



# Protein phylogenetic analysis of $\text{Ca}^{2+}$ /cation antiporters and insights into their evolution in plants

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Cation transport is a critical process in all organisms and is essential for mineral nutrition, ion stress tolerance, and signal transduction. Transporters that are members of the  $\text{Ca}^{2+}$ /cation antiporter (CaCA) superfamily are involved in the transport of  $\text{Ca}^{2+}$  and/or other cations using the counter exchange of another ion such as  $\text{H}^+$  or  $\text{Na}^+$ . The CaCA superfamily has been previously divided into five transporter families: the YRBG,  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX),  $\text{Na}^+/\text{Ca}^{2+}$ ,  $\text{K}^+$  exchanger (NCKX),  $\text{H}^+/\text{cation}$  exchanger (CAX), and cation/ $\text{Ca}^{2+}$  exchanger (CCX) families, which include the well-characterized NCX and CAX transporters. To examine the evolution of CaCA transporters within higher plants and the green plant lineage, CaCA genes were identified from the genomes of sequenced flowering plants, a bryophyte, lycophyte, and freshwater and marine algae, and compared with those from non-plant species. We found evidence of the expansion and increased diversity of flowering plant genes within the CAX and CCX families. Genes related to the NCX family are present in land plant though they encode distinct MHX homologs which probably have an altered transport function. In contrast, the NCX and NCKX genes which are absent in land plants have been retained in many species of algae, especially the marine algae, indicating that these organisms may share “animal-like” characteristics of  $\text{Ca}^{2+}$  homeostasis and signaling. A group of genes encoding novel CAX-like proteins containing an EF-hand domain were identified from plants and selected algae but appeared to be lacking in any other species. Lack of functional data for most of the CaCA proteins make it impossible to reliably predict substrate specificity and function for many of the groups or individual proteins. The abundance and diversity of CaCA genes throughout all branches of life indicates the importance of this class of cation transporter, and that many transporters with novel functions are waiting to be discovered.

**Keywords:** calcium transport, cation transport, evolution,  $\text{H}^+/\text{Ca}^{2+}$  exchanger,  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, phylogeny, CaCA

## INTRODUCTION

The importance of transporters as “gatekeepers” of the cell is exemplified in recent genomic studies. For example, the central role of transporters in controlling cell growth was indicated in a study showing that increased yeast growth correlates with increased cell surface area, and thus increased nutrient uptake (Groeneveld et al., 2009). Indeed, of the 1312 distinct reaction steps in a reconstruction of yeast metabolism, 401 involve transport (Herrgard et al., 2008). Likewise, the analysis of fully sequenced photosynthetic eukaryotes finds that transporters consistently make up a significant proportion (~5%) of the genome (Mäser et al., 2001; Merchant et al., 2007; Rensing et al., 2008). Calcium ( $\text{Ca}^{2+}$ ) is a critical element in all organisms; it is an essential nutrient which also has a conserved signaling role (Hirschi, 2004; Case et al., 2007). Transporters that mediate the movement of this ion are therefore particularly important. These  $\text{Ca}^{2+}$  transporters impact upon cell division, growth, development, and adaptation to environmental conditions. Three classes of membrane transporters help mediate  $\text{Ca}^{2+}$  flux across a membrane and regulate cytosolic  $\text{Ca}^{2+}$  levels:  $\text{Ca}^{2+}$ -permeable channels,  $\text{Ca}^{2+}$ -ATPases, and  $\text{Ca}^{2+}$ /cation

antiporters (CaCAs). Biochemical identification and functional analysis of CaCAs in an array of organisms have helped conceptualize the functional properties and important physiological roles of these exchangers in cellular ion homeostasis (Lyttton, 2007; Manohar et al., 2011).

The majority of the ion-coupled  $\text{Ca}^{2+}$  exchange activity within a cell is encoded by members of the  $\text{Ca}^{2+}$ /cation antiporter (CaCA) gene superfamily which are present widely in archaea, bacteria, fungi, plants, and animals (Saier et al., 1999; Cai and Lyttton, 2004a; Table 1). These proteins serve as essential components in  $\text{Ca}^{2+}$  cycling systems. CaCA proteins promote  $\text{Ca}^{2+}$  efflux across membranes, normally against its concentration gradient, by using a counter-electrochemical gradient of other ions such as  $\text{H}^+$ ,  $\text{Na}^+$ , or  $\text{K}^+$  to energize the process. Animal proteins principally use  $\text{Na}^+$  gradients as the driving force while plant and bacterial exchangers exclusively utilize  $\text{H}^+$ . Based on functional and phylogenetic analysis, the CaCA superfamily is composed of at least five families (Cai and Lyttton, 2004a): the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) family, the  $\text{Na}^+/\text{Ca}^{2+}$ ,  $\text{K}^+$  exchanger (NCKX) family and the CCX family, the  $\text{H}^+/\text{cation}$  exchanger (CAX) family, and the YRBG family

**Table 1 | Definition of the five major gene families that make up the CaCA superfamily.**

CaCA gene family	Described major function	Species domain distribution	Key selected members
YRBG	Putative Na <sup>+</sup> /Ca <sup>2+</sup> exchanger	Bacteria, archaea	<i>EcyrbG</i> (bacteria)
NCX	Na <sup>+</sup> /Ca <sup>2+</sup> exchanger	Eukaryotes	<i>NCX1</i> – 3 (mammalian), <i>AtMHX</i> (plant)
NCKX	K <sup>+</sup> -dependent Na <sup>+</sup> /Ca <sup>2+</sup> exchanger	Eukaryotes – excluding land plants	<i>NCKX1</i> – 5 (mammalian)
CCX	Cation/Ca <sup>2+</sup> exchanger	Eukaryotes	<i>NCKX6</i> (mammalian), <i>AtCCX3</i> (plant)
CAX	Ca <sup>2+</sup> /H <sup>+</sup> exchange	Bacteria, eukaryotes – excluding mammals, insects, nematodes	<i>ScVCX1</i> (yeast), <i>AtCAX1</i> (plant), <i>EcchaA</i> (bacteria)

**Table 2 | Summary of the major properties and functions of selected CaCA genes.**

Gene name (species)	CaCA family	Transport function	Sub-cellular location	Main function	Reference
<i>yrbG</i> ( <i>E. coli</i> )	YRBG	Putative Na <sup>+</sup> /Ca <sup>2+</sup> exchange – unconfirmed	Inner membrane	Unknown; not involved in regulating cytosolic Ca <sup>2+</sup> ; not essential	Naseem et al. (2008)
<i>NCX1</i> (human, mouse)	NCX	Na <sup>+</sup> /Ca <sup>2+</sup> exchange; stoichiometry: 3Na <sup>+</sup> :1Ca <sup>2+</sup>	Plasma membrane	Ca <sup>2+</sup> efflux from cell in heart, brain, kidney	Blaustein and Lederer (1999)
<i>AtMHX</i> ( <i>Arabidopsis</i> )	NCX (MHX)	Mg <sup>2+</sup> /H <sup>+</sup> and Zn <sup>2+</sup> /H <sup>+</sup> exchange; electrogenic	Vacuole	Vacuolar Mg <sup>2+</sup> sequestration; Mg <sup>2+</sup> homeostasis	Shaul et al. (1999)
<i>NCKX1</i> (bovine, human)	NCKX	K <sup>+</sup> -dependent Na <sup>+</sup> /Ca <sup>2+</sup> exchange; stoichiometry: 4Na <sup>+</sup> :1Ca <sup>2+</sup> , 1K <sup>+</sup>	Plasma membrane	Ca <sup>2+</sup> efflux in rod photoreceptor retina cells to allow light-triggered signal generation	Reilander et al. (1992), Schnetkamp (1995)
<i>NCKX6/NCLX</i> (human, mouse)	CCX	Na <sup>+</sup> /Ca <sup>2+</sup> and Li <sup>+</sup> /Ca <sup>2+</sup> exchange, K <sup>+</sup> -independent?	Mitochondria	Mitochondrial Ca <sup>2+</sup> efflux by Na <sup>+</sup> /Ca <sup>2+</sup> exchange	Cai and Lytton (2004b), Palty et al. (2004, 2010)
<i>AtCCX3</i> ( <i>Arabidopsis</i> )	CCX	K <sup>+</sup> /H <sup>+</sup> , Na <sup>+</sup> /H <sup>+</sup> , and Mn <sup>2+</sup> /H <sup>+</sup> exchange	Endo-membrane	Vesicle ion uptake; K <sup>+</sup> homeostasis in flower tissues	Morris et al. (2008)
<i>chaA</i> ( <i>E. coli</i> )	CAX (CHAA)	Ca <sup>2+</sup> /H <sup>+</sup> , Na <sup>+</sup> /H <sup>+</sup> , and K <sup>+</sup> /H <sup>+</sup> exchange	Inner membrane	Outward and inward cation flux; ion homeostasis	Ivey et al. (1993), Ohyama et al. (1994), Radchenko et al. (2006)
<i>VCX1</i> (yeast)	CAX	Ca <sup>2+</sup> /H <sup>+</sup> exchange; stoichiometry: 2H <sup>+</sup> :1Ca <sup>2+</sup>	Vacuole	Vacuolar Ca <sup>2+</sup> loading; regulates cytosolic Ca <sup>2+</sup>	Cunningham and Fink (1994), Denis and Cyert (2002)
<i>AtCAX1</i> ( <i>Arabidopsis</i> )	CAX	Ca <sup>2+</sup> /H <sup>+</sup> exchange; stoichiometry: 3H <sup>+</sup> :1Ca <sup>2+</sup> (based on red beet studies); can also mediate Mn <sup>2+</sup> /H <sup>+</sup> and Cd <sup>2+</sup> /H <sup>+</sup> exchange	Vacuole	Vacuolar Ca <sup>2+</sup> loading; provides Ca <sup>2+</sup> tolerance; determines cellular Ca <sup>2+</sup> concentration; involved in response to stress	Blackford et al. (1990), Hirschi et al. (1996), Cheng et al. (2003), Conn et al. (2011)
<i>CrCAX1</i> (green microalgae)	CAX	Ca <sup>2+</sup> /H <sup>+</sup> and Na <sup>+</sup> /H <sup>+</sup> exchange	Vacuole (predicted)	Provides vacuolar Ca <sup>2+</sup> sequestration and Ca <sup>2+</sup> tolerance when expressed in yeast	Pittman et al. (2009)
<i>DrCAX</i> (zebra fish)	CAX	Ca <sup>2+</sup> /H <sup>+</sup> exchange	Endo-membrane	Neural crest development	Manohar et al. (2010)

named after the *Escherichia coli* gene *yrbG* (Table 1). Detailed characterization of selected isoforms from each of these families has begun to shed light on their functions (summarized in Table 2).

The YRBG family has been the least characterized. Sequence similarity suggests *E. coli* YrbG may function as a NCX but the transporter is not essential for bacterial growth and it is unknown whether YrbG is a Ca<sup>2+</sup> transporter (Cai and Lytton, 2004a; Naseem et al., 2008). In *E. coli*, pH and monovalent cations can regulate cytosolic free Ca<sup>2+</sup> through Ca<sup>2+</sup> influx and efflux, but direct

measurement of cytosolic Ca<sup>2+</sup> levels using the Ca<sup>2+</sup> reporter aequorin suggests that YrbG does not play a role in regulating cytosolic Ca<sup>2+</sup> (Naseem et al., 2008). Orthologs of *yrbG* are present in other prokaryotes but do not appear to be present in eukaryotes (Cai and Lytton, 2004a).

Na<sup>+</sup>/Ca<sup>2+</sup> exchangers, present at the plasma membrane of most animal cells, are a fast and high-capacity Ca<sup>2+</sup> transport system that are important regulators of cellular Ca<sup>2+</sup> homeostasis by causing efflux of Ca<sup>2+</sup> from the cell. Detailed functional and

molecular studies have revealed two distinct families of  $\text{Na}^+/\text{Ca}^{2+}$  exchange proteins (Lytton, 2007). The first of these, the NCX transporters are composed of three distinct types in mammals: NCX1, NCX2, and NCX3. All three exchangers share about 70% overall amino acid identity and have identical transport function. Invertebrates have a single NCX gene, whereas vertebrate species have multiple NCX genes as a result of at least two duplication events (On et al., 2008). The presence of NCX genes in all animal species' genomes allowed the construction of a phylogenetic tree that correlated to animal evolution and revealed that the origin of NCX duplication was initiated at the emergence of vertebrate organisms (On et al., 2008). A plant  $\text{Mg}^{2+}/\text{H}^+$  exchanger termed *Arabidopsis thaliana*  $\text{Mg}^{2+}/\text{H}^+$  exchanger (AtMHX), is also a member of the NCX family. This transporter concentrates  $\text{Mg}^{2+}$  into vacuoles and has no apparent  $\text{Na}^+/\text{Ca}^{2+}$  exchange activity (Shaul et al., 1999). Another putative  $\text{Mg}^{2+}/\text{H}^+$  exchanger (XNTA) has been discovered in *Paramecium*, but although it appears to be a member of the CaCA superfamily, it is distinct from AtMHX and does not appear to fall within the NCX clade (Haynes et al., 2002).

The second  $\text{Na}^+/\text{Ca}^{2+}$ -transporting exchanger family, the NCKX, operate in cellular  $\text{Ca}^{2+}$  efflux by extruding one  $\text{Ca}^{2+}$  ion and one  $\text{K}^+$  ion in exchange for four  $\text{Na}^+$  ions (Lytton, 2007). Like the NCX transporters, the NCKX play an important role in  $\text{Ca}^{2+}$  homeostasis, notably in retinal and neuronal cells, and are important in various roles including photoreceptor function and skin pigmentation (Table 2). Also like NCX transporters, NCKX are reversible and can import  $\text{Ca}^{2+}$  into the cell upon reversal of the transmembrane  $\text{Na}^+$  gradient. NCKX proteins differ from NCX proteins in their requirement for  $\text{K}^+$ , their  $\text{Ca}^{2+}$  kinetics, and sequence divergence. To date there has been no evidence of NCKX activity or NCKX-like genes present in any plants.

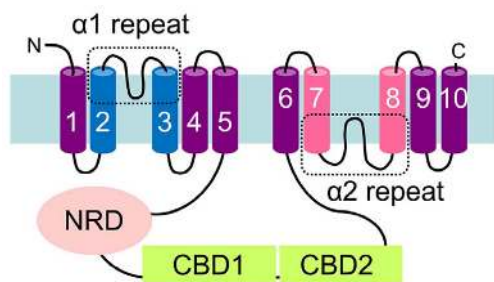
Initial analyses of the CaCA superfamily did not identify the CCX family and this family has only recently been created and considered a stand-alone group. A novel gene from mammals, originally thought to be a NCKX gene, named NCKX6, has significant sequence variation from other known NCX and NCKX genes (Cai and Lytton, 2004b). Subsequent phylogenetic analysis has grouped NCKX6 separately from the NCX and NCKX family in the CCX family, which includes members from vertebrates, invertebrates, fungi, protozoa, and plants (Cai and Lytton, 2004a). NCKX6 (also named NCLX) appears to be a bona fide NCX but it is slightly contentious as to whether it is  $\text{K}^+$ -dependent or not; other studies have suggested that this transporter is  $\text{K}^+$ -independent and can also transport  $\text{Ca}^{2+}$  by  $\text{Li}^+$  exchange (Palty et al., 2004; Table 2). Several transporters in plants, some formerly referred to as CAX, have been reassigned to the CCX family (Shigaki et al., 2006). For example, *Arabidopsis* CCX3 (formerly CAX9) is a  $\text{H}^+$ -dependent cation transporter but with no apparent  $\text{Ca}^{2+}$  transport properties (Morris et al., 2008).

Members of the CAX family have been identified from many species including plants, algae, some invertebrate animals, fungi, protozoa, and bacteria, but are absent from the genomes of mammals, insects, and nematodes (Shigaki et al., 2006; Manohar et al., 2011). CAXs have been classified into three major categories based on phylogenetic analysis: Type 1, 2, and 3 (Shigaki et al., 2006). Several Type 1 and 3 CAXs have been characterized across species, particularly in plants and yeast, and have been shown to

be important for  $\text{Ca}^{2+}/\text{H}^+$  exchange, predominantly at the vacuolar membrane (Manohar et al., 2011; Pittman, 2011). In particular, many plant and fungal CAXs are important for providing tolerance to excess  $\text{Ca}^{2+}$ , for regulating  $\text{Ca}^{2+}$  homeostasis and nutrition, and the modulation of environmental or developmental events via  $\text{Ca}^{2+}$  signal generation (McAinsh and Pittman, 2009; Cunningham, 2011). However, to date only two Type 2 CAXs, the *S. cerevisiae* Vnx1 and zebrafish DrCax1 have been characterized (Cagnac et al., 2007, 2010; Manohar et al., 2010).

The Type 1 plant CAXs have been further divided into two phylogenetically distinct groups (Type 1A and Type 1B; Shigaki et al., 2006). This distinction suggests functional differentiation between the two groups; however, no clear-cut functional differences have so far been identified. *Arabidopsis* and rice (*Oryza sativa*) each have six CAX ORFs equally divided between the two phylogenetic groups. One suggestion has been that there are differences in ion substrate selectivity between members of the two groups; for example, Type 1B proteins such as AtCAX2 and AtCAX5 have been shown to transport multiple ions including  $\text{Mn}^{2+}$  and  $\text{Cd}^{2+}$  in addition to  $\text{Ca}^{2+}$  (Hirschi et al., 2000; Shigaki et al., 2003; Edmond et al., 2009) while Type 1A members such as AtCAX1 and AtCAX3 are important for  $\text{Ca}^{2+}$  homeostasis and were originally thought to be specific for  $\text{Ca}^{2+}$  (Shigaki et al., 2003; Conn et al., 2011). However, Type 1A CAXs can mediate the transport of multiple ions *in planta* and when heterologously expressed in yeast (Kamiya et al., 2005; Shigaki et al., 2005, 2010; Koren'kov et al., 2007; Mei et al., 2009). Thus plant CAXs have a broad cation specificity and hence the CAX family has more recently been referred to as  $\text{H}^+$ /cation exchangers rather than just  $\text{H}^+/\text{Ca}^{2+}$  exchangers. The Type 1B sub-group may contain more diverse CAX proteins. Two non-canonical Type 1B CAXs with an additional transmembrane (TM) insertion between TM4 and TM5 were recently reported from plants in the Asteraceae family (Jain et al., 2009), although the relevance of this alteration is unknown. Recently, a CAX gene from the chlorophyte microalgae *Chlamydomonas reinhardtii* was identified and suggested to group within the Type 1C sub-group alongside protozoan CAX genes (Pittman et al., 2009). Like the higher plant CAXs, CrCAX1 appears to have broad substrate specificity, and in addition to  $\text{Ca}^{2+}$  can also transport the monovalent ion  $\text{Na}^+$ .

All CaCA proteins share a similar topological model: on average 10 TM helices are predicted to form a  $\text{Ca}^{2+}$  transduction pathway which includes two conserved  $\alpha$ -repeats regions within TM helices 2–3 and 7–8. These two  $\alpha$ -repeats are important for ion selectivity, binding, and transport in diverse CaCA members (Kamiya and Maeshima, 2004; Ottolia et al., 2005; Shigaki et al., 2005) and are predicted from topology models to be orientated on opposite sides of the membrane (Figure 1), and in the folded structure of the protein face toward each other to form an ion conductance channel (Nicoll et al., 2007). This topology is exemplified by NCX (Figure 1). The sizes of individual members of the CaCA superfamily vary from ~300 to 1000 amino acids, and although the core topological structure is conserved there are some exceptions; for example, the CCX proteins have additional TM domains within the C-terminal half of the protein (Cai and Lytton, 2004b). The clusters of 5 + 5 TM helices within both halves of a typical CaCA are separated by a large cytosolic loop (Figure 1) that regulates



**FIGURE 1 | Topological model of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) protein.**

Barrel structures denote the transmembrane spanning domains identified by hydropathy analysis using TMHMM version 2. The conserved  $\alpha 1$ - and  $\alpha 2$ -repeat regions which span TM 2 and 3, and TM 7 and 8 are highlighted. The  $\text{Ca}^{2+}$ -binding domains (CBD) and the predicted  $\text{Na}^+$  regulatory domain (NRD) present on the large central cytosolic loop are highlighted.

exchange activity in some members. It is likely that both halves of the protein formed following an ancient duplication event. While split halves of NCX or CAX proteins are not able to function alone (Ottolia et al., 2001; Zhao et al., 2009), it is not inconceivable that an ancestral form was useful as part of a related protein domain if this event occurred very early in the evolution of the gene family.

In most genomes putative membrane transporters abound with few transporters representing unique sequences in the genome (Bock et al., 2006). Despite fundamental differences in organismal complexity, an emerging theme is that diverse species have similar protein-coding potentials and alterations in CaCA transporters among taxa may be associated with modifications in function. The recent sequencing of genomes from diverse photosynthetic eukaryotes, including various higher plants, bryophytes, lycophytes, unicellular and multi-cellular green, red, and brown alga, and diatoms, act as powerful genomic resources for continued comparative analyses of CaCA proteins. Evolutional relationships among the characterized and uncharacterized CaCAs will lead to a better understanding of their potential roles. In this study we have performed a multi-genome identification of CaCA genes and a phylogenetic analysis that have provided insights into the evolution of CaCA transporters within higher plants and the green plant lineage.

## MATERIALS AND METHODS

### DATA SOURCES

Predicted protein-coding genes from complete and near complete genome sequences were downloaded for 74 species in May 2011 from a variety of databases (Table S1 in Supplementary Material). Species were selected for their broad taxonomic range in order to capture the full diversity of CaCA sequences, and where possible, photosynthetic organisms were included. The species examined included 12 land plants (including one lycophyte and one bryophyte), and 12 algae of various phyla and class (Table S1 in Supplementary Material), four fungi, eight other unicellular eukaryotes (including a variety of protists and oomycetes), seven animals (including two mammals), 21 bacteria (of which 10 were members of the cyanobacteria), and 10 archaea.

### CaCA IDENTIFICATION

Putative CaCA proteins were identified using the basic local alignment search tool (BLAST; Altschul et al., 1997). A local database was constructed from the predicted protein sequences for the 74 species examined. The database was queried with 147 previously identified CaCA protein-coding sequences (Cai and Lytton, 2004a) including all known CaCA proteins from *A. thaliana*. The initial results contained a variety of putative ion transporter sequences from the CaCA superfamily and from other families, in particular those with known functions in  $\text{Na}^+/\text{H}^+$ , arginine, and citrate transport, which were discarded. To obtain sequences specific to the CaCA transporter family, only those matches with an  $e$ -value of  $<1$ , that spanned a region of  $>100$  bp in length, with no less than 15% sequence identity, and where the predicted coding sequence corresponding to the match was  $>280$  bp in length were considered for further analysis. The remaining 1328 candidate CaCA homologs were then screened by hydropathy analysis using TMHMM v2.0<sup>1</sup> (Krogh et al., 2001) to remove all non-TM proteins and to remove those whose hydropathy profile was clearly dissimilar to a selection of representative CaCA genes from the NCX, NKCX, CCX, CAX, and YRBG families from human, *A. thaliana*, *O. sativa*, *S. cerevisiae*, *E. coli*, and *Synechocystis* sp. Further screening of candidate sequences was performed by aligning them with a small selection of well-characterized representative CaCA proteins, and visual inspection was used to remove those that were clearly not members of the CaCA family. Finally 308 putative CaCA homologs were retained for phylogenetic analysis, plus 34 novel CaCA-like proteins (named EF-CAX) that were shown by Pfam analysis<sup>2</sup> (Finn et al., 2010) to contain putative  $\text{Ca}^{2+}$ -binding EF-hand domains (Pfam ID: PF00036) and so-called “ $\text{Na}_\text{Ca\_ex}$ ” (consensus CaCA) domains (Pfam ID: PF01699; Table S2 in Supplementary Material).

### PHYLOGENETIC ANALYSES

Phylogenetic analysis of full-length CaCA proteins was not suitable because their diversity makes sequence alignment difficult. Several steps were taken in order to obtain reasonable alignments. Firstly, poorly conserved and extended N- and C-terminal regions of the predicted proteins were removed. TMHMM was then used to identify and extract putative TM domains for each protein. Furthermore, the greatest sequence distance between two TM domains was recorded and inferred to be the length of the central loop region. Visual inspection of preliminary alignments suggested that the number of TM domains can be variable between species, which may affect the accuracy of sequence alignment. To account for these differences we used MAFFT v6.0 (Katoh et al., 2002) under the E-INS-i option, which is specifically designed to cope with homologous regions separated by unalignable regions. Other approaches were also investigated for aligning these regions, and all yielded comparable results. The final phylogeny was estimated using maximum likelihood under the WAG + F model of amino acid substitution with  $\Gamma$ -distributed rates across sites, as implemented in RAxML v7.1 (Stamatakis, 2006). Other substitution models were examined and found to yield very similar or

<sup>1</sup>[www.cbc.dtu.dk/services/TMHMM](http://www.cbc.dtu.dk/services/TMHMM)

<sup>2</sup><http://pfam.sanger.ac.uk>

substantially worse log-likelihood values. Confidence in the tree was assessed using the fast bootstrap approach (Stamatakis et al., 2008). The tree was viewed using the FigTree program<sup>3</sup>. The resulting phylogeny appeared to be broadly consistent with previous findings, with five major clades corresponding to the CAX, CCX, NCX, NCKX, and YRBG genes. Thus gene products were classified according to their position in the phylogeny. For the phylogenetic analysis of the EF-CAX, due to poor sequence similarity between the N-terminal half of these proteins with N-terminal sequence of the other CaCA proteins, the conserved  $\alpha 2$ -repeat region sequence within the C-terminal half of CaCA and EF-CAX proteins was used instead. This sequence was identified using the consensus  $\alpha 2$ -repeat region sequence as determined previously for CaCA proteins (Cai and Lytton, 2004a), and extracted from each CaCA protein. The  $\alpha 2$ -repeat sequences were realigned to confirm that the correct consensus sequence was obtained and were then used to generate trees using maximum likelihood, as described above.

### AMINO ACID SEQUENCE COMPARISONS

For the visual analysis and comparison of CaCA protein sequences, multiple sequence alignments were performed using ClustalW2<sup>4</sup> (Larkin et al., 2007) and shaded using the BOXSHADE v.3.2 viewer and manually annotated.

## RESULTS AND DISCUSSION

### IDENTIFICATION OF GENES ENCODING CaCA HOMOLOGS IN PHOTOSYNTHETIC EUKARYOTES

To examine the evolution and potential diversification of the CaCA superfamily in photosynthetic eukaryotes and throughout the land plant lineage, we screened the sequenced genomes of 10 flowering plant species. These included six dicots: *A. thaliana*, a tree species – poplar (*Populus trichocarpa*), grape (*Vitis vinifera*); and three legumes: soybean (*Glycine max*), *Medicago truncatula*, and *Lotus japonica*, and four monocots: rice (*O. sativa*), corn (*Zea mays*), sorghum (*Sorghum bicolor*), and *Brachypodium distachyon*. The genomes of two non-flowering land plants were available for analysis; the bryophyte moss *Physcomitrella patens*, and the lycophyte spikemoss *Selaginella moellendorffii*. A range of diverse algal species were also chosen. These comprised four freshwater algae: the model unicellular chlorophyte alga *C. reinhardtii*, the unicellular chlorophyte *Coccomyxa* sp. C-169 (formerly named *Chlorella* sp. C-169), and the multi-cellular chlorophyte *Volvox carterii*; and a selection of marine algae: the prasinophytes *Ostreococcus tauri* and *Micromonas pusilla*, the rhodophyte *Cyanidioschyzon merolae*, the haptophytes *Emiliania huxleyi*, the brown alga *Ectocarpus siliculosus*, the pelagophyte *Aureococcus anophagefferens*, and photosynthetic members of the stramenopiles, including the diatoms *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, and *Fragilariopsis cylindrus*. In addition, two non-photosynthetic stramenopiles, the oomycetes *Phytophthora sojae*, and *Pythium ultimum* were examined. A selection of mammalian, invertebrate, fungal, protozoan, bacterial, cyanobacterial, and archaeal species with completed genome sequences were chosen that provide a cross section of life for comparison with the plant and algal species

(Table S1 in Supplementary Material). Using a dataset of previously identified CaCA query sequences, the genomes of all these species were screened by BLAST analysis and the results were further screened by hydrophobicity analysis, sequence alignment, and individual protein BLAST to validate each of the outputs and generate a final list of putative CaCA proteins (see Materials and Methods). In 13 of the species screened (including five bacteria and seven archaea) no obvious CaCA genes were identified (Table S1 in Supplementary Material). We were also unable to identify a clear-cut CaCA gene from the eukaryotic protozoan parasite *Giardia lamblia*. In contrast, the remaining 18 prokaryote species and all other eukaryotes examined including all of the plant and algal species possessed CaCA gene members (Figure 2). In all 342 CaCA genes were identified from 74 species (including the 13 species with no CaCA genes), giving an average of 4.6 CaCA genes per species. From the 12 land plants, 159 genes were determined (13.25 genes per species) and from the 12 algal species, 70 genes were identified (5.8 genes per species). The search method used multiple queries for each genome examined with the robust search method employed suggesting confidence that the search returned all expected genes and that CaCA genes are truly lacking in 13 of the species.

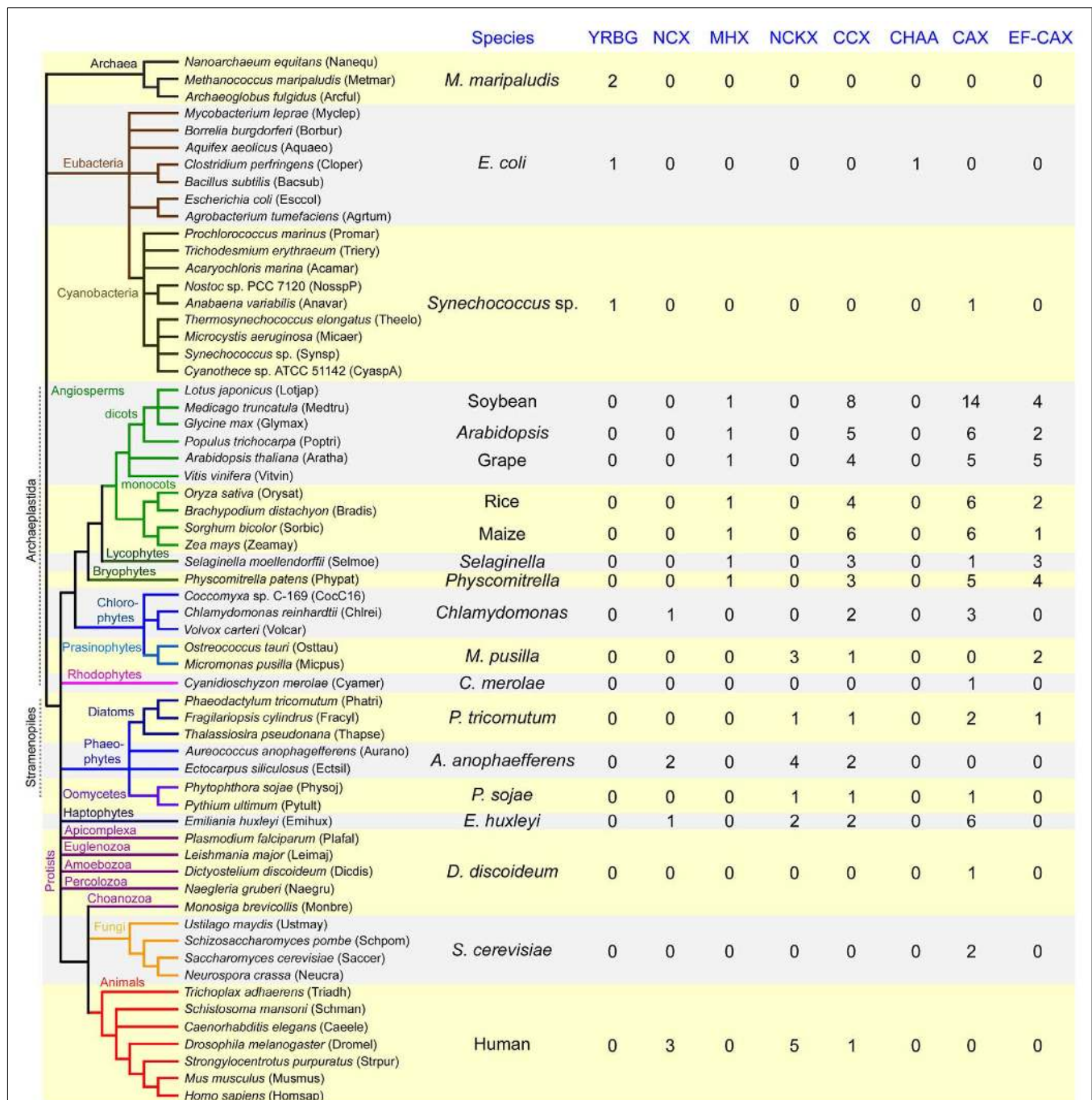
The classification of the genes into one of the five CaCA families was determined by phylogenetic analysis of the full superfamily. Initial amino acid sequence alignments using full-length sequence generated poor alignments and resulted in a low confidence tree. To improve the sequence alignments and generate the tree, the highly variable N- and C-terminal tail sequences and the hydrophilic loop sequences were therefore removed. The predicted size of the central loop region, the longest hydrophilic loop region of the CaCA protein, situated between TM5 and 6 of the consensus NCX (Figure 1), NCKX, CCX, and YRBG proteins, and between TM6 and 7 of the consensus CAX proteins, varied largely between the CaCA sequences, ranging from 19 amino acids in one of the CAX proteins to 602 amino acids in one of the CCX proteins. The NCX, NCKX, and CCX proteins all had on average a longer central loop region than the CAX and YRBG proteins; however, within all of these families there was significant variation in central loop length and the predicted number of TM domains (Table S3 in Supplementary Material). In particular, there was large variation within the NCX, NCKX, CCX, and CAX families. The significant variation within the CAX family was caused in part by the topological variation and variation in protein size between the Type 1 and Type 2 CAX protein sub-types (Shigaki et al., 2006). There was also large variation in the size of the N-terminal regions before the first TM span. Many of the animal NCX, NCKX, and CCX proteins are predicted to possess an N-terminal signal peptide (Lytton, 2007), while many of the plant CAX proteins possess a long N-terminal tail that has been shown to play a post-translational regulatory role (Pittman et al., 2002; Pittman, 2011), but which is absent in the fungal and bacterial CAX proteins.

The resulting tree indicates the presence of the five main CaCA families identified in previous analysis (Cai and Lytton, 2004a) although the bootstrap values at the base of the NCKX, NCX, and CCX branches were low (Figure 3). Likewise, some of the exact details for this tree and the individual family trees should be viewed with caution because long branches could lead to phylogenetic

<sup>3</sup><http://tree.bio.ed.ac.uk/software/figtree>

<sup>4</sup><http://www.ebi.ac.uk/Tools/msa/clustalw2>



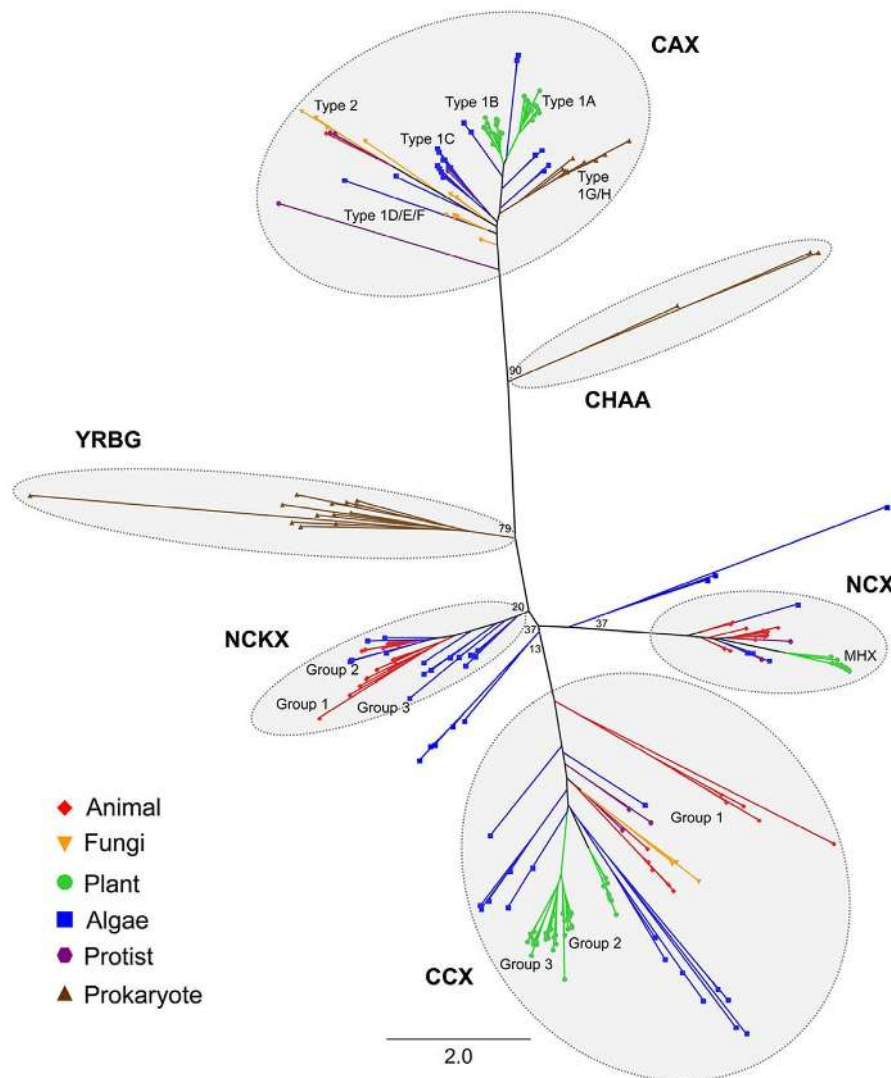


**FIGURE 2 | Variation in the distribution of genes within each of the major CaCA gene families (YRBG, NCX, NCKX, CCX, CHAA, CAX) and sub-families (MHX, EF-CAX) across eukaryotic and prokaryotic organisms.** Gene numbers determined by phylogenetic analysis from representative species for each of the classes of organisms examined are shown. The species from which

CaCA genes were identified are listed (left hand side) with the species name abbreviation in parentheses. The evolutionary relationships of the species is shown, generated using iTOL (<http://itol.embl.de>) and based on the NCBI taxonomy tree. For the full gene distribution for each of the species analyzed see Table S1 in Supplementary Material.

artifacts such as long branch attraction. The YRBG family contains only prokaryotic genes, including *E. coli yrbG* and genes from other bacteria (including cyanobacteria), but also archaea (Figure 3). None of the prokaryotic species analyzed here contained genes

that fell within the NCX, NCKX, and CCX families. These families possess higher plant (in the case of NCX and CCX), algal, and animal genes, with some fungal-derived genes in the CCX family. The algal genes are widely dispersed amongst these three



**FIGURE 3 | A phylogenetic tree of the CaCA superfamily.** The tree was constructed using the maximum likelihood method and derived from alignments of conserved hydrophobic region sequences identified from the genomes of selected animal, fungal, land plant, algae, protist, bacterial, and archaeobacterial species, listed in **Figure 2**. Six major groups are highlighted.

The sub-groups determined for the CAX, CCX, NCX, and NCKX families as shown in **Figures 4, 6, 7, and 9**, are also given. Line colors and symbols denote the species class. Bootstrap values are indicated at the nodes of major branches. The branch length scale bar indicates the evolutionary distance of two amino acid substitutions per site.

families and it is mostly the algal gene product sequences that yield the longest branch lengths within the tree indicating significant sequence variation. Furthermore, algal genes were also identified which were basal to the NCX and NCKX groups and could not be distinguished between the two families based on phylogeny (**Figure 3**). The largest proportion of CaCA genes identified was from the CAX family (120 of the 342 genes, which is 1.6 CAX genes per species; Table S1 in Supplementary Material). These genes were from all the classes of organisms examined including some invertebrate animals, as shown previously (Shigaki et al., 2006), but there were no mammalian or insect CAX genes. The CAX family included some bacterial and cyanobacterial genes but none from any of the archaeal species examined. As with the CCX

family, the size and branching of the CAX family indicates significant sequence variation and potential diversification. The *E. coli* protein ChaA, originally identified as a  $H^+/Ca^{2+}$  exchanger (Ivey et al., 1993) and subsequently suggested to function as a  $H^+/Na^+$  exchanger (Ohya et al., 1994), was not identified as a CaCA homolog by the BLAST search performed by Cai and Lyttton (2004a). These authors commented that the ChaA protein had limited sequence similarity with other CaCA proteins. More recent phylogenetic analysis of the CAX family suggested that *chaA* falls within the Type 3C branch of the CAX tree (Shigaki et al., 2006). The BLAST search in this study did identify *E. coli chaA* and our global CaCA phylogenetic analysis indicates that *chaA* and other related genes from *Agrobacterium tumefaciens* and *Mycobacterium*

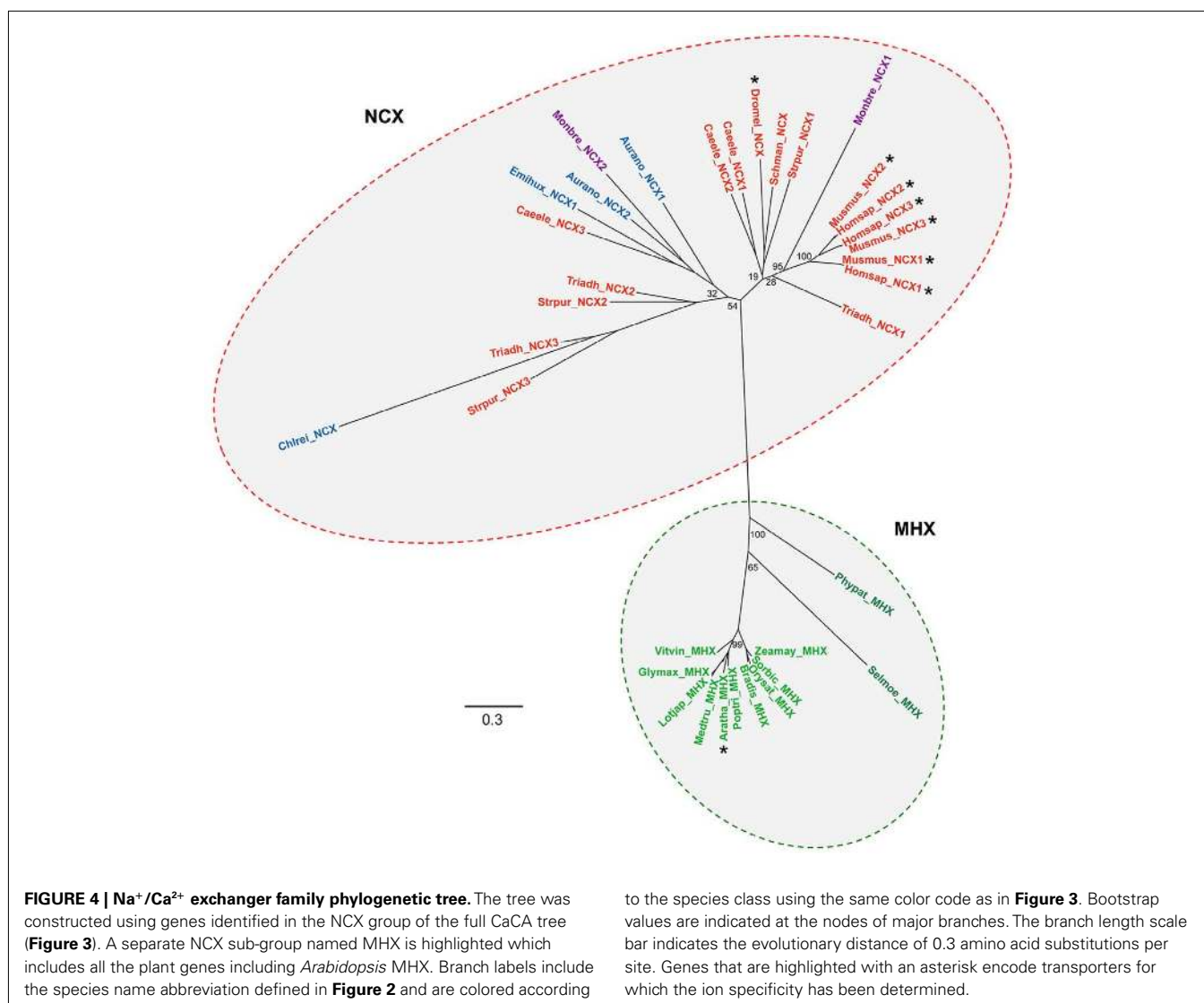
*leprae* are distinct from the other CAX genes and form a separate group which we have named here CHAA (Figure 3). Like the YRBG group, the CHAA group is specifically prokaryotic.

#### NCKX AND “ANIMAL-TYPE” NCX GENES ARE ABSENT IN LAND PLANTS BUT PRESENT IN ALGAE

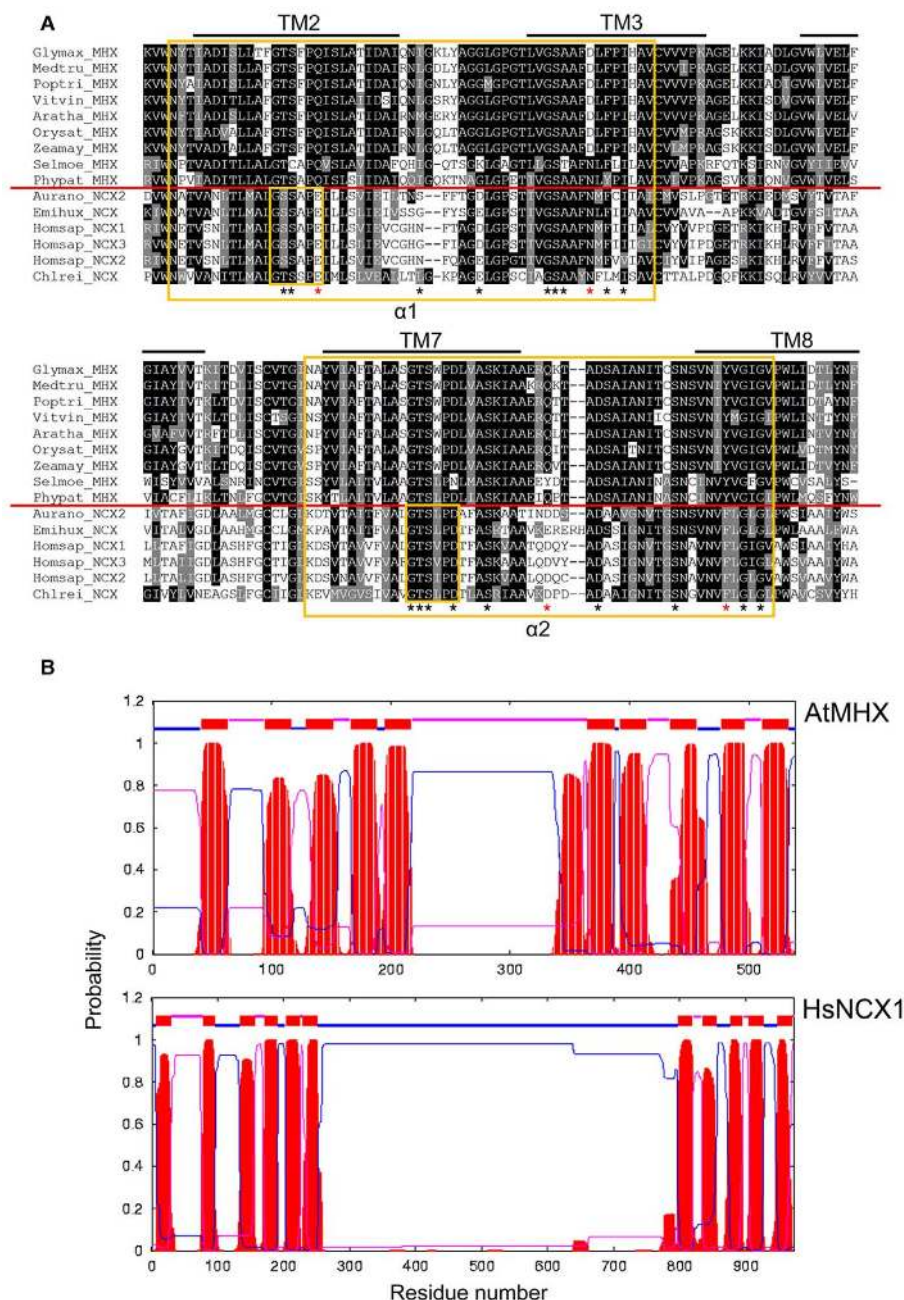
Our analysis of the selected genomes indicated that NCX and NCKX genes appear to be ubiquitous in vertebrate and invertebrate species, as previously determined (Cai and Lytton, 2004a; On et al., 2008). In contrast, no genes from any of the higher plants, bryophyte, or lycophyte species were identified that group within the NCKX family (Figure 3). Likewise, no genes from any of the land plants were found to cluster closely with the NCX genes from animals (Figure 4), although more distantly related NCX-like genes were present (see below). Higher plants probably lack sufficient transmembrane  $\text{Na}^+$  gradients to energize the counter transport of  $\text{Ca}^{2+}$  or other cations and hence have retained  $\text{Ca}^{2+}/\text{H}^+$  exchangers instead of  $\text{Na}^+/\text{Ca}^{2+}$  exchange activity (Hirschi, 2001).

NCX genes were however, detected in algae including the chlorophyte *Chlamydomonas*, the pelagophyte *A. anophagefferens*, and the haptophyte *E. huxleyi* (Figure 4). These algal NCX homologs have high sequence similarity with mammalian NCX proteins including conservation of the key residues in the  $\alpha 1$ - and  $\alpha 2$ -repeat regions (Figure 5) that are critical for  $\text{Na}^+/\text{Ca}^{2+}$  exchange activity (Lytton, 2007). Furthermore, the genes from both of the marine algae, which are more distantly related from the land plants and chlorophyte algae, cluster more closely with animal NCX genes (Figure 4), and we would predict them to have a similar function.

The NCKX tree also contains genes from various algal species (Figure 6). The NCKX tree has three main groups with Group 1 being animal-specific, containing invertebrate, insect, and mammalian genes, including human NCKX3, NCKX4, and NCKX5. Group 2 has animal genes including human NCKX1 and NCKX2. Algal NCKX genes from the two Stramenopile species *A. anophagefferens* and *P. tricornutum*, and the prasinophyte *Micromonas* cluster close to Group 2 but the lower bootstrap values and longer branch lengths of these genes do not allow us to confidently assign







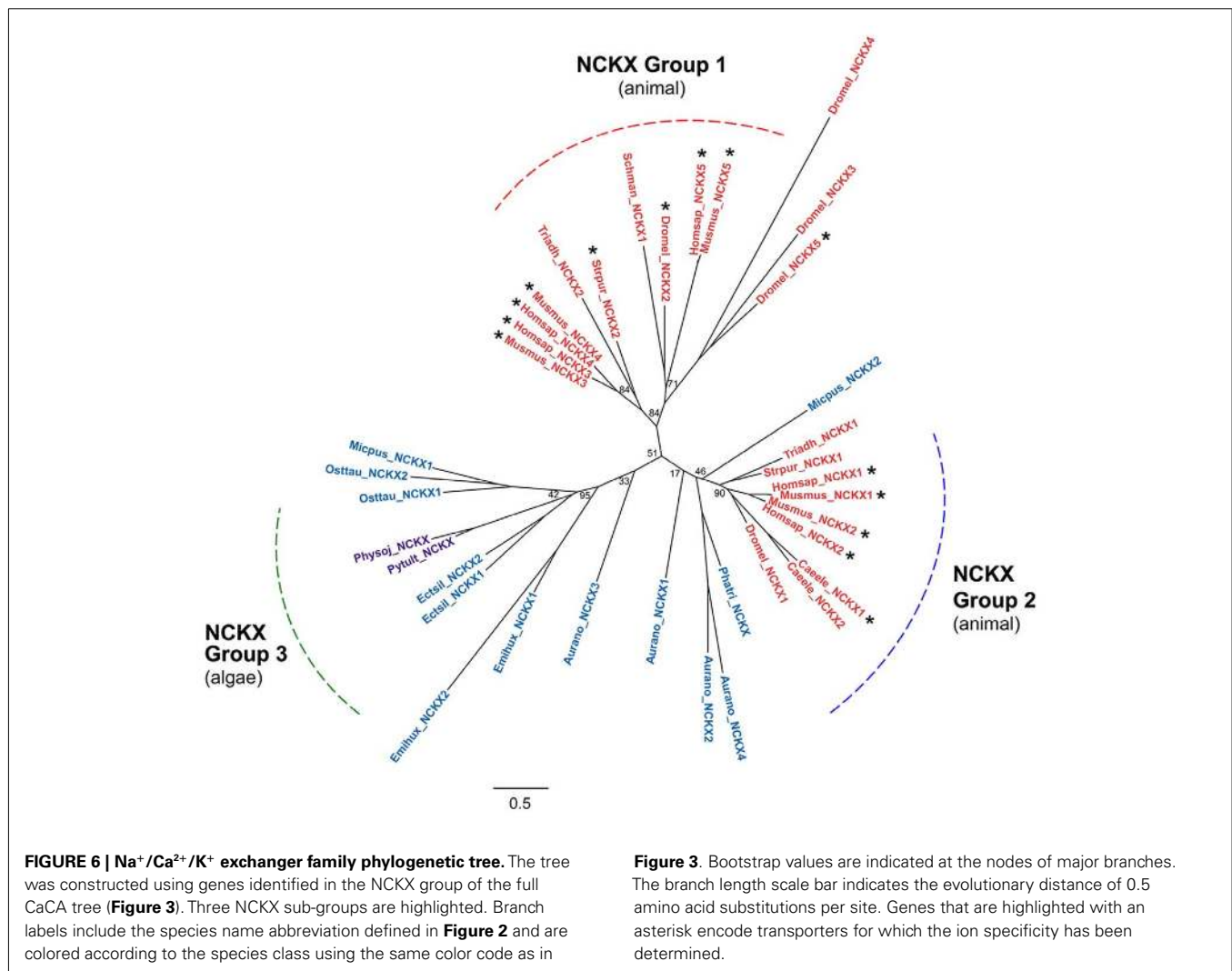
**FIGURE 5 | Sequence and structural variation of NCX and MHX**

**homologs. (A)** Multiple amino acid sequence alignments of the  $\alpha$ 1- and  $\alpha$ 2-repeat region sequence from selected plant MHX, human NCX, and algal NCX proteins. Alignments were performed using ClustalW. Amino acids that are identical or similar are shaded black or gray, respectively. Predicted hydrophobic regions and putative transmembrane spans are

over-lined. The  $\alpha$ -repeat regions and signature residues are boxed in yellow. The red line separates the MHX and NCX sequences. Asterisks indicate residues shown to be important for  $\text{Na}^+/\text{Ca}^{2+}$  exchange activity in NCX1, with those in red being not conserved in the MHX sequences. **(B)** Topology models of AtMHX and HsNCX1 generated by TMHMM. Red areas indicate predicted TM spans.

them to Group 2 rather than Group 3. Group 3 is an algal NCKX group, including another *Micromonas* gene and two genes from the related *O. tauri* picoalgae, two genes from *E. huxleyi*, and two genes from the brown alga *E. siliculosus*, plus genes from the two oomycete species, that are related to the Stramenopile algae (Figure 6). It is notable that all of the algal NCKX genes

are from marine species and from non of the freshwater species, suggesting a potential link between the high  $\text{Na}^+$  and  $\text{K}^+$  concentration in seawater and the presence of a putative  $\text{K}^+$ -regulated  $\text{Na}^+$ -coupled  $\text{Ca}^{2+}$  transporter. Indeed all of the marine algae examined here, with the exception of the red algae *C. merolae*, either possessed an NCKX gene or a gene that was basal to the



NCKX group (Figure 2), suggesting that these transporters may be critical for life in a marine environment. The apparent absence of NCX and NCKX genes from all land plants but their presence in various classes of algae, including the more closely related chlorophytes and prasinophytes, may suggest that they have been lost in all plants. However, it is also possible that these genes were also absent from the common ancestor species in the green eukaryote lineage and that the algae lineage subsequently acquired these genes through horizontal transfer.

Algal NCX and NCKX proteins have not yet been cloned and characterized, further work is needed to determine if these transporters encode Na<sup>+</sup>/Ca<sup>2+</sup> and K<sup>+</sup>-dependent Na<sup>+</sup>/Ca<sup>2+</sup> exchange activities. However, plasma membrane Na<sup>+</sup>/Ca<sup>2+</sup> exchange activity has been detected in some algae, such as the marine chlorophyte *Dunaliella*, and in the freshwater chlorophyte *Chlamydomonas* when assayed in the presence of external Na<sup>+</sup>, with a predicted ratio of 3 Na<sup>+</sup>:1 Ca<sup>2+</sup> (Karimova et al., 2000), equivalent to the stoichiometry of animal NCX. While the presence of a high external Na<sup>+</sup> concentration in marine algae and therefore a steep Na<sup>+</sup> gradient across the plasma membrane might be expected to make the presence of a Na<sup>+</sup>-coupled ion transporter energetically

favorable, it is perhaps somewhat surprising that a freshwater alga like *Chlamydomonas* possesses a putative NCX in addition to CAX genes. However, *Chlamydomonas* is also a soil-living microorganism and may encounter saline conditions in some environments. NCX and NCKX genes did not appear to be ubiquitous in all classes of algae, particularly other freshwater algae. They could not be identified in the freshwater chlorophytes *V. carteri* and *Coccomyxa* sp., or in the genome of the red alga *C. merolae*, although future analysis of these genomes will be needed to confirm that these gene losses are not due to poor genome sequence quality.

#### ADAPTIVE EVOLUTION OF MHX GENES IN LAND PLANTS

While no obvious NCX genes were detected across the plant genes analyzed, distantly related NCX-like genes were detected in all plant species, and formed a tight monophyletic group which includes the *A. thaliana* AtMHX gene, hence we named this group MHX (Figure 4). AtMHX was previously found to be closely related to mammalian NCXs but was shown to function as a H<sup>+</sup>-coupled Mg<sup>2+</sup> and Zn<sup>2+</sup> exchanger and not a NCX (Shaul et al., 1999). A single AtMHX homolog was identified from the

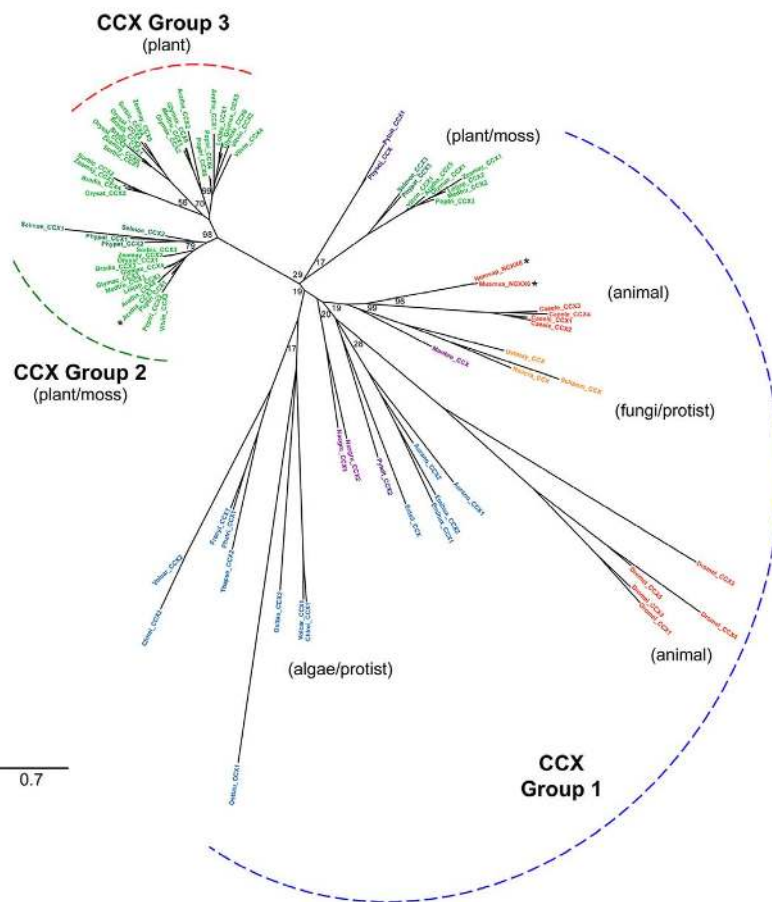
closely related zinc-hyperaccumulator species *Arabidopsis halleri* (Elbaz et al., 2006) but the extent of MHX genes within plants and other species has not been previously examined. The putative *Paramecium*  $Mg^{2+}$  exchanger *XNTA* appears to be a CaCA homolog but has significant divergence from the NCX gene family and therefore is not regarded as a MHX homolog (Haynes et al., 2002). An NCX-like gene was identified in each of the flowering plant genomes examined. Each of these gene sequences had high identity to *AtMHX* and formed a clear MHX sub-group within the NCX family (Figure 4). None of the plants examined had more than one MHX gene in contrast to the variation in numbers of CAX and CCX genes between plant species. Single MHX genes were also present in the lower land plants *Physcomitrella* and *Selaginella* but MHX genes do not appear to be present in algae as none of the NCX or NCX-related genes from any of the algal species grouped with the plant MHX genes (Figures 3 and 4). Furthermore, MHX-like genes were not present in any non-plant species. The birth of MHX genes appears to be an example of adaptive evolution which has taken place most probably in the common ancestor of land plants.

There is 20–22% amino acid sequence identity (31–33% similarity) between the plant MHX sequences and human NCX1, and both sets of proteins share a number of conserved residues particularly within the hydrophobic regions and in the  $\alpha 1$ - and  $\alpha 2$ -repeat regions, yet there are clear distinctions (Figure 5A). Many of the residues that have been shown to be critical for  $Na^+/Ca^{2+}$  exchange activity in NCX1 (Nicoll et al., 1996; Iwamoto et al., 2000; Philipson and Nicoll, 2000; Ottolia et al., 2005) are conserved in the MHX sequences but there are some specific differences. Key residues Glu-113 and Asp-130 in the  $\alpha 1$ -repeat region of NCX1, and Asp-825 and Phe-844 in the NCX1  $\alpha 2$ -repeat region, are substituted in the equivalent positions in each of the plant MHX homologs (Figure 5A). For example, Glu-113 is replaced by a Gln residue in each MHX protein. Such a G113Q substitution was shown to abolish  $Na^+$ -dependent  $Ca^{2+}$  transport activity of NCX1 (Nicoll et al., 1996). Mutations of Asp-130 and Asp-825 of NCX1 were found to substantially reduce the affinity of the exchanger for  $Ca^{2+}$  (Iwamoto et al., 2000). Asn-143 of NCX1 is also a critical residue and is substituted by Asp in the higher plant MHX proteins but not the lower plant MHXs (Figure 5A). Interestingly, the N143D mutation of NCX1 showed wild type activity (Ottolia et al., 2005). These amino acid changes in the  $\alpha 1$ -repeat region of *AtMHX* compared to NCX1 therefore appear to explain the lack of  $Na^+/Ca^{2+}$  exchange activity by *AtMHX* and probably by the other plant MHX transporters. The MHX genes also encode proteins with a much shorter central loop than the NCX genes (Figure 5B; Table S3 in Supplementary Material) and lack the  $Ca^{2+}$  binding domain sequence motifs present on the NCX proteins in this region (Lytton, 2007), further explaining the loss of  $Ca^{2+}$  activity and regulation by the *AtMHX* transporter. The residues in the  $\alpha 1$ -repeat region are thought to be involved in ion selectivity (Shigekawa et al., 2002; Ottolia et al., 2005) and therefore we might presume that the residues within the MHX  $\alpha 1$ -repeat region encode the  $Mg^{2+}$  and  $Zn^{2+}/H^+$  exchange activity observed for *AtMHX*. Whether each of the plant MHX transporters is involved in  $Mg^{2+}$  transport or in the transport of other cations remains to be seen.

## THE DIVERSIFICATION OF CCX GENES IN LAND PLANTS

To date only two CCX members have been functionally characterized in depth, mammalian NCKX6/NCLX, which has been shown to have  $Na^+(Li^+)/Ca^{2+}$  exchange activity (Cai and Lytton, 2004b; Palty et al., 2004) and CCX3 from *Arabidopsis* which appears to function as a  $H^+/K^+$  exchanger and which can also transport  $Na^+$  and  $Mn^{2+}$  but not  $Ca^{2+}$  (Morris et al., 2008). Thus, there is clearly variation in function within the CCX transporter family as indicated by the CCX tree (Figure 7). Apart from the duplication of insect and nematode CCX genes (four to five genes), single genes are present in mammal and fungi species, and one or two CCX genes were present in each algal species examined, except in *Coccomyxa* sp. and *C. merolae* where CCX genes could not be identified. In contrast, there has been a significant diversification in CCX genes within land plants, with the bryophyte and lycophyte species each possessing three genes, and individual flowering plant species possessing three to eight CCX genes (Figure 2), suggesting that these are important genes within plants. There is significant variation between these plant CCX genes which are found within each of the three main groups of this tree (Figure 7). There are two plant-specific groups; Group 3 contains solely angiosperm genes and includes *Arabidopsis* CCX1 and CCX2, while Group 2, which includes *Arabidopsis* CCX3 and CCX4, also contains CCX genes from *Physcomitrella* and *Selaginella*. Each of the 10 flowering plant species analyzed has at least one member in each of these two groups. There is evidence of recent gene duplication within both groups, particularly within Group 3 where there are mostly two genes for each dicot species and three genes for each monocot species, while within Group 2 three of the dicot species (*Arabidopsis*, soybean, and poplar), plus *Physcomitrella* and *Selaginella*, have two CCX genes. Group 1 contains all the algal and non-plant genes, including NCKX6/NCLX, single CCX genes from *Physcomitrella* and *Selaginella*, and single CCX genes each from seven of the 10 higher plant species, including CCX5 from *Arabidopsis*, but CCX5-like genes from the remaining three monocot species (*Brachypodium*, rice, and sorghum) appear to have been lost (Figure 7). There is considerable variation among the algal CCX genes which are widely dispersed throughout Group 1, as indicated by their long tree branch lengths, but as yet nothing is known about these genes or their function to explain this variation.

Further biochemical analysis of the plant CCX proteins is required before we can discern whether the CCX members from each group differ in substrate specificity. However, it is tempting to speculate that members of Group 2 may share *AtCCX3*  $H^+/K^+$  exchange activity and that the plant members of Group 1 like *AtCCX5* may be more likely to have  $Ca^{2+}$  transport activity as they are more closely grouped to NCKX6/NCLX. A closer look at the amino acid sequence of selected plant, algal, and human CCX sequences from each of the three groups shows that they all have strong sequence conservation within the  $\alpha 1$ - and  $\alpha 2$ -repeat regions, including 100% identity in the  $\alpha 1$  signature motif GNGAPD and the  $\alpha 2$  signature motif G(N/D)SxGD (Figure 8). Yet there are still some obvious sequence differences in both  $\alpha$ -repeat regions between the Group 1 and the Group 2/3 CCX proteins, in particular between the  $Ca^{2+}$  transporting NCKX6/NCLX and the non- $Ca^{2+}$  transporting *AtCCX3*, that might determine cation specificity.



**FIGURE 7 | Cation/ $\text{Ca}^{2+}$  exchanger family phylogenetic tree.** The tree was constructed using genes identified in the CCX group of the full CaCA tree (Figure 3). Three CCX sub-groups are highlighted. Branch labels include the species name abbreviation defined in Figure 2 and are colored according to the species class using the same color code as in

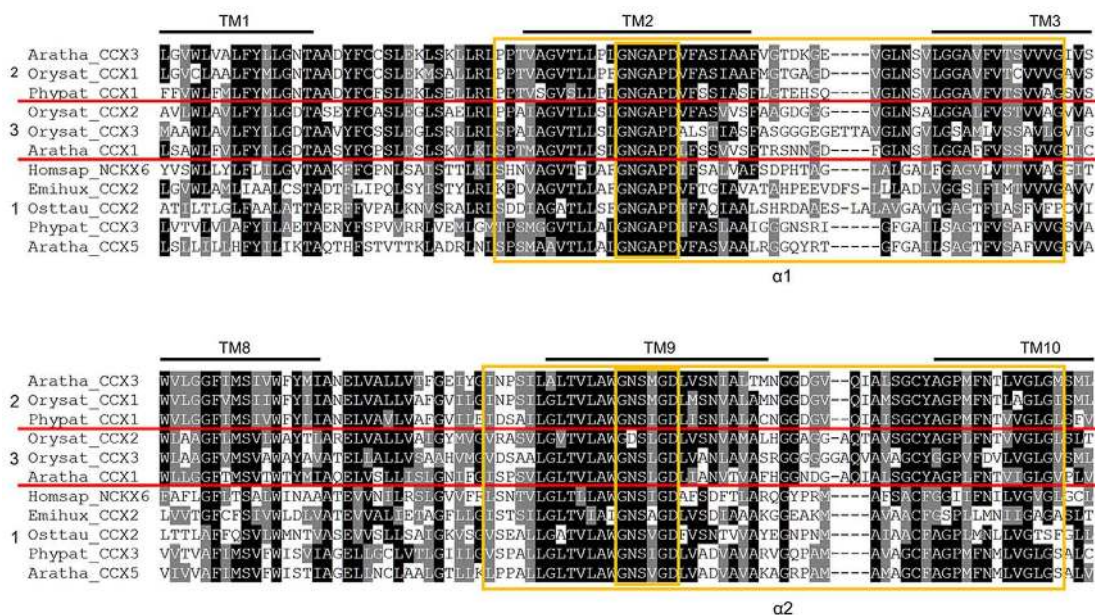
**Figure 3.** Bootstrap values are indicated at the nodes of major branches. The branch length scale bar indicates the evolutionary distance of 0.7 amino acid substitutions per site. Genes that are highlighted with an asterisk encode transporters for which the ion specificity has been determined.

## EXPANSION OF THE CAX GENE FAMILY THROUGHOUT THE PLANT KINGDOM

Previous analysis has examined the diversification and evolution of CAX genes and identified distinct phylogenetic groups that encompass genes from bacterial, fungal, plant, and invertebrate species (Shigaki et al., 2006). Here we have screened through a wider range of flowering plant genomes and a wide selection of algal species, from which CAX genes have not been examined in detail. Shigaki et al. (2006) identified a number of distinct Type 1 phylogenetic groups, many of which were comprised of fungal (Type 1D, 1E, and 1F) and bacterial/cyanobacterial (Type 1G and 1H) CAX genes. As this study has focused predominantly on plant and algae species, only a small number of selected fungal and bacterial species were examined and therefore the CAX tree generated here lacks distinction between the fungal and bacterial Type 1 groups (Figure 9A). The *Leishmania* parasitic protist gene stands out with an unusually long branch that may be reflective of the effect of the parasitic lifestyle on molecular evolution. The Type 2 group which contains distinct CAX genes including yeast *VNX1* and invertebrate CAX genes was also clearly observed in this

tree, but this group contains no plant or algal genes. A number of CAX genes were identified from various algal species, although three of the species, *O. tauri*, *M. pusilla*, and *A. anophagefferens* appeared to lack CAX homologs (Figure 2). A previously cloned CAX gene from *Chlamydomonas* was shown to cluster within the Type 1C group which includes CAX genes from parasitic protists such as *Plasmodium falciparum* (Pittman et al., 2009). Genes from other freshwater chlorophyte algae including a second gene from *Chlamydomonas*, likewise fall within this group, plus genes from some of the marine algae and the oomycetes. However, five CAX genes from the haptophyte alga *E. huxleyi*, two stramenopile algae genes, and two chlorophyte algae genes from *Chlamydomonas* and *Volvox*, cluster more closely to the land plant CAX genes and might be considered as Type 1B-like genes. It might therefore be expected that these algal CAX homologs share some functional characteristics of the plant Type 1B CAX transporters. Despite the sequence divergence between *Chlamydomonas* CAX1 in the Type 1C group and *Arabidopsis* CAX1 in the Type 1A group, both of these transporters have very similar function; when expressed in a yeast heterologous expression





**FIGURE 8 | Sequence variation of CCX homologs.** Multiple amino acid sequence alignments of the  $\alpha$ 1- and  $\alpha$ 2-repeat region sequence from selected plant, moss, algal, and human CCX proteins. Alignments were performed using ClustalW. Amino acids that are identical or

similar are shaded black or gray, respectively. Predicted transmembrane spans are over-lined. The  $\alpha$ -repeat regions and signature residues are boxed in yellow. The red lines separate the Group 1, 2, and 3 CCX sequences.

system both function as vacuolar-localized  $H^+/Ca^{2+}$  exchangers with equivalent regulatory characteristics (Pittman et al., 2009). The main functional difference appears to be that CrCAX1 can also transport  $Na^+$ . To date all of the biochemically characterized CAX transporters show  $Ca^{2+}$  transport activity, including those in the Type 2 group (Manohar et al., 2010), therefore it is not clear why there is so much sequence diversity within the CAX family compared to the NCX or NCKX families which yield much tighter families (Figure 3).

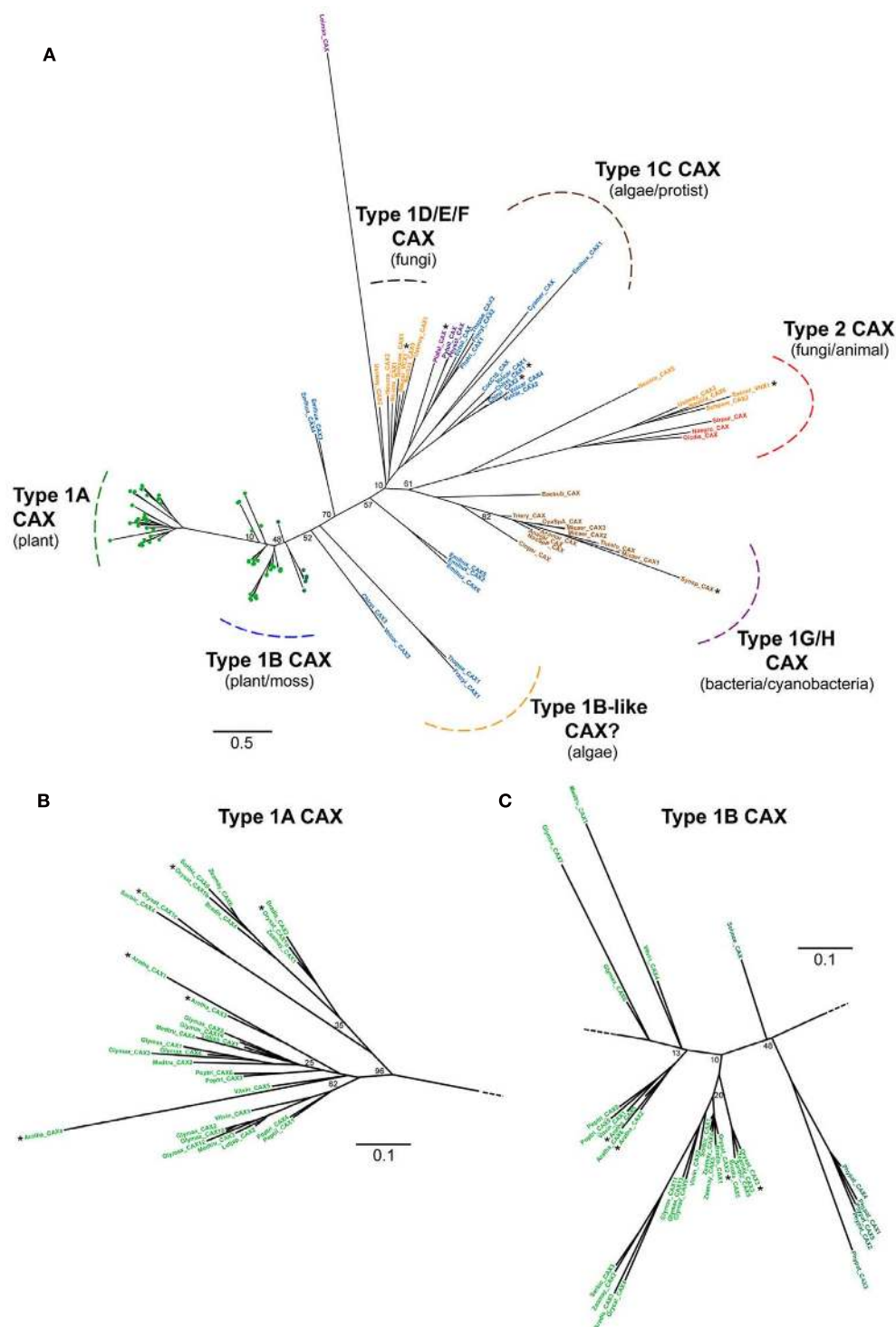
The previously analyzed flowering plant CAX genes were found to cluster into either the Type 1A or Type 1B groups, and this was consistently seen here with all the flowering plant genomes examined. Within the Type 1A group, clear distinction between the genes from monocot and dicot plants was observed (Figure 9B) but this was not as obvious within the Type 1B group which showed greater overall divergence and also included the CAX genes from *Physcomitrella* and *Selaginella* (Figure 9C). This analysis indicates that there has been a diversification of CAX genes within land plants. Within the Chlorophyta, the species possess one to three CAX genes; in contrast, in the land plants each species has on average five to six CAX genes (Figure 2). This expansion may have occurred prior to the evolution of the flowering plants as the bryophyte *Physcomitrella* has five CAX genes. The most parsimonious explanation is that there was likely to have been at least one duplication event in the flowering plant ancestor. However, the evolution of CAX genes in plants seems to have been a dynamic process with many apparent instances of gains and losses, for example, the lycophyte *Selaginella* appears to have just a single CAX gene. With two exceptions, all the monocots and dicots analyzed here have between four and six CAX genes, generally equally divided between the Type 1A and Type 1B groups. 14

CAX genes were identified from soybean, also spread between both groups, but we could only identify two CAX genes from *L. japonica*, both within the Type 1A group. The genes in most of these species await future studies to see whether any novel functional traits will be uncovered or whether the range of characteristics determined for the *Arabidopsis* CAX proteins is conserved for the other flowering plants.

#### IDENTIFICATION OF GENES ENCODING CAX-LIKE PROTEINS CONTAINING EF-HAND DOMAINS IN SOME PLANTS AND ALGAE

In addition to the CaCA genes described, the BLAST search identified a number of genes which were clearly distinct from the others. Further analysis indicated that they were most closely related to the plant CAX genes. Amino acid sequence alignment showed that these gene products have higher similarity with selected plant CAX sequences in the C-terminal half of the proteins but the sequences aligned poorly against the N-terminal half of the CAX proteins (Figure 10) despite having an overall CAX-like topology including 6 + 5 TM domains separated by a central loop, although this loop is longer than in most CAX proteins (Figure 11A). Pfam analysis (Finn et al., 2010) was performed on these proteins and confirmed that they contain a “Na\_Ca\_ex” domain (Pfam ID: PF01699) in the C-terminal half of the protein. This domain denotes the core consensus 5 TM hydrophobic region present in NCXs and in all other CaCA proteins<sup>5</sup> including CAX proteins (Figure 11A). A closer look at the sequence of these proteins found that there was a high number of similar residues shared with the  $\alpha$ 2-repeat region of the CAX sequences including the presence of residues conserved throughout CaCA proteins (Cai and

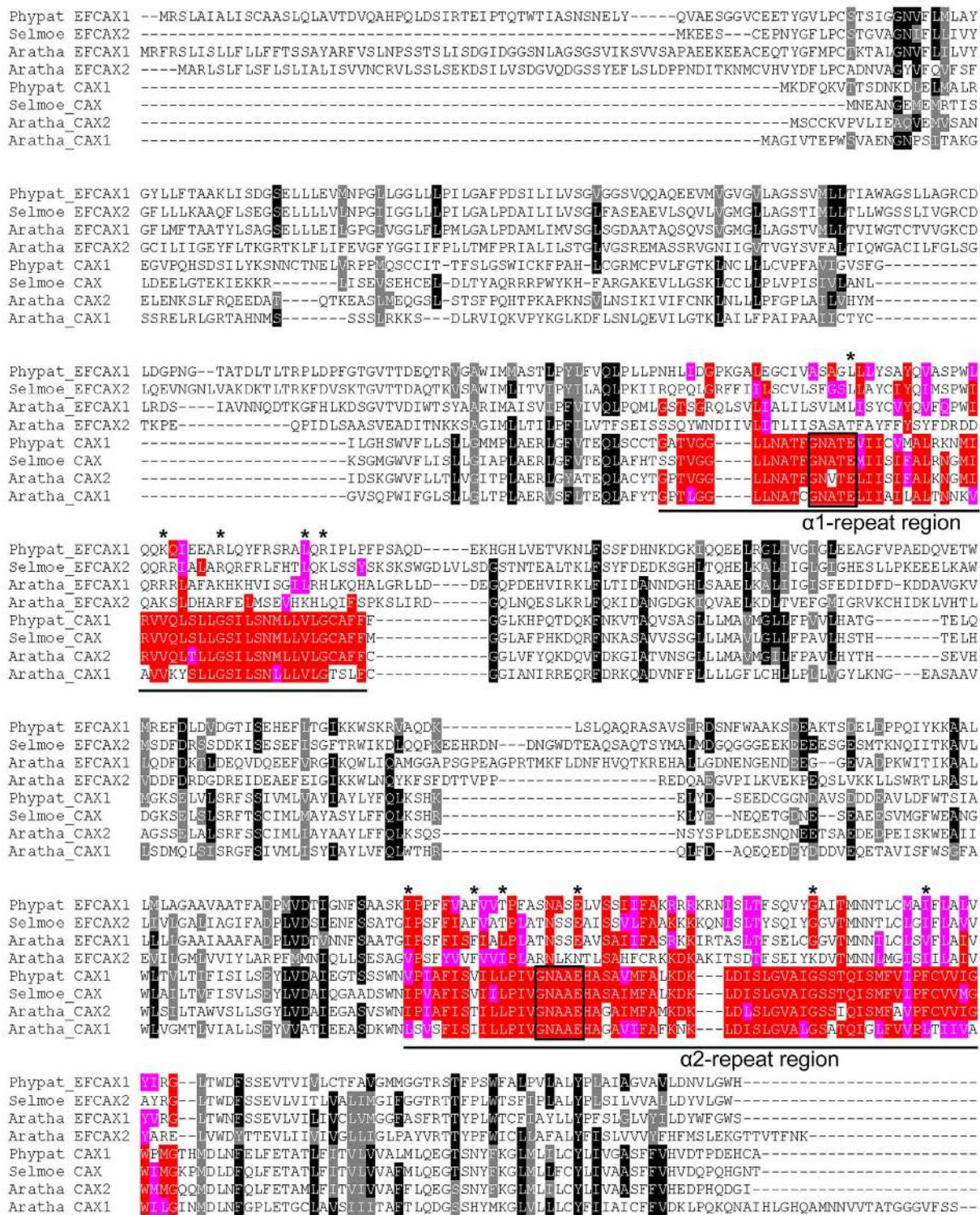
<sup>5</sup>[http://pfam.sanger.ac.uk/family/Na\\_Ca\\_ex](http://pfam.sanger.ac.uk/family/Na_Ca_ex)



**FIGURE 9 | CAX family phylogenetic tree. (A)** The tree was constructed using genes identified in the CAX group of the full CaCA tree (Figure 3). The Type 1A-H and Type 2 CAX sub-groups are highlighted and determined using the nomenclature described previously (Shigaki et al., 2006). Branch labels include the species name abbreviation defined in Figure 2 and are colored according to the species class using the same color code as in Figure 3. The individual gene labels for the

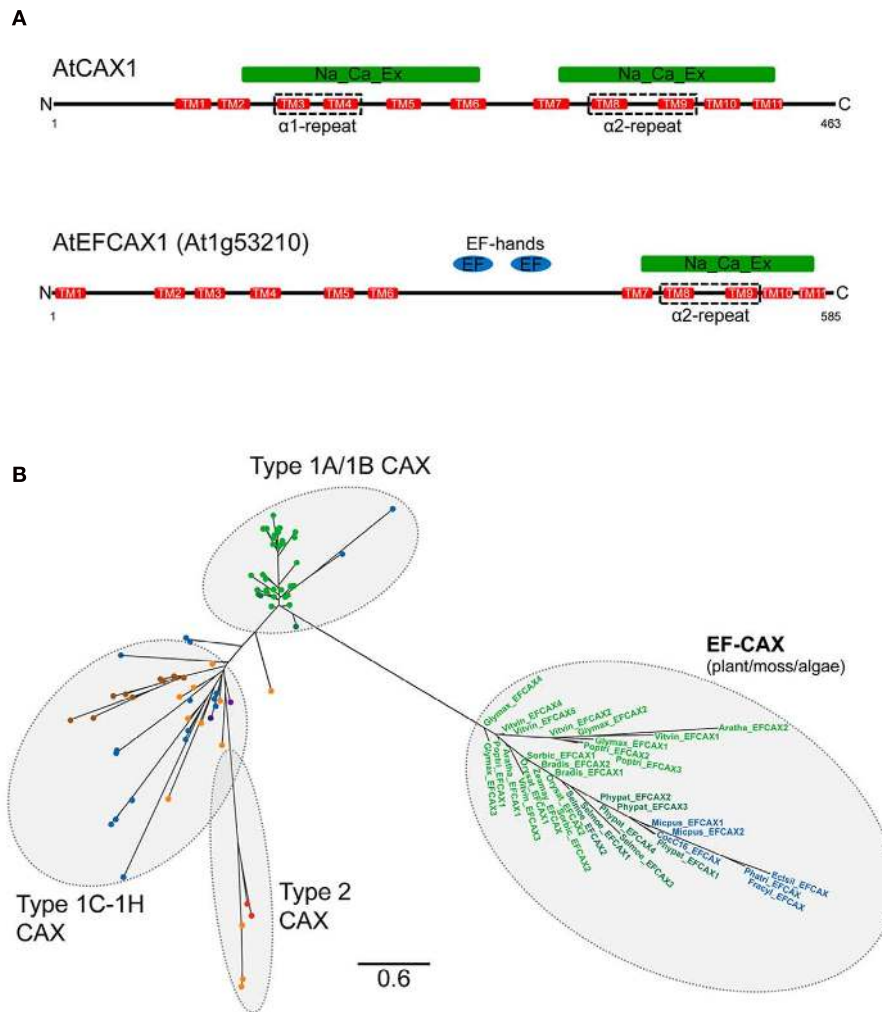
Type 1A and 1B groups are replaced by circles and the gene name labels are given in the enlarged tree sections in (B,C). Bootstrap values are indicated at the nodes of major branches. The branch length scale bar indicates the evolutionary distance of 0.5 amino acid substitutions per site [0.1 amino acid substitutions per site in (B,C)]. Genes that are highlighted with an asterisk encode transporters for which the ion specificity has been determined.





**FIGURE 10 | Multiple sequence alignment of CAX and EF-CAX sequences.** Full-length amino acid sequences of selected *Arabidopsis*, *Physcomitrella*, and *Selaginella* CAX and EF-CAX proteins were aligned. The alignment was performed using ClustalW. Amino acids that are identical or

similar are shaded black/red or gray/pink, respectively. The α-repeat regions and signature residues are highlighted. Amino acids that are conserved in >90% of CaCA proteins (Cai and Lytton, 2004a) are highlighted (asterisk) within the α1- and α2-repeat regions.



**FIGURE 11 | Structural and phylogenetic comparisons of CAX and EF-CAX proteins. (A)** Schematic representation of the topology structure of a typical CAX protein (*Arabidopsis* CAX1) and a representative EF-CAX protein (*Arabidopsis* EFCAX1). The black line denotes the protein from N-terminus to C-terminus, red bars denote the TM domains as predicted by TMHMM, and the numbers indicate the first and last amino acid residues. The locations of the  $\alpha$ 1- and  $\alpha$ 2-repeat regions are highlighted. The identified Pfam domains are indicated above the protein schematic. **(B)** CAX gene family phylogenetic tree including the EF-CAX genes. The tree was

constructed using the maximum likelihood method and derived from alignments of  $\alpha$ 2-repeat region sequences extracted from genes identified in the CAX group of the full CaCA tree (**Figure 3**) and the EF-CAX genes as determined by Pfam analysis. The CAX sub-groups are highlighted in addition to the EF-CAX sub-group. Branch labels include the species name abbreviation defined in **Figure 2** or are replaced by a circle and are colored according to the species class using the same color code as in **Figure 3**. The branch length scale bar indicates the evolutionary distance of 0.6 amino acid substitutions per site.

Lytton, 2004a) but poor sequence similarity with the  $\alpha$ 2-repeat region (**Figure 10**). In addition the CAX-like proteins possess one or more EF-hand-type  $\text{Ca}^{2+}$ -binding domains (Pfam ID: PF00036) within the long central loop (**Figure 11A**). One of these genes from *Arabidopsis* (At1g53210) has been identified from a genome search (Shigaki et al., 2006), but as yet they have not been cloned or characterized. From all of the genomes analyzed here, 34 of these CAX/EF-hand (EF-CAX) genes were identified, predominantly from land plants (*Arabidopsis*, poplar, grape, soybean, *Brachypodium*, maize, rice, and sorghum) but also from *Physcomitrella*, *Selaginella*, and from some diverse algae species: the chlorophyte *Coccomyxa* sp., the prasinophyte *M. pusilla*, and

three of the stramenopile algae (*E. siliculosus*, *F. cylindrus*, and *P. tricornutum*). Interestingly, *Selaginella* has more EF-CAX genes (three genes) than CAX genes (one gene), while *M. pusilla*, which has two EF-CAX genes, has no identifiable CAX genes. These genes were not identified from any of the bacterial, fungal, or animal genomes, but the presence of these genes in these select algae species suggests that they either evolved once in an ancestral organism from an ancestral CAX gene, or on at least two occasions, such as within the Stramenopiles and within the Archaeplastida. Using the  $\alpha$ 2-repeat region sequence alone to generate a phylogenetic tree, we confirmed that the EF-CAXs are more closely related to the CAX family members (**Figure 11B**) than to any of the other



CaCA families (data not shown). There are some long branch lengths within the EF-CAX clade indicating a degree of divergence between these genes, although the divergence between plant and algae EF-CAX genes appears to be less than between plant and algae CAX genes. Furthermore, the analysis demonstrated that the EF-CAX genes are more distant from the plant Type 1A and Type 1B CAX genes than the plant Type 1A/1B CAX genes are from the non-plant CAX genes (**Figure 11B**).

The functions of the EF-CAX proteins are unknown and future work will be needed to determine if these proteins function as a transporter or merely as a  $\text{Ca}^{2+}$  binding protein. It is unclear how effective a CaCA transporter would be with a single  $\alpha$ -repeat region. Experiments with animal NCX proteins indicate that both  $\alpha$ -repeat regions are required for transport function and it has been suggested that together these regions interact within the tertiary structure to form an ion conductance pore (Nicoll et al., 2007). For the *Arabidopsis* CAX proteins, it has been shown that separated N- or C-terminal halves of CAX1 or a mixture of N-terminal CAX1 with N-terminal CAX3 lack transport activity (Zhao et al., 2009). In contrast, a mutated NCKX6/NCLX protein containing either a single  $\alpha 1$ - or  $\alpha 2$ -repeat region appears to function; however, oligomerization between two copies of these  $\alpha 1$ - or  $\alpha 2$ -repeat proteins thus yielding a protein with two  $\alpha$ -repeat regions is required (Palty et al., 2006). If the EF-CAX proteins were to function as a transporter, these proteins may need to form oligomers to allow transport activity to occur. Alternatively, one possibility might be that the N-terminal half of the EF-CAX protein contains a region which can function as an  $\alpha$ -repeat region but that the sequence has changed significantly from the consensus  $\alpha$ -repeat sequence and is no longer recognizable. Clearly future functional analysis is needed to understand these novel proteins.

## CONCLUSION

$\text{Ca}^{2+}$  homeostasis is critical for all organisms and therefore a suite of  $\text{Ca}^{2+}$  regulators including transporters must be present. The CaCA superfamily represents an ancient class of genes which have members present in most species. A major function of these transporters is in  $\text{Ca}^{2+}$  transport as is evident from the functional analysis of CaCA gene products from different classes of organisms and from each of the different CaCA families: for example, *E. coli* yrbG, *Drosophila* NCX, *C. elegans* NCKX1, human NCKX6, and rice CAX1a all appear to mediate  $\text{Ca}^{2+}$  transport activity (Schwarz

and Benzer, 1997; Szerencsei et al., 2000; Cai and Lytton, 2004b; Kamiya and Maeshima, 2004; Naseem et al., 2008), yet the analysis here shows that they have minimal sequence conservation. It is therefore difficult to predict the function and ion specificity of these many putative transporters, yet some of them are certainly likely to function in the transport of ions other than  $\text{Ca}^{2+}$ . This divergence in function is particularly apparent in higher plants, such that the *Arabidopsis* NCX and CCX proteins AtMHX and AtCCX3 possess  $\text{Mg}^{2+}$  transport and  $\text{K}^{+}$  transport activity, respectively, rather than  $\text{Ca}^{2+}$  transport (Shaul et al., 1999; Morris et al., 2008). Overall, the CaCA complement and function in plants is different than in most animals. The plants possess a wide diversity of CAX genes, some or all of which encode proteins that will have  $\text{Ca}^{2+}$  transport and probably other cation transport function, a diversity of CCX genes with mostly unknown function, and a single MHX genes. This diversification appears to mirror the evolution of plants on land, as shown by the analysis of *Physcomitrella* and *Selaginella* in this study, which also multiple CAX and CCX genes plus single MHX genes. In contrast, many of the algal species have few or no CAX and CCX genes, which are divergent from those of the land plants, and animal-like NCX and NCKX genes. Recent analysis of  $\text{Ca}^{2+}$  channels in algal species has likewise found that many of the animal  $\text{Ca}^{2+}$  channel genes that are lost in land plants are present in algae (Wheeler and Brownlee, 2008; Verret et al., 2010), indicating that many algal species possess animal-like systems for  $\text{Ca}^{2+}$  signaling and suggesting that there are significant differences in  $\text{Ca}^{2+}$  signaling and homeostasis mechanisms between algae and land plants. The motile lifestyle of most algae species compared to the sessile land plants plus the significant differences in environmental conditions between the sets of organisms may in part explain the presence of very different  $\text{Ca}^{2+}$  regulatory mechanisms. Algae may therefore represent an excellent model for studying the evolution of  $\text{Ca}^{2+}$  signaling and aspects of animal-like signaling.

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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.2012.00001/abstract](http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2012.00001/abstract)

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