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SHORT COMMUNICATION

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Hypothesis paper: the development of a regulatory layer in P2B autoinhibited Ca^{2+}-ATPases may have facilitated plant terrestrialization and animal multicellularization

Anett Stéger and Michael Palmgren

Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg, Denmark

ABSTRACT

With the appearance of plants and animals, new challenges emerged. These multicellular eukaryotes had to solve for example the difficulties of multifaceted communication between cells and adaptation to new habitats. In this paper, we are looking for one piece of the puzzle that made the development of complex multicellular eukaryotes possible with a focus on regulation of P2B autoinhibited Ca^{2+}-ATPases. P2B ATPases pump Ca^{2+} out of the cytosol at the expense of ATP hydrolysis, and thereby maintain a steep gradient between the extra- and intracytosolic compartments which is utilized for Ca^{2+}-mediated rapid cell signaling. The activity of these enzymes is regulated by a calmodulin (CaM)-responsive autoinhibitory region, which can be located in either termini of the protein, at the C-terminus in animals and at the N-terminus in plants. When the cytoplasmic Ca^{2+} level reaches a threshold, the CaM/Ca^{2+} complex binds to a calmodulin-binding domain (CaMBD) in the autoinhibitor, which leads to the upregulation of pump activity. In animals, protein activity is also controlled by acidic phospholipids that bind to a cytosolic portion of the pump. Here, we analyze the appearance of CaMBDs and the phospholipid-activating sequence and show that their evolution in animals and plants was independent. Furthermore, we hypothesize that different causes may have initiated the appearance of these regulatory layers: in animals, it is linked to the appearance of multicellularity, while in plants it co-occurs with their water-to-land transition.

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Introduction

Autoinhibited Ca^{2+}-ATPases (ACAs) belong to the P2B subfamily of P-type ATPases[1]. In animals, they are called plasma membrane Ca^{2+}-ATPases (PMCAs), which however does not define the subfamily, as in plants, members also locate to the vacuolar membrane[2,3,4].

By exporting Ca^{2+} from the cytosol at the expense of ATP, P2B Ca^{2+}-ATPases are responsible for keeping the cytosolic Ca^{2+} concentration in the sub-micromolar range while its external level is usually in the millimolar range[5]. Keeping the intracellular Ca^{2+} concentration low is essential to prevent cell damage as even µM concentrations would trigger toxic effects[6,7]. The resulting steep Ca^{2+} concentration difference between the extra- and intracellular compartments is utilized for Ca^{2+}-mediated rapid cell signaling, a phenomenon common for various organisms from prokaryotes to complex eukaryotic organisms. Calcium signaling mechanisms across kingdoms were recently reviewed by Luan and Wang[8].

Different internal and external signals lead to a rapid increase in cytoplasmic Ca^{2+} concentration. The maximal magnitude of the appearing Ca^{2+} spike is different from external signal to signal; thus, the cells know how to differentiate and respond to them[5]. Such a regulation enables greater communication and coordination between cells. Ca^{2+} based signaling is basically involved in all important cell functions and became universally important with the appearance of multicellular organisms about 600 million years ago[9].

At very low Ca^{2+} concentrations, an autoinhibited P2B Ca^{2+}-ATPase is practically inactive because a terminal tail acts as an inhibitor by folding over its catalytic domain[10,11]. When the cytoplasmic Ca^{2+} concentration increases, Ca^{2+} forms a complex with CaM which causes conformational changes and results in the exposure of CaM hydrophobic sites[12]. Thereby the CaM/Ca^{2+} complex can interact with the calmodulin-binding domain (CaMBD) which is located at the C- or N-termini of P2B ATPases. The CaMBD overlaps with the autoinhibitory sequence, and upon binding of CaM/Ca^{2+} the autoinhibitory effect of the tail is neutralized[13,14].

Plasma membrane (PM) Ca^{2+}-ATPases were shown to be similarly activated by acidic phospholipids[15]. In PMCAs, one of their binding sites interferes with the terminal CaMBD, while the other is located in the loop connecting transmembrane domain 2 and 3 (the A domain) close to an alternative splicing site[16–18].

The aim of this study was to identify the evolutionary history of P2B ATPase regulation with a broader phylogenetic analysis and to hypothesize what initiated it. Our previous paper showed that the appearance of the autoinhibitory regulatory (R) domain of plasma membrane H^+-ATPases co-occurred with the water-to-land transition of plants more than 450 million years ago[19]. Such a development might have

CONTACT Michael Palmgren palmgren@plen.ku.dk Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, Frederiksberg DK-1871, Denmark

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been necessary for plants to adapt to harsh and changing terrestrial conditions. Did the same happen in case of the regulatory domain of plant P2B ATPases? Or is its evolution connected to the shift to multicellularity? And when did it develop in animals? Such questions are to be discussed in the paper.

Materials and methods

Identification of P2B sequences

The search for P-type P2B ATPase sequences was based on the strategy from Stéger et al.[19]. In brief, P-type P2B ATPase protein sequences were identified in the following databases using the Basic Local Alignment Search Tool (BLAST) program: the NCBI protein database (http://blast.ncbi.nlm.nih.gov/), the KEGG: Kyoto Encyclopedia of genes and genomes (https://www.genome.jp/tools/blast/), the PhycoCosm Algal Genomics Resource (https://phycocosm.jgi.doe.gov/phycocosm/home), the MycoCosm Fungal Genomic Resource (https://mycocosm.jgi.doe.gov/mycocosm/home) and the Figshare repository (https://figshare.com). For each species, BLAST searches were carried out using the *A. thaliana* ACA8 (P-type P2B pump, NP_001331281) protein sequence. All hits in these species with significant similarity to the query (expected value < e – 90) were analyzed by exploring their relationship to the P2B-type ATPase subfamily. It was carried out by constructing phylogenetic trees for all candidate amino acid sequences in each individual genome together with a set of known P-type ATPases (P2B ATPases: PorgiPMCA, OxaviPMCA, ArathACA8, HomsaAT2B1; not P2B ATPases: SaccePmc1, HomsaSPCA1, SaccePmr1, SacceENA1, SaccePma1, ArathAHA2, HomsaSERCA2, HomsaATP1A1; for full names and accession numbers see Table S1). If sequences in the phylogenetic tree were affiliated to the P2B clade, they were considered as potential P2B ATPases. The individual sequences were subsequently confirmed by manual inspection for conserved sequence motifs characteristic of P-type ATPases in general (TGES, DKTGT, GDG, KGA) and P2B ATPases (PEGLP). As a result, 153 P2B ATPases from a total of 62 species were collected from major eukaryotic phyla and prokaryotes.

Phylogenetic analysis of P2B ATPases

The phylogenetic analysis of P2B ATPase sequences was based on Stéger et al.[19]. In brief, the alignment of the potential amino acid sequences was performed using MUSCLE[20] implemented in MEGA11. This resulted in approximately 1100 amino acid residue positions in the final datasets. Phylogenetic analyses were subsequently carried out using both Bayesian inference and maximum likelihood methods. Bayesian inference was conducted with MrBayes 3.2.6[21] and maximum likelihood analyses with RAxML 8.2.9[22] or in initial analyses, with MEGA11[23]. To get statistical reinforcement for the placement of nodes in the RAxML analyses, clade robustness was estimated with 1000 rapid bootstrap inferences followed by a thorough analysis of maximum likelihood. The MrBayes analyses were performed using the following settings: eight chains of Markov chain Monte-Carlo iterations and a heated parameter of 0.05 with trees sampled every 1000 generations. The average standard deviations of split frequencies at termination of the analyses after 1 000 000 generations were below 0.01. The MrBayes and RAxML analyses were run on the CIPRES Science Gateway[24] in the Extreme Science and Engineering Discovery Environment (XSEDE). Sequence characteristics for the different clades were detected manually.

Results

Predicted CaMBD sequences

Following the initial survey of genomes by BLAST searches, candidate P2B ATPases were defined phylogenetically. A sequence in the predicted proteome of a selected species was characterized as a P2B ATPase if, in a maximum likelihood phylogenetic tree, it ended up in a monophyletic clade that included well-characterized animal PMCAs and plant ACAs but excluded P2A (SERCA-type and secretory-type Ca^{2+}-ATPases, P2C (Na^+/K^+-ATPases), P2D (ENA-type Na^+-ATPases), P3A (plasma membrane H^+-ATPases) and P3B (Mg^{2+}-ATPases) ATPases.

The identified P2B Ca^{2+}-ATPase sequences were aligned to identify potential CaMBDs based on criteria defined by Mantilla et al.[25]. CaMBDs share some common characteristics such as a positive net charge, an alpha helix propensity, moderate hydrophobicity, two bulky hydrophobic anchor residues (the first a Trp and the second typically either Phe or Leu) and a length of 15–30 residues[26–28]. By searching for these features, we identified potential CaMBDs in Holozoa, Streptophyta and also Amoebozoa P2B ATPases whereas they were absent from prokaryotes and most unicellular protists (Figures 1–3). Most of these CaMBDs are still waiting to be confirmed experimentally to see whether they are functional, especially in case of Amoebozoa since to our knowledge a CaMBD has not been reported in this lineage before.

The CaMBD developed independently in plants and animals

Animal and plant CaMBDs are markedly different (Figure 3). First, in plants, the CaMBD is located at the N-terminus, while it is located at the C-terminus in animals. Second, the two CaMBD sequences do not show much resemblance. Furthermore, we show an amoebozoan CaMBD sequence similar to that of holozoan sequences, while it is absent in all investigated chlorophyte sequences. This could mean that in terms of animals the common ancestor of Amoebozoa and Holozoa was already equipped

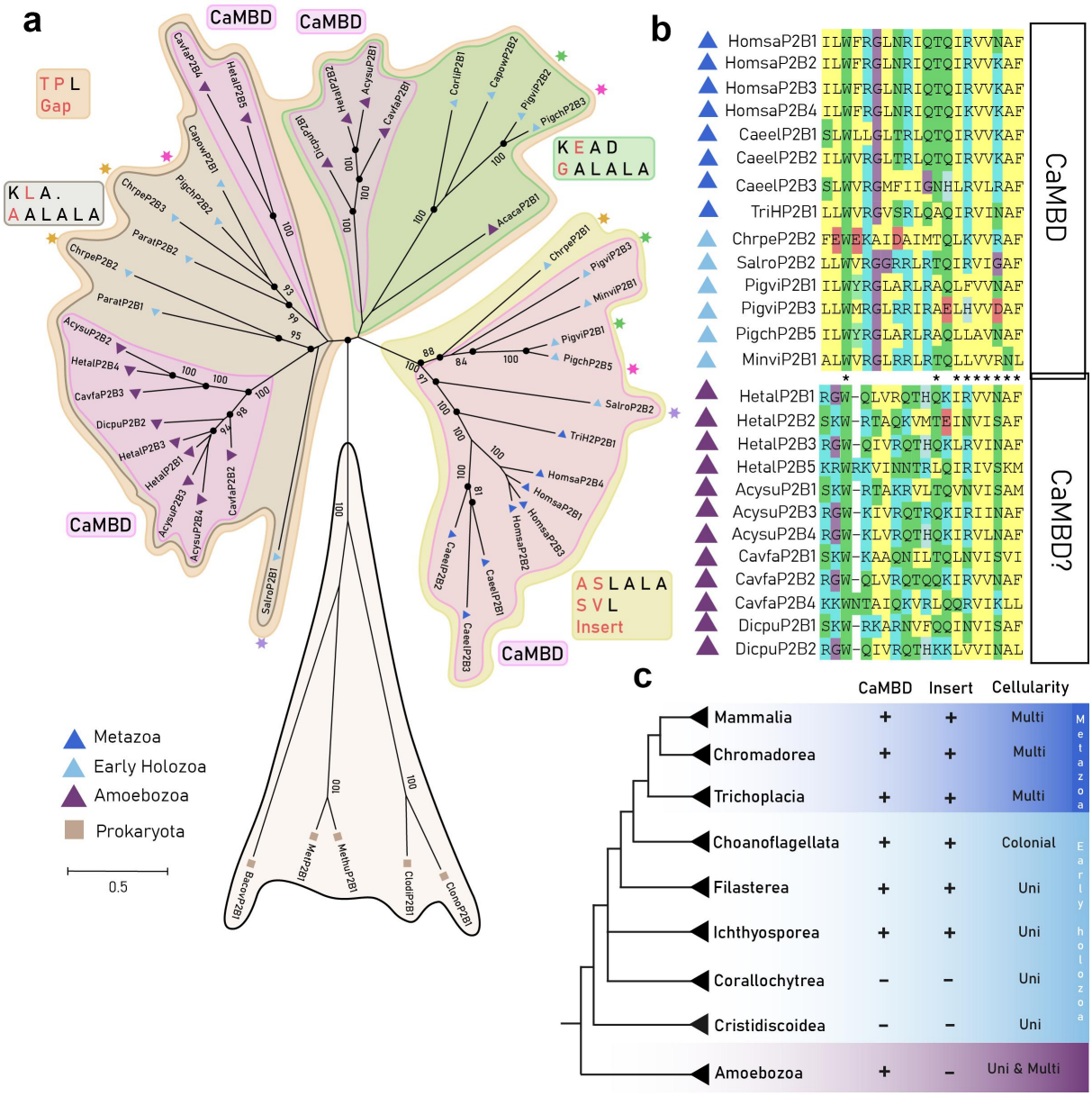

Figure 1. The CaMBD in animals might have developed with the appearance of multicellularity. (a): Phylogenetic tree of Metazoa, early Holozoa and Amoebozoa P2B ATPases. The tree was rooted with some Prokaryota P2B ATPases. Each P2B ATPase in the tree is marked with a colored shape according to its taxonomy. P2B ATPases with a CaMBD are marked with a pink area. Colored stars indicate species with P2B ATPases in both major clades. Synapomorphies are given in colored boxes corresponding to the color of the clade. In these boxes, "insert" and "gap" indicate the potential phospholipid-binding site or the lack of it. The exact sequence of it is shown in Supplementary Figure 1. Full names of species and accession numbers are given in Supplemental Table 1. The tree is a result of maximum-likelihood analysis using RAxML with 1000 bootstrap rounds. Numbers at nodes indicate bootstrap support values. Scale bar: 0.5 amino acid substitution per site. Bayesian inference analysis was also conducted with 1,000,000 generations and resulted in an average standard deviation of split frequencies below 0.01. Black dots at notes of the tree show maximum statistical support in the Bayesian inference analysis. The details of the analysis are described in the Methods section. (b): Sequence alignment showing the CaMBD sequence found in Holozoa and the potential CaMBD sequence in Amoebozoa. Black stars on the top of the Holozoa sequences indicate a high similarity between the Amoebozoal P2B ATPase sequences and Holozoal CaMBDs. Dark blue triangle: Metazoan, bright blue triangle: early Holozoan, purple triangle: Amoebozoan. (c): The simplified phylogenetic figure summarizes in which class we could identify a CaMBD or the phospholipid-binding sequence shown in Supplementary Figure 1. It also indicates the most common cellularity form. In Amoebozoa the CaMBD was only identified in species that can become multicellular.

with a CaMBD while in plants it developed only after Chlorophyta and Streptophyta diverged.

The development of animal CaMBD co-occurred with the appearance of multicellularity

Most amoebozoan species are unicellular, but the group also includes some clades of slime molds with an interesting morphology. They can be unicellular but they can also form multicellular structures where in some cases the cells start to differentiate. We failed to identify a CaMBD in the P2B ATPases of the amoeba *Acanthamoeba castellanii*, which is only present in a unicellular form. However, all investigated Amoebozoae which can be multicellular were equipped with a CaMBD resembling sequence in their C-terminal region

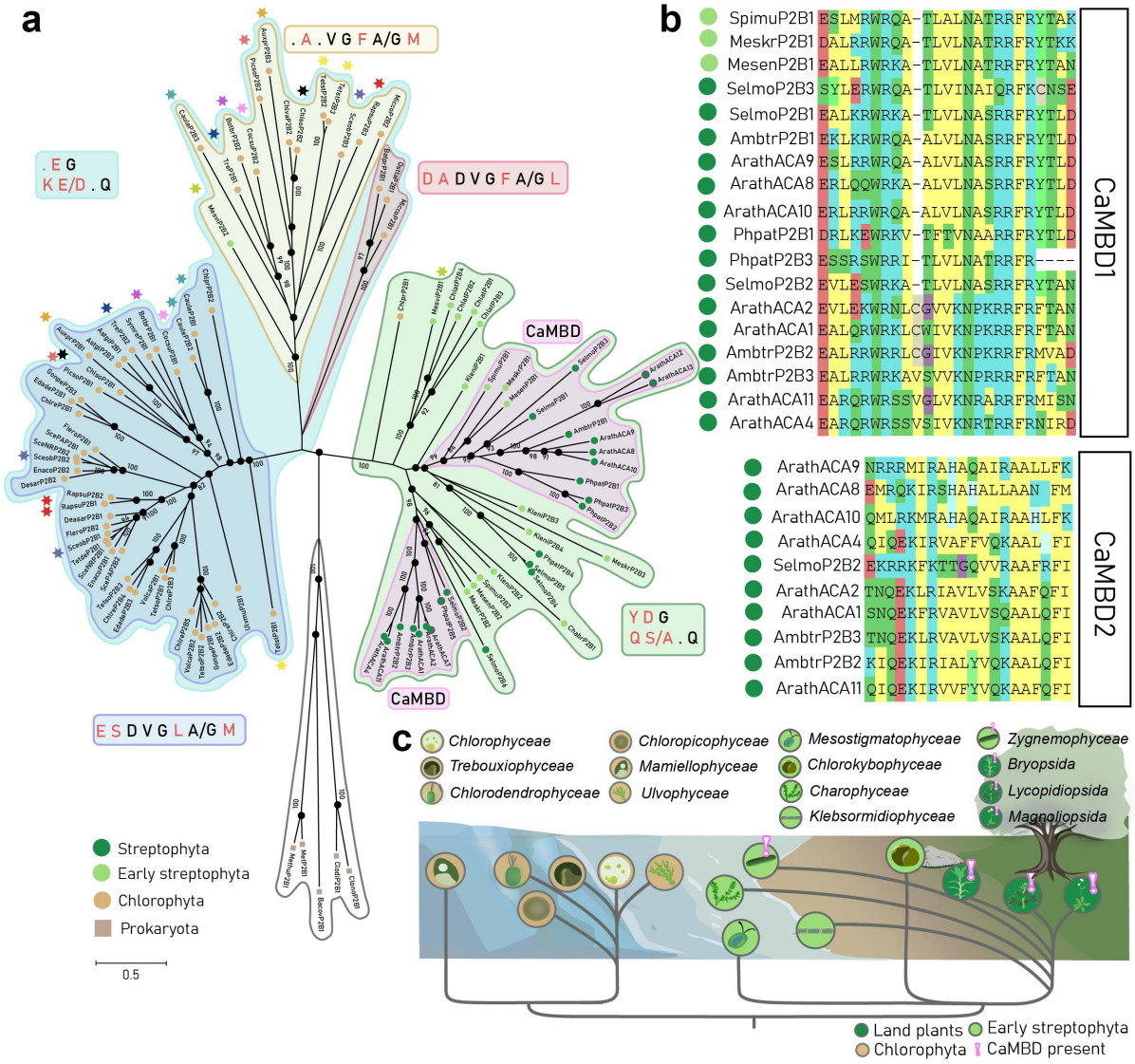

Figure 2. The CaMBD in plants might have co-incided with their water-to-land transition. (a): Phylogenetic tree of streptophyte and chlorophyte P2B ATPases. The tree was rooted with a number of prokaryote P2B ATPases. Each P2B ATPase in the tree is marked with a colored shape according to its taxonomy. P2B ATPases with a CaMBD were found only in the green clade and are marked with a pink area. A grey outline around the symbol indicates an exception in the CaMBD clade. Colored stars indicate species with P2B ATPases in different clades. Synapomorphies are given in colored boxes corresponding to the color of the clade. The meaning of the abbreviations is given in Supplemental Table 1. The tree is a result of maximum-likelihood analysis using RAxML with 1000 bootstrap rounds. Numbers at nodes indicate bootstrap support values. Scale bar: 0.5 amino acid substitution per site. Bayesian inference analysis was also conducted with 1,000,000 generations and an average standard deviation of split frequencies below 0.01. Black dots at notes of the tree show maximum statistical support in the Bayesian inference analysis. The details of the analysis are described in the Methods section. (b): Sequence alignment showing the CaMBDs found in Streptophyta. (c): The figure shows the most typical habitat of the different classes. Pink exclamation mark indicates the presence of CaMBD sequence in the class. The color codes are given in the figure. The figure is adapted from[19].

(Figure 1). Thus, the CaMBD might have been present already in those Amoebozoae that can develop multicellularity.

Similarly, the CaMBD was found to be absent in P2B ATPases of the earliest diverging unicellular Holozoa but appeared in those groups of unicellular Holozoa, which are the closest predecessors of Metazoa (such as *Ministeria vibrans, Pigoraptor chileana* and *Pigoraptor vietnamica*) (Figure 1). The colony forming choanoflagellate *Salpingoeca rosetta* was also found to be equipped with a CaMBD in its C-terminal sequence and in all investigated metazoan P2B-ATPases a CaMBD was identified (Figure 1). Likewise, Mantilla et al.[25] identified CaMBDs in all the 16 analyzed metazoan species. The findings that the CaMBD of P2B ATPases is present only in those unicellular organisms, which are the closest predecessors of multicellular metazoa and is ubiquitous in multicellular or colonial holozoa lead us to hypothesize that the CaMBD sequence was an advantage for multicellular organisms and might have been necessary for this evolutionary step.

The development of plant CaMBDs co-occurred with terrestrialization

Viridiplantae (green plants) are divided in Chlorophyta (green algae) and Streptophyta (including all land plants). We were not able to identify a CaMBD in any of the chlorophyte P2B ATPases irrespectively of whether they were unicellular, multicellular or

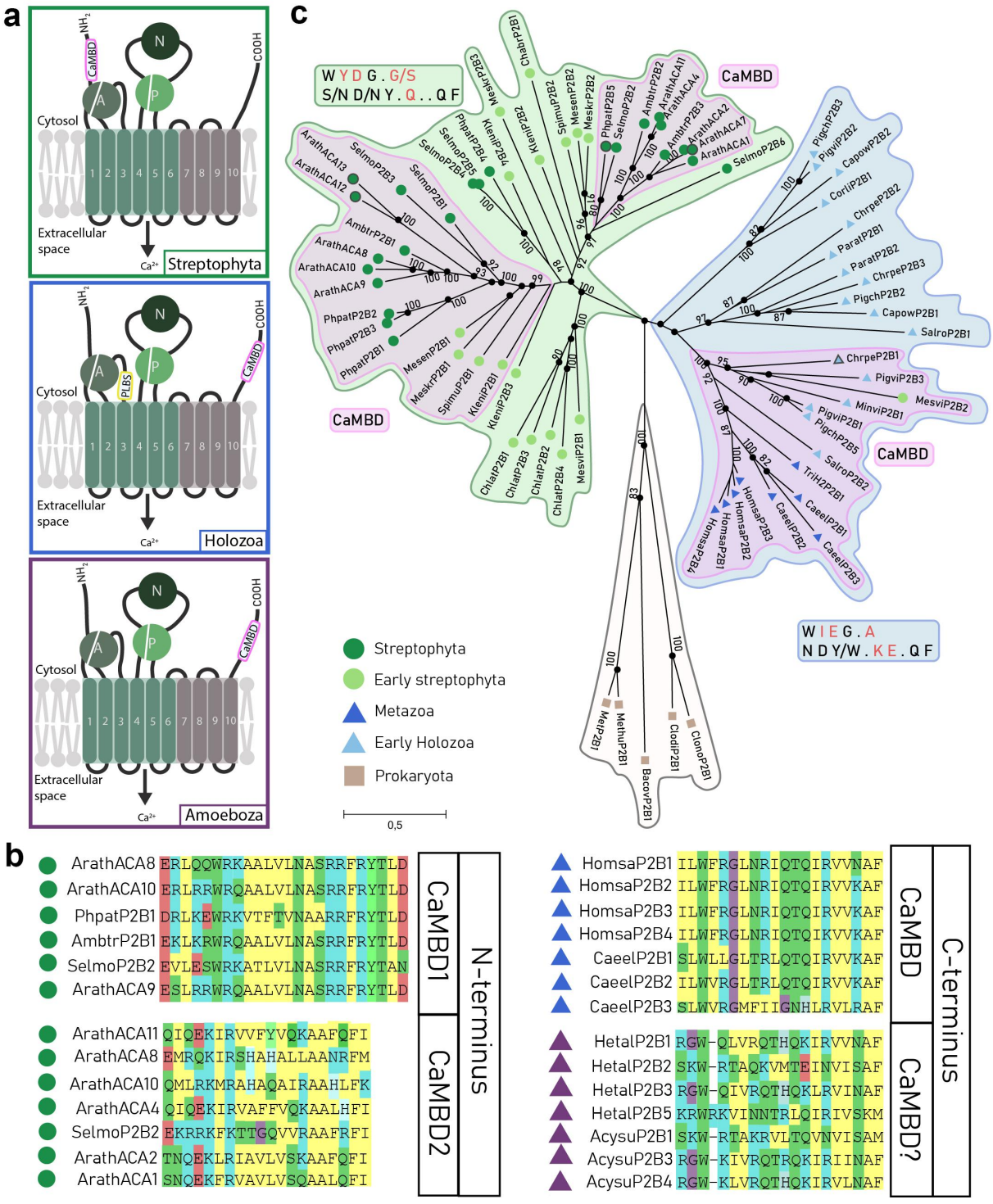

Figure 3. The CaMBDs of animals and plants developed independently. (a): Schematic representation of P2B ATPases from Streptophyta, Holozoa and Amoebozoa showing where the presumed CaMBD and phospholipid-binding site (indicated with PLBS) are located. (b): CaMBD sequences in P2B ATPases of animal and plant origin do not show much resemblance. (c): Phylogenetic tree of streptophyte and holozoan P2B ATPases. The tree was rooted with a number of prokaryote P2B ATPases. Each P2B ATPase in the tree is marked with colored shape according to its taxonomy. P2B ATPases with a CaMBD are marked with a pink area. Synapomorphies are given in colored boxes corresponding to the color of the clade. The meaning of the abbreviations is given in the Supplemental Table 1. The tree is a result of maximum-likelihood analysis using RAxML with 1000 bootstrap rounds. Numbers at nodes indicate bootstrap support values. Scale bar: 0.5 amino acid substitution per site. Bayesian inference analysis was also conducted with 1,000,000 generations and an average standard deviation of split frequencies below 0.01. Black dots at notes of the tree show maximum statistical support in the Bayesian inference analysis. Details of the analysis are described in the Methods section.

colonial. However, we detected such sequences in the Zygnemophyceae class of early Streptophytes (including *Spirogloea muscicola, Mesotaenium endlicheranum* and *Mesoteanium kramstae*), which comprises one of the closest predecessors of land plants. In more advanced land plants, CaMBDs were present in all investigated species (Figure 2). In summary, the CaMBD sequence seems to have appeared in some unicellular early streptophytes, and was not present in other earlier

multicellular streptophytes or in any of the investigated chlorophytes regardless of the cell-type. Thus, in plants, the development of the CaMBD might not be connected to multicellularization.

But then what initiated its development? Chlorophytes live in water or on damp surfaces, while some of the early Streptophytes also appear in soil or on rock surfaces. Interestingly, the appearance of the CaMBD resembles the appearance of the R domain in P3A plasma membrane H^+-ATPases that coincided with terrestrialization of plants[19]. Thus, we hypothesize that the CaMBD domain facilitated the survival of plants when facing the harsh conditions on land.

The appearance of a putative phospholipid-binding insert

We identified a non-conserved insert of 38 to 88 residues rich in acidic amino acid residues, which was specific for all holozoan P2B ATPases in Clade II and situated in the loop between transmembrane (TM) segments 2 and 3, very close to TM3 (Suplementary Figure S1). In the human pump PMCA1 (ATP1B1; NP_001673) the insert comprises 62 residues (34 of which are charged) and is situated between Gly292 and Pro355. A binding site for activating phospholipids has been localized in the same loop near TM3 but could not be defined precisely[16–18]. Strikingly, we found that this insert is present only in those holozoan sequences which are equipped with a CaMBD, except in the case of *Chromosphaera perkinsii*, a unicellular early holozoan species. This non-conserved region rich in acidic and basic amino acid residues might be the phospholipid-binding site, which could mean that these pumps have evolved another layer of regulation besides the CaMBD.

Signs of early gene duplication in animal and plant P_{2B} ATPases

The structure of phylogenetic trees makes us believe that P2B ATPases underwent gene duplications at an early stage of eukaryotic evolution. This hypothesis is supported by the fact that the different clades of P2B ATPases from Chlorophyta and Holozoa each include isoforms from the same species (marked by stars in Figure 1A, 2A). Similar signs of ancient gene duplications were reported previously in P3A, P2A and P5A-ATPases[19,29].

Discussion

CaMBD sequences are hard to identify due to their diverse, less conserved nature. Some P2B ATPases (for example Arabidopsis ACA12[30]) have a degraded CaMBD that is not functional due to partial deletion. Therefore, the nature of the presumed CaMBDs identified here should be considered with caution until the CaMBD sequences are confirmed experimentally.

In case of animal P2B ATPases, the CaMBD sequence and the phospholipid-binding sequence first evolved in a unicellular organism. The development of regulation in unicellular organisms enabled better communication and coordination between individual cells, which could have been a key to the evolution of multicellularity approximately 600 million years ago[31]. Thus, the development of these regulatory layers in P2B ATPases might have been a contributing factor to allowing Holozoa to become multicellular. It was not enough for this step as some unicellular organisms also have it, but it was needed since all multicellular animal species are equipped with it.

We have proposed a CaMBD sequence in multicellular Amoebozoa that resembles the one found in Holozoa. It could suggest that the CaMBD was already developed in Amoebozoa and because of the sequence similarities maybe already in the common predecessor of Holozoa and Amoebozoa.

In plants, we could not find a connection between the development of multicellularity and the appearance of a CaMBD. It was not seen in colonial or multicellular Chlorophyta and in early multicellular Streptophyta, but appeared in the closest predecessors of land plants, in early unicellular streptophytes, the Zygnemophyceae algae. Furthermore, it was detectable in all of the investigated embryophytes (land plants). As chlorophytes and early streptophytes typically live in water, while embryophytes are found on land, there might be a connection between terrestrialization and the appearance of this regulatory layer.

A similar observation has been made regarding the regulatory domain of P3A plasma membrane H^+-ATPases[19]. In P3A ATPases, the 14-3-3 protein binds to a phosphorylated Thr residue at the C-terminus of the pump, which is necessary for pump activation. Interestingly, 14-3-3 protein is present already in chlorophytes but their number expanded with the appearance of land plants[32]. Likewise, the protein kinase superfamily is present in chlorophytes but has expanded during streptophyte evolution[33]. CaM appears to be present in all eukaryotic organisms[34]. Thus, this could mean that the different parts of regulatory pathways were already present before the evolution of the regulatory domain in P3A and P2B ATPases but only with the appearance of these domains could plants make the evolutionary transition toward land about 450 million years ago.

To summarize, in this hypothesis paper, we propose that the animal and plant CaMBD sequences developed independently and because of different major evolutionary challenges. The animal CaMBD may have developed with multicellularity while the plant one seems to co-appear with terrestrialization. We believe that the development of these regulatory layers enabled the P2B pumps to fulfill their current role in animal and plant signaling pathways[35].

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Anett Stéger http://orcid.org/0000-0003-1627-7584
Michael Palmgren http://orcid.org/0000-0002-9982-6114

Author contributions

M.P. developed the core idea, A.S. did the phylogenetic analysis, M. P. supervised the work, A.S. and M.P. wrote the manuscript.

Data availability statement

All data supporting the findings of this study are included in this article, its Supplementary Information, and the Supplementary Data file. Any other data will be made available upon reasonable request.

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