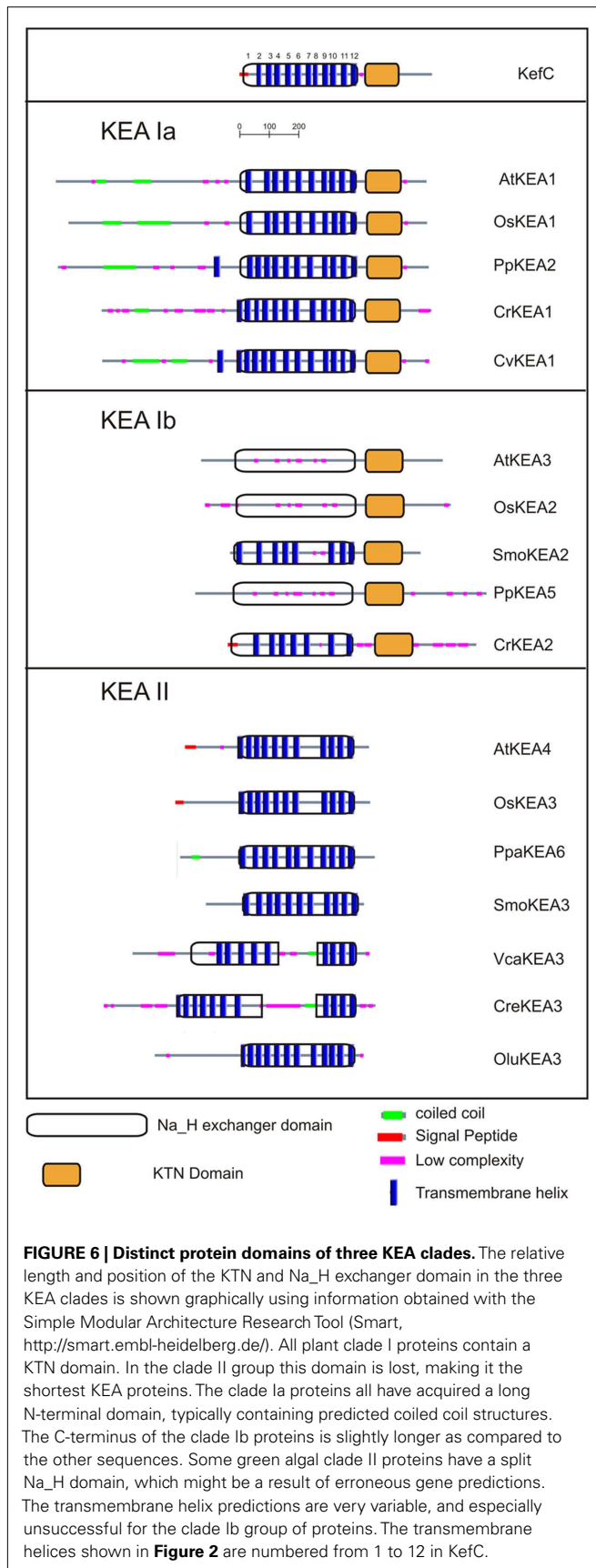


nucleomorph of a non-photosynthetic *Cryptomonas paramecium*, but not in nucleomorphs of green algal origin (Tanifuji et al., 2011).

Evolution of KEA in plants. Protein phylogenetic analyses showed that all plants, from unicellular algae to flowering plants, had three types of KEAs classified as clades Ia, Ib, and II (Figure 5). Green algae, like *C. reinhardtii* and *V. carteri*, contain only one gene in each group. In other plants, diversification occurred in clades KEA-Ia, KEA-II, or both.

Clade Ia KEAs possess a long N-terminal domain. Phylogenetic analysis showed that plant KEAs in clade I diverged into two

types that differ in their protein organization. Within clade Ia, all plant sequences from moss to monocot and dicots have a long hydrophilic N-terminal domain of ~570–770 amino acids (Figure 6) that is often missed in preliminary gene annotations. This domain is typically rich in negatively charged glutamate residues, and predicted coiled coil structures (Figure S4 in Supplementary Material). The role of this unusual extra sequence is unknown (Rose et al., 2004). The N-terminal domain of green algae Cre and Cva share some similarity (46%) and are slightly shorter than that of higher plants (Figure 6). The absence of this region in prokaryotes would indicate that the N-terminal domain is a recent acquisition that gained increasing relevance in land plants.



In contrast, proteins in clade Ib, such as AtKEA3, do not harbor the N-terminal extension, instead they have gained specific insertions between predicted membrane helices 4 and 5 and in the Rossman fold glycine motif of the KTN domain (**Figures 4 and 6**). Although all hydrophobic regions are conserved in the clade Ib proteins, transmembrane helices are poorly identified by transmembrane prediction programs (**Figure 6**). In general, only one gene of this type is present per plant, except in *G. max* and *P. patens*, where two genes are present (**Table 4**; Table S3 in Supplementary Material; **Figure 5**).

Recent diversification of clade II KEAs. Genes encoding AtKEA4–6 have a hydrophilic N-terminal domain of ~157–235 residues that is predicted to include a signal peptide (**Figures 4 and 6**). Green algal sequences found in *V. carteri* and *C. reinhardtii* have an anomalous Na_H exchanger domain that is interrupted by a similar sequence in both species (**Figure 5**). Interestingly, clade KEA-II has diversified further in angiosperms where AtKEA5 is clearly separate from AtKEA4 and AtKEA6 indicating this occurred recently after the evolution of early land plants.

Function of KEA. Nearly nothing is known about plant KEA function, so bacterial homologs could provide insights. KefB and KefC in *E. coli* are glutathione regulated K⁺ efflux transporters, that are important for bacterial survival during exposure to toxic metabolites (Booth, 2003). Electrophiles react with glutathione to form glutathione adducts, thereby releasing KefC and KefB inhibition by GSH, and eliciting K⁺ efflux and H⁺ uptake. Cytoplasmic acidification is suggested to prevent DNA damage (Ferguson et al., 2000; Miller et al., 2000). Reduced GSH stabilizes the association of the two KTN domains, whilst the larger adducts disrupt this interaction (Roosild et al., 2010). Based on mutagenesis studies, a short central hydrophilic regulatory loop was proposed to interact with the KTN domain. This regulatory loop is partially conserved in the plant KEA1–3 sequences, but the residues forming the glutathione-binding pocket are less conserved in the plant sequences (**Figure 2**), indicating that plant KEA1–3 are regulated in a different way (Roosild et al., 2010). Although originally proposed to function as K⁺ channels, recent studies indicate KefC has K⁺/H⁺ antiport activity (Fujisawa et al., 2007).

Recently, AtKEA1 and AtKEA3 peptides were detected by mass spectroscopy in *Arabidopsis* chloroplast preparations (Zybailov et al., 2008). Moreover, localization to thylakoid membrane of SynNha3 (Tsunekawa et al., 2009), a *Synechocystis* protein closely related to KEA-I and KEA-II proteins (**Figure 1**), would suggest a role of plant KEA in chloroplast ion homeostasis. So far only one cyanobacterium, *Gloeobacter violaceus*, has a gene encoding a KEA-I homolog (**Figure 3**). Perhaps ancestral primary plastids originated just after the separation of primitive cyanobacterium, like *Gloeobacter*, from present-day lineages (Crisuolo and Gribaldo, 2011). In *Gloeobacter*, thylakoids are absent and photosystems reside at the plasma membrane (Vothknecht and Westhoff, 2001). We hypothesize that the long N-terminal sequence of AtKEA1/2 has a role in chloroplast-specific processes in plant (Vothknecht and Westhoff, 2001). These and other possibilities need to be tested.

Evolutionary origin of CHX clade in plants

The CHX clade is populated by genes from higher plants, but none from metazoa so far. Nearly all deduced proteins from plants have ~800 residues (Table S4 in Supplementary Material) that consist of a hydrophobic Na⁺/H⁺ exchanger domain of ~400 residues at the amino terminus, and a carboxyl hydrophilic domain of 300–400 residues. The long hydrophilic C-tail does not contain any known conserved motifs, so its role is a complete mystery. NhaS4 from the cyanobacterium *Synechocystis* PCC 6803 encodes a much shorter protein of 410 residues, though the predicted protein from *Synechococcus elongatus* PCC 6301 has 715 residues.

Based on analyses using the TM domain alone, we show that the present-day NhaS4 from cyanobacteria, shares the highest similarity with AtCHX16–CHX20 (Figure 1; Sze et al., 2004). NhaS4 gene is found in single-celled *Synechocystis* PCC 6803 (Inaba et al., 2001) as well as filamentous *Anabaena variabilis* (Ava1632). Many non-cyanobacteria do not have genes that cluster with the CHX/NhaS4 clade. One exception is MyxaNhaS4 (Table S1 in Supplementary Material) from the gliding bacterium *Myxococcus xanthus* (a member of Myxococcales) which form fruiting body in response to nutrient starvation (Mauriello et al., 2010). Intriguingly, the mechanisms of motility and establishment of cell polarity in *M. xanthus* and eukaryotes share several similarities (Leonardy et al., 2010). Although genes from metazoa are absent from this clade, *Dictyostelium discoideum* (a protist and slime mold) has two members, Nhe3 and Nhe4, of unknown function that cluster with NhaS4 (Figure 1). The function and membrane location of cyanobacterial NhaS4 are still unclear. Interestingly, all fungi examined have a single gene *KHA1* that encodes a homolog of *Synechocystis* NhaS4 as seen in Figure 1. Fungi include unicellular *Saccharomyces cerevisiae*, and filamentous fungi such as *Neurospora crassa*, *Aspergillus oryzae*, and *Ustilago maydis* (Figure 1; Table S1 in Supplementary Material). Mutants of *ScKHA1* have served as a useful heterologous

system to test functions of *Arabidopsis* CHX16–20 (Chanroj et al., 2011; Table 3).

Comparative analyses of full-length protein sequences revealed additional insights on the origin of plant CHX members. One CHX homolog was found in *Spirogyra pratensis*, a Zygnematales, though none was detected in *Chlamydomonas reinhardtii*, *Volvox carteri* (Figures 1 and 7), or red algae (not shown). Early land plants, like *Physcomitrella* and *Selaginella*, had four and three genes, respectively encoding CHX homologs (Figure 1). Interestingly, all these proteins cluster in one subclade closest to *Arabidopsis* CHX20 (Figure 7) and CHX16–19. Results suggest that AtCHX16–CHX20 genes arose early in CHX gene family diversification. In *Arabidopsis*, these genes are expressed in leaves and roots, especially in dermal tissues, such as root epidermis (Cellier et al., 2004; Chanroj, 2011), root cap, and guard cells (Padmanaban et al., 2007; see Table 3).

Multiple subclades within the CHX family. Analysis of CHX homologs from 14 higher plants unveiled the diversification of this family in flowering plants (Figure 7). In general, CHX genes diverged less in monocots than in dicots. First, the average number of CHX genes (16) per monocot genome is less than that seen in many dicots (>26; Table 4). Second, Figure 7 shows that CHX members in *Arabidopsis* and other dicots can be separated into eight subclades, and five of these are conserved in monocots (Table 5). For example, orthologs of AtCHX20 and AtCHX16–CHX19 were evident in rice, corn, and sorghum, as shown by the blue branches in Figure 7 (Table S4 in Supplementary Material). Furthermore, monocot orthologs of AtCHX15, AtCHX1/2, and AtCHX28 were suggested by their close association with these subclades. Many of these genes in *Arabidopsis* are preferentially expressed in pollen (Sze et al., 2004; Table 5). Oddly, homologs of AtCHX21/23 were not obvious in monocot

Table 3 | Functions of characterized CHX genes (CPA2) in *Arabidopsis thaliana*.

Plant isoform	Tissue expression	Membrane localization (aa)	Main functions and phenotype	Reference
AtCHX13	Pollen, induced by K ⁺ starvation in root tip and elongation zone	Plasma membrane in plant and yeast cells (832)	K ⁺ uptake in yeast and plant cells, restore growth of K ⁺ -uptake deficient yeast, <i>chx13</i> mutant plants are sensitive to low K ⁺ , essential for K ⁺ acquisition	Zhao et al. (2008), Sze et al. (2004)
AtCHX17	Pollen (microspore), root epidermis, root cortex, leaf, induced by salt stress, K ⁺ starvation, acidic pH, ABA	PVC in plant, PM in plant, endomembrane in yeast (820)	K ⁺ transport and pH homeostasis, <i>chx17</i> mutants have less K ⁺ in roots when K ⁺ starved, restore growth of <i>kha1</i> yeast at alkaline pH, membrane trafficking: reduce CPY secretion in KTA40–2 yeast; confer HygB tolerance in <i>kha1</i> mutant	Cellier et al. (2004), Sze et al. (2004), Maresova and Sychrova (2006), Chanroj et al. (2011), Chanroj (2011)
AtCHX20	Guard cells, root tip/cap	Reticulate endomembrane (842)	K ⁺ transport and pH homeostasis, guard cell movement and osmoregulation, <i>chx20</i> mutants show reduced stomatal aperture, pH homeostasis in yeast	Padmanaban et al. (2007), Chanroj et al. (2011)
AtCHX23	Pollen grain, pollen tube	Reticulate endomembrane: ER in pollen tube (867)	K ⁺ transport (E coli), <i>chx21chx23</i> mutant pollen tube failed to target the ovule, signaling: unresponsive to female cues	Sze et al. (2004), Lu et al. (2011)

(aa) refers to total residues in protein.

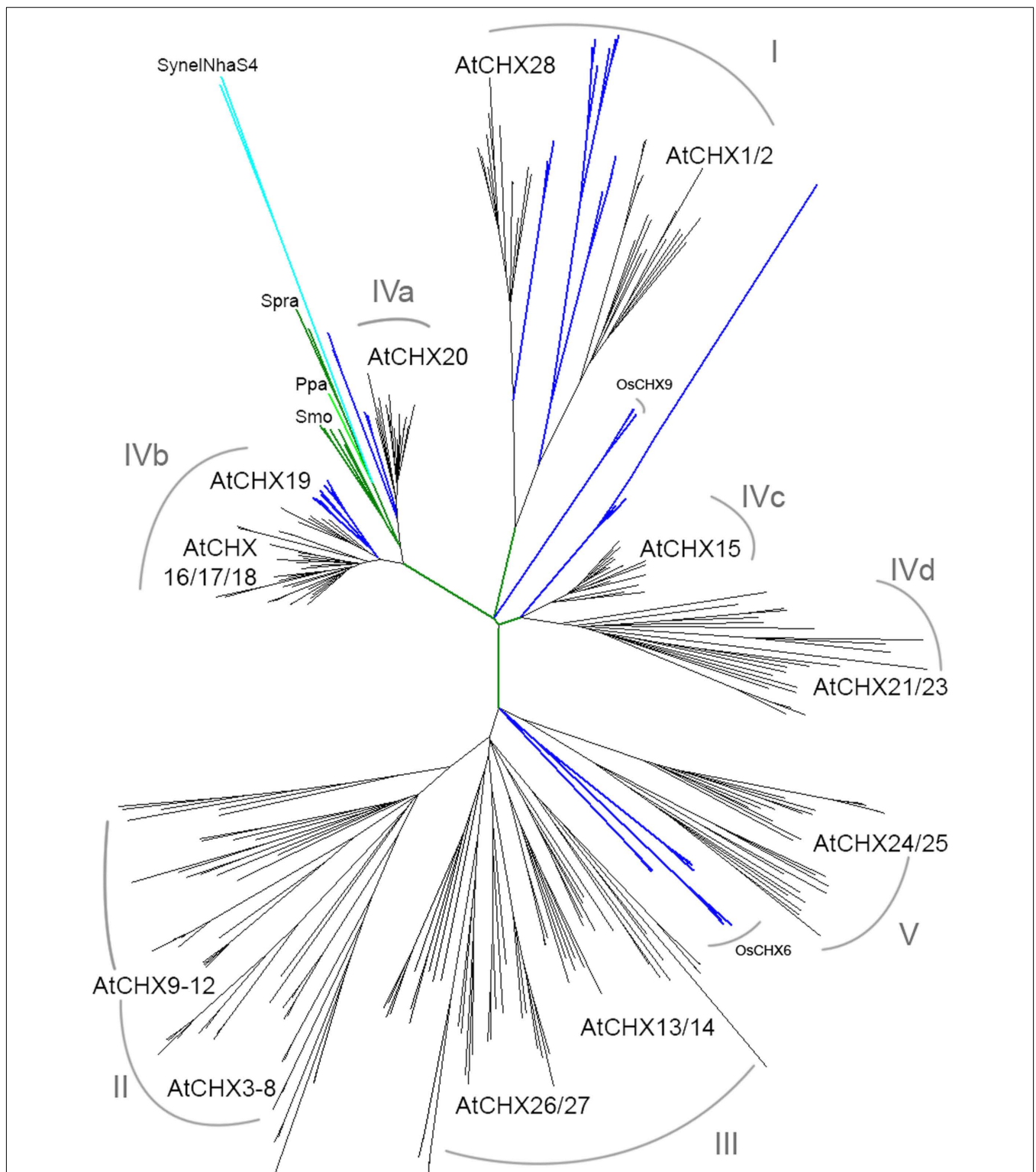


FIGURE 7 | CHX homologs diversified from moss to flowering plants.

Full-length CHX proteins from 15 species were aligned and then analyzed by maximum likelihood. *Spirogyra* (Spr), moss (Ppa), and club moss (Smo; green) CHXs clustered with AtCHX20 (subclade IVa). Both monocot (blue) and dicot (black) plants had orthologs of AtCHX20, AtCHX16–19 (IVb), AtCHX28 (II), AtCHX1/2 (I), and AtCHX15 (IVc). Corn, sorghum, or rice had two to four genes encoding OsCHX6 homologs in a monocot cluster near AtCHX24/25.

Most dicots had additional CHX homologs that clustered with AtCHX24/25 (V), 26/27, and 13/14 (III). AtCHX3–12 proteins (subclade II) are specific to *A. thaliana* and *A. lyrata*. A cluster of CHX resulting from multiple gene duplications is specific to *Medicago truncatula* (Mtr). See summary in **Table 5**. Species, protein accession numbers and protein properties are described in Table S4 in Supplementary Material, and unrooted rectangular tree is in Figure S4 in Supplementary Material.

genomes. However, rice had four other genes (OsCHX5–8) that clustered near AtCHX24/25, though none were closely related to 16 additional *Arabidopsis* gene products, CHX3–12, 13/14, or 26/27. Three to four CHX genes from other monocot plants including *Brachypodium*, a model of an energy grass, sorghum, and corn also clustered with rice OsCHX5–8, suggesting these represent a monocot subclade of CHX homologs (Figure 7).

In contrast, some dicot plants shared additional genes that are potential orthologs of AtCHX24/25, 13/14, and 26/27. Other dicot CHXs from poplar, *Citrus clementina*, *Mimulus*, and *Manihot esculenta* (cassava) formed clusters that were separate from AtCHX13/14 or 24/25, 26/27 subclades, suggesting these genes had diverged later in speciation. Notably, AtCHX3–8 and AtCHX9–12 proteins were in general specific to *Arabidopsis* genus, indicating diversification of CHX genes during *Arabidopsis* speciation. *Medicago truncatula* also had a cluster of 12 genes that could be a result of additional duplications during speciation.

Functions of plant CHX proteins. Functional studies of plant CHX proteins are just beginning and so far limited to *Arabidopsis thaliana*. According to results from the founding members of the family, AtCHX16–AtCHX20, these proteins are implicated in K⁺ and pH homeostasis of dynamic endomembranes. First, expression of AtCHX17 or its homologs CHX16–CHX20, rescued alkaline-sensitive growth phenotype of the host yeast strain (Chanroj et al., 2011). Second, CHX20 caused acidification and alkalization of the cytosol and vacuole of yeast, respectively; although CHX17 did not alter cytoplasmic or vacuolar pH (Chanroj et al., 2011). Third, AtCHX17 restored growth of bacteria defective in several K⁺ uptake systems, and tracer studies demonstrated that CHX17, CHX20, and CHX23 mediated monovalent cation transport with a preference for K⁺ over Na⁺ (Table 3). Thus CHXs, including plasma membrane-localized CHX13, transport K⁺ (Zhao et al., 2008), though their ability to restore yeast growth depends on different external pH's. Interestingly, these findings of plant CHX are consistent with mutant studies that suggest *Synechocystis* NhaS4 probably transported K⁺, but not Na⁺ (Inaba et al., 2001).

The association of AtCHX proteins with endomembranes and their role in pH and cation homeostasis would suggest they perform important roles in membrane trafficking events that affect protein and cargo sorting. Two lines of evidence support this idea. First, AtCHX17, AtCHX18, or AtCHX19, but not CHX20, conferred tolerance to hygromycin B in yeast lacking multiple cation-handling mechanisms (Chanroj et al., 2011). Sensitivity to aminoglycosides in yeast has been related to altered intracellular pH homeostasis and membrane trafficking. Second, Chanroj et al. (2011) showed that yeast mutants expressing CHX17 secreted less vacuolar-destined carboxypeptidase Y (CPY) to the external medium similar to cells expressing NHX1. Thus cation and pH homeostasis in endosomes, such as PVC, appeared to influence sorting and trafficking of proteins in the endomembrane system. Based on these findings and analysis of *Atchx20* mutants (Padmanaban et al., 2007), we propose that CHX20 affects endomembrane dynamics and osmoregulation needed for guard cell swelling and stomatal opening.

The multiplicity of CHX genes in *Arabidopsis* pollen and possibly other plants is surprising and not understood. The first study on pollen CHX proteins showed that AtCHX21 and AtCHX23 are functionally related and that they are involved in either the reception or the transduction of female signals that target pollen tube to the ovule (Lu et al., 2011). Pollen carrying double mutant *chx21chx23* developed into mature grains, extended a tube into the transmitting tract, however the tube failed to find the egg (Lu et al., 2011). Pollen tube elongates by tip growth and failure to shift the axis of polarity in the double mutant suggests that localized cation and pH homeostasis by CHX has a role in the establishment, or maintenance of polarity or both. Homologs of *Arabidopsis* CHX21 and CHX23 are present in dicot, but not in monocot, plants (Figure 7; Table 5; Table S4 in Supplementary Material). Whether they influence pollen tube guidance of dicots in general or have other roles in reproductive fitness will need to be investigated.

DISCUSSION

ORIGIN OF PLANT KEA AND CHX GENES FROM CYANOBACTERIA

Our phylogenetic analyses confirm that plant KEA and CHX genes of the CPA2 superfamily most likely originated from ancestral NhaA/NapA genes of prokaryotes (Chang et al., 2004; Brett et al., 2005a) of cyanobacterial origin. Although many bacteria, such as *E. coli* and *K. pneumonia* possess both NhaP and NhaA genes, CPA2 genes related to KEA/KefB/C are detected in a subset of bacteria, including Cyanobacteria *Gloeobacter* and *Anabaena variabilis* (Figure 3). Oddly, KEA or Kef-like genes have not been found in any fungi yet. These results suggest that plant CPA2 genes were derived from a cyanobacterium after it formed an endosymbiotic relationship with a primitive non-photosynthetic eukaryote cell. *Gloeobacter violaceus* may be a descendant of that primitive cyanobacterium (Criscuolo and Gribaldo, 2011), as it has genes encoding proteins homologous to NhaP, NhaS3, NhaS4, and KefB (KEA1/2). Similarly, NhaS4 from predominantly Cyanobacteria is highly homologous to plant CHX (Figure 1). However, NhaS4 also shares high similarity with fungi KHA1 and Nhe3/4 from a protist *Dictyostelium discoideum*.

Our analysis suggests that KEA transporters in eukaryotes serve functions preferentially in plant cells, and possibly in chloroplasts or plastids. First, we find that all plants sequenced to date possess three subtypes of KEA (Table 4). Green algae, *Chlamydomonas* and *Volvox*, have one KEA in each subtype, while higher plant KEAs diversified in subtype KEA1/2 or KEA4–6 (Figure 3). Second, KEAs are found mainly in plastid-containing organisms, including red algae and secondary endosymbiont like Chromista, but are not detected in fungi. Third, peptide sequences of KEA-Ia subtype were identified in proteomic analysis of isolated *Arabidopsis* plastids (Zybailov et al., 2008). Fourth, a functional study showed that *Synechocystis* NhaS3 was localized to thylakoid membranes and that it conferred tolerance to high Na and low K⁺ when expressed in a salt-sensitive *E. coli* (Inaba et al., 2001; Tsunekawa et al., 2009). As NhaS3 is related to KefB and KEAs, plant KEA-I subtype may participate in monovalent cation and pH homeostasis of thylakoid membranes in chloroplasts. Plant KEA-Ia proteins are distinguished by a long N-terminal domain. The roles of this unusual domain and of KEA subtypes in plants need to be determined.

Table 4 | Overview of genes encoding cation–proton antiporters (CPA) genes in plants.

Classification	Species	CPA1			CPA2			CHX
		NHX subtypes			KEA subtypes			
		NHX1-4	NHX5/6	NHX7/8	KEA-Ia	KEA-Ib	KEA-II	
					KEA1/2	KEA3	KEA4/5/6	
EurosidI	<i>M. esculenta</i>				1	1	4	28
	<i>R. communis</i>	4	2	1	2	1	3	26
	<i>P. trichocarpa</i>	2	4	2	2	1	4	30
	<i>M. truncatula</i>	5	1	?	1	1	2	45
	<i>G. max</i>	7	3	1	4	2	6	46
	<i>C. sativus</i>				1	1	3	19
EurosidII	<i>A. thaliana</i>	4	2	2	2	1	3	28
	<i>A. lyrata</i>	4	2	2	2	1	4	28
	<i>C. papaya</i>				1	1	2	28
	<i>E. grandis</i>	6	1	2	1	1	2	19
Eudicot	<i>V. vinifera</i>				1	1	2	15
	<i>M. guttatus</i>				1	1	3	17
Monocots	<i>S. bicolor</i>	4	2	1	1	1	2	16
	<i>Z. mays</i>	6	2	1	2	1	3	15
	<i>O. sativa</i>	4	2	1	1	1	2	17
	<i>B. distachyon</i>	4	2	2	1	1	2	15
Lycophyte	<i>S. moellendorffii</i>	2	2	2	1	1	2	3
Bryophyte	<i>P. patens</i>	5	2	2	3	2	2	4
Zygnematales	<i>S. pratensis*</i>	1	1	1	1	1	1	1
Volvocales	<i>V. carteri</i>	1–2	1	2	1	1	1	0
Volvocales	<i>C. reinhardtii</i>	1	3	1	1	1	1	0
Fungi	<i>S. cerevisiae</i>	0	1	1	0	0	0	1 KHA1
Cyanobacteria	<i>Synechocystis</i>	0	0	NhaP1/2	(NhaS3/5)			NhaS4
Bacteria	<i>E. coli</i>	0	0	1 NhaP	2 KefB/C		1 YBaI	0

Gene numbers are taken from the data in PHYTOZOME (<http://www.phytozome.net/>) from genomes of *Manihot esculenta*, *Ricinus communis*, *Populus trichocarpa*, *Medicago truncatula*, *Glycine max*, *Cucumis sativus*, *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Carica papaya*, *Eucalyptus grandis*, *Vitis vinifera*, *Mimulus guttatus*, *Sorghum bicolor*, *Zea mays*, *Oryza sativa*, *Brachypodium distachyon*, *Selaginella moellendorffii*, *Physcomitrella patens*, *Volvox carteri*, and *Chlamydomonas reinhardtii*. *Data for *Spirogyra pratensis* are unpublished results of C. Delwiche. Gene numbers from *Saccharomyces cerevisiae*, *Escherichia coli* K12, and *Synechocystis* PCC 6803 are provided for comparison. Blanks indicate “not analyzed.”

VACUOLAR NHX1–4 CLADE IS SPECIFIC TO PLANTS

The finding that single-celled algae and early land plants have homologs of plasma membrane NhaP/SOS1, endosomal AtNHX5/6, and vacuolar AtNHX1–4 suggest these genes were present in ancestral algae before plants colonized land (Table 4). Furthermore, their roles are conserved and fundamental to plant cells, as the three NHX subtypes have persisted from single-celled algae to flowering plants. Endosomal AtNHX5/6 is part of the IC-NHE clade found in all eukaryote, including protist, fungi, and metazoa (Brett et al., 2005a). It is noteworthy that yeast NHX1 is localized to PVC and shares more similarity to endosomal NHX5/6 than to plant vacuolar NHX1. Importantly, our analysis extends a previous study (Brett et al., 2005a) that a clade of “vacuolar” AtNHX1–4 is specific to plants as seen from *Chlamydomonas* and moss to rice and *Arabidopsis* (Figure 2). Importantly, gene diversification of the “plant vacuolar NHX” in monocots and dicots (Table 4) underscores the diverse and critical

roles vacuoles play in plant life (Martinoia et al., 2007; Muntz, 2007).

DIVERSIFICATION OF CHX GENE FAMILY DURING PLANT EVOLUTION

The multiplicity of the CHX gene family as plants colonized terrestrial habitats is striking. Phylogenetic analysis demonstrated this gene family in plants was founded by members similar to AtCHX20 and AtCHX16–19. First, cyanobacterium SynNhaS4 shares highest similarity with AtCHX17–20. Second, one CHX from a Charophyte, *Spirogyra*, is homologous to AtCHX20. Third, CHX genes from *Selaginella* and *Physcomitrella* encode proteins that are most homologous to AtCHX20 and AtCHX16–19, and fourth all flowering plants sequenced so far have CHX genes orthologs to guard cell-specific AtCHX20 and AtCHX16–19 (Figure 7). Thus a CHX member in *Spirogyra*, an alga that is closely related to land plants (Karol et al., 2001), is conserved in early land plants. In *Arabidopsis*, AtCHX20 is preferentially expressed in guard cells (Padmanaban

Table 5 | CHX homologs from algae to angiosperms cluster into distinct subclades.

Subclade	IVa	IVb	IVc	IVd	I	I-m	V	III	II
<i>A. thaliana</i>	CHX20	CHX16-19	CHX15	CHX21/23	CHX1/2/28	OsCHX9	CHX24/25	CHX13/14, 26/27	CHX3-12
Expression in <i>At</i> only	Guard	Root/leaf	Pol/Veg	Pollen	Pollen		Pollen	Pollen	Pollen
ALGAE									
Cre	0								
Spra	1								
EARLY LAND PLANT									
Ppa moss	4								
Smo	3								
MONOCOT									
Osa	1	3	2		4	(2)	(4)		
Bdi	2	3	2		4	(1)	(3)		
Zma	1	3	3		4	(1)	(3)		
Sbi	2	4	2		4	(2)	(2)		
DICOT									
Aco	1	2	3					11	
Mgu	1	3	3	1	2		2	3	1
Vvi	2	4	1	1	4		1	2	
Egr	2	1	1	1	2		4	3	3
Ccl	2	4	1	3	3		6	5	4
Ath	1	4	1	2	3		2	4	11
Aly	1	4	1	2	3		2	4	11
Csa	1	4	1	1	2		1	5	3
Mtr	3	5	2	5	2		2	6	12
Ptr	1	4	1	4	4		2	5	7
Mes	n.d.	3	2	3	4		3	5	2

Number of genes affiliated with each subclade is shown. The AtCHX family is grouped into five subclades, I–V (Sze et al., 2004), and subclade IV splits in four groups, IVa–IVd, according to phylogenetic analysis (Figure 7) and functional studies. Expression refers only to CHX genes of *Arabidopsis thaliana*. The founding members are likely genes in subclade IVa. CHX gene family diversified in flowering plants, especially in dicots. Genes in two clusters specific to monocots are in parenthesis. See text for full names of plant species. Aco, *Aquilegia coelulea*; Ccl, *Citrus clementina*.

et al., 2007), and CHX16–19 are expressed in various root cells, leaf hydathodes, as well as at the micropylar end of developing seeds (Chanroj, 2011). It would be interesting to determine if one or more of moss CHX genes are expressed in cells forming leaf pores and/or stomata which first appeared in mosses as they developed specialized epidermal cells to prevent desiccation (Freeman, 2008; Peterson et al., 2010).

Analysis of higher plants showed several monocot CHXs are orthologous to AtCHX15, AtCHX1/2, and AtCHX28. Thus these genes were present before the diversification of dicots and monocots. As these *Arabidopsis* genes are expressed in pollen (Sze et al., 2004⁵), we suggest CHX genes evolved as plants developed pollen grains to protect and transfer sperms for successful reproduction on land. Several rice CHX genes are expressed in developing anthers when pollen is at the bicellular and tricellular stages (Fujita et al., 2010⁶), supporting this idea. In general, monocots have less CHX genes than dicots encoding proteins homologous to pollen-expressed AtCHXs (Table 4). Thus CHX genes further diversified as dicot plants evolved as seen in AtCHX3–12 (Sze et al., 2004)

from *Arabidopsis thaliana* or *A. lyrata*. Several homologs exist in *Medicago truncatula* in a sister clade to pollen-expressed *Arabidopsis* CHX. Appearance of pollen is an important innovation for successful reproduction on land (Freeman, 2008). Whether CHX homologs are related to development of monocolpate versus tricolpate pollen seen in monocots and dicots, respectively, remains to be seen.

STRUCTURAL AND FUNCTIONAL INNOVATIONS FOR SURVIVAL ON LAND

Based on functional studies of CHXs and phylogenetic results, we propose a simple model. The emergence of CHX genes in early land plants and their multiplicity in flowering plants, but not in higher metazoa, suggest CHX function is tied to land plant-specific cellular processes. Their roles in guard cell movement (Padmanaban et al., 2007) and pollen tube guidance (Lu et al., 2011) would support this idea. Based on these two studies, it is obvious that endomembrane-associated NHX members cannot substitute for CHX functions (Chanroj et al., 2011), even though vacuolar and endosomal NHX homologs are expressed in most cell types. Studies showed that AtCHX17 and AtCHX20 are associated with dynamic endomembranes of the endocytic and exocytic pathways suggesting roles in

⁵www.geneinvestigator.com/

⁶http://www.plexdb.org/

osmoregulation, remodeling of membranes as well as in secretion. Land plants secrete a variety of compounds for growth, development, and defense, including signaling molecules, soluble proteins, membrane complexes, extracellular and wall components. We propose that CHX transporters modulate the cation and pH environment of diverse intracellular compartments for protein activities within the lumen and/or for directed vesicle trafficking to deliver cargo in flowering plants to designated locations.

SUMMARY

Based on the prevalence of CPA1 genes in all organisms, we suggest that plant NHX gene family evolved to serve fundamental needs for cation and pH homeostasis in all eukaryote cells. NhaP/SOS1 regulates cation/H⁺ fluxes at the plasma membrane as in prokaryotes, and endosomal NHX appeared later to modulate homeostasis and support functions of multiple intracellular compartments in primitive eukaryote cells. Further diversification of plant-specific vacuolar AtNHX1–4 type genes occurred in algal ancestors and in multicellular plants as vacuolar functions increased. In contrast, plant CPA2 genes likely originated from an ancestral cyanobacterium, where NhaS3- or NhaS5-like genes were transferred to the nuclear genome of ancestral plant cells. Three subtypes of KEA proteins are conserved from algae to flowering plants. Although their functions are still unknown, the evolutionary history would implicate a role of some KEA in ion homeostasis of plastids. A NhaS4-like gene of an ancient cyanobacterium is a likely progenitor of the CHX gene family which has multiplied and diversified specifically in flowering plants. Their functions in guard cell movement and pollen tube guidance determined so far would indicate they evolved to support vegetative and reproductive innovations required by plants to adapt and survive on land.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2012.00025/abstract

Table S1 | Cation–proton antiporters 1 and CPA2 genes and proteins from prokaryotes, algae, protist, metazoa, and plants used in Figure 1.

Table S2 | Gene descriptions of selected CPA1 proteins used in Figure 2.

Table S3 | Gene descriptions of selected KEA/Kef proteins in Figure 3.

Table S4 | Description of CHX genes and proteins from Figure 7.

Table S5 | XIs. Gene descriptions of selected KEA/Kef proteins in Figure 3 with hyperlinks.

Figure S1 | Phylogenetic tree showing CPA1 and CPA2 proteins used in Figure 1.

Figure S2 | Tree showing all CPA1 and NHX proteins used in analysis of Figure 2.

Figure S3 | Tree of KEA/Kef proteins used in Figure 3.

Figure S4 | Alignment of the N-terminal domain of KEA clade Ia sequences. The N-terminal domain of the clade Ia protein sequences found in the different plant taxa were aligned by MUSCLE (Edgar, 2004). Conserved residues are shown in orange. Regions of predicted coiled coil structures are underlined in blue. The database references for the used species are listed in Table S3.

Figure S5 | Tree showing all CHX proteins used in analysis of Figure 7.

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