Plant TPK Ion Channel Evolution

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Potassium (K) is a crucial element of plant nutrition, involved in many physiological and molecular processes. K+ membrane transporters are playing a pivotal role in K+ transport and tissue distribution as well as in various plant stress responses and developmental processes. Two-pore K+-channels (TPKs) are essential to maintain plant K+ homeostasis and are mainly involved in potassium transport from the vacuoles to the cytosol. Besides vacuolar specialization, some TPK members display different membrane localization including plasma membrane, protein storage vacuole membrane, and probably the organelles. In this manuscript, we elucidate the evolution of the voltage-independent TPK (two-pore K+-channels) family, which could be represented in some species by one pore, K+-inward rectifier (Kir)-like channels. A comprehensive investigation of existing databases and application of modern bioinformatic tools allowed us to make a detailed phylogenetic inventory of TPK/KCO3 (KCO: potassium channel, outward rectifying) channels through many taxa and gain insight into the evolutionary origin of TPK family proteins. Our results reveal the fundamental evolutional difference between the first and second pores, traced throughout multiple taxa variations in the ion selection filter motif, presence of thansposon, and methylation site in the proximity of some KCO members and suggest virus-mediated horizontal transfer of a KCO3-like ancestor by viruses. Additionally, we suggest several interconnected hypotheses to explain the obtained results and provide a theoretical background for future experimental validation.

KCO3 TPK two pore potassium channel molecular phylogeny molecular evolution

1. Introduction

Potassium (K) is one of the essential nutrients required for plant growth and development. This mineral is the most abundant cation of plants and K^+ is crucial for a multitude of physiological processes such as protein translation, enzymatic catalysis, screening of negative charges, and providing turgor [1][2][3]. Cytosolic K^+ also plays an important role in plant adaptive responses to environmental stresses such as drought and salinity [4][5][6] and plant disease [6][7].

Plant tissue K^+ content is typically in the order of ~100 mM on a fresh weight basis and sustaining this level requires a sophisticated array of membrane transporters. K^+ transport is mediated by K^+ transporters that include active K^+ carriers and passive K^+ channels. The former can be classified as belonging to the CPA, Trk/Ktr/HKT, or KT/HAK/KUP families [8][9] whereas K^+ channels are encoded by members of the voltage-gated Shaker channel family or the voltage-independent two-pore K^+-channel (TPK) family, which in some species includes a one pore, K^+-inward rectifier (Kir)-like channel named KCO3 in Arabidopsis (**Figure 1**) [10][11][12].

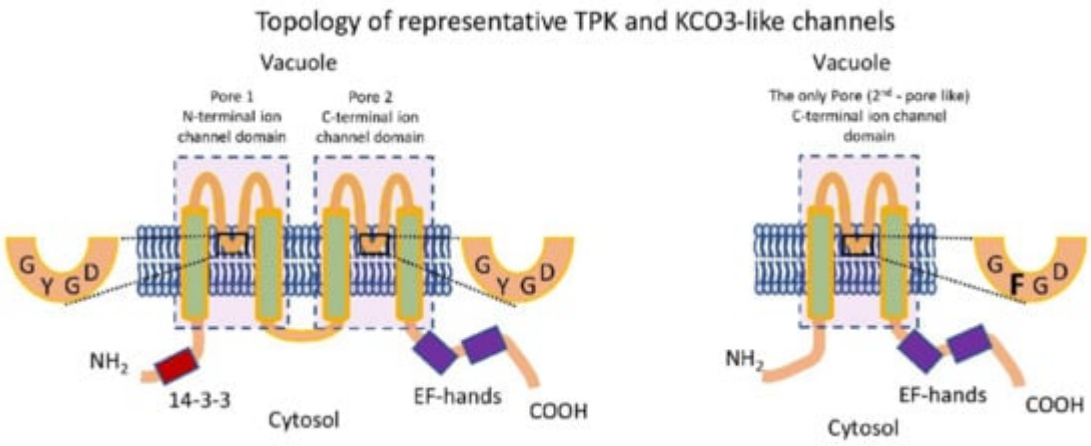

Figure 1. Representative topology of TPK and KCO3 channels. A tandem arrangement of two 'ion channel' domains (PF07885) (for TPK) and a single ion channel domain (for KCO3) is presented. Each domain (rectangle) contains two transmembrane helices, an intermediate pore loop, and a selectivity filter. KCO type channels lack the N-terminal domain.

TPKs activity is insensitive to membrane voltage. However, many TPK channels contain one or two Ca^{2+} binding EF-hands in the C-terminus and channel activity has been shown to depend on cytoplasmic Ca^{2+} levels [13][14][15][16]. TPKs can have domains for the binding of 14-3-3 proteins in their N terminus. Phosphorylation of this domain by kinases such as KIN7 [17] and subsequent 14-3-3 binding greatly affects TPK activity [17][18][19]. Cytoplasmic pH is another factor impacting channel open probability [14] as is interaction with various kinase isoforms of the CIPK (CBL-interacting kinase) family [20][21]. Furthermore, TPK channels can alter activity upon membrane stretching or the creation of trans-tonoplast osmotic gradients, indicating a role in intracellular osmosensing [22].

Extensive studies have shown that the majority of TPKs and KCO3 is targeted to the tonoplast (i.e., the membrane of the lytic and/or storage vacuole) [14][18][23][24][25][26][27]. However, isoforms have also been detected in the plasma membrane (e.g., AtTPK4) and possibly the thylakoid membrane [28]. However, the questions of AtTPK3 thylakoid localization as well as physiological function remain open and need further experimental support [29]. Functional TPK channels contain four pore domains (e.g., [12][30]) and the application of FRET and BiFC techniques revealed the existence of homodimeric forms of AtTPK1 and AtTPK5 [17][23]. In addition, the formation of heterodimers between AtTPK1 and AtTPK3 has been suggested [31]. Formation of stable dimers of AtKCO3 also occurs [32], but not surprisingly, did not show functionality. TPK channels are probably involved in the maintenance of K^+ homeostasis in plant cells by controlled intracellular K^+ transport from and into organelles, particularly the vacuoles [14][33]. Furthermore, a recent study on *A. thaliana* suggests that TPKs and TPC1 channels may interact at the tonoplast to endow excitability to this membrane [31]. Nevertheless, functional aspects of many TPK/KCO channels remain largely unexplored.

The panoply of regulatory mechanisms, membrane localizations, and potential functions impinges on the intriguing question of how various KCO and TPK isoforms relate to each other in an evolutionary sense. Phylogenetic studies of TPK/KCO channels have been conducted in the past decades [19][25][34], but were based on a limited number of sequences and species. Recent progress in the genome sequencing of plant species from various taxonomic

groups as well as data from other taxa has greatly facilitated comparative studies across a much wider variety of species. In this study, we compiled a comprehensive TPK/KCO inventory and carried out phylogenetic and structural analyses on both the first and second pore domains of the TPK channels. Our detailed domain architecture analysis suggests that the two-pore domains of plant TPKs have a distinct evolutionary antecedent. The first pore exhibits a highly conserved sequence in the pore loop and a TxGYGD selectivity filter analogous to that found in archeal KcsA channels and subsequently in Shaker type voltage-gated (VG) channels. The second pore has an altered selectivity filter (TxGFGD) and is more likely to have ancestry based on a KCO lineage, since our phylogenetic analyses showed the occurrence of KCO type channels in a far wider number of species than hitherto reported. Additionally, obtained results suggest virus-mediated transfer of KCO-like ancestral proteins from several potential hosts.

2. Analysis on Results

2.1. Analysis of the Arabidopsis C- and N-Terminal Pore Regions

TPK and KCO type channels contain two and one 'cation channel domain' (pFAM PF07885), respectively (**Figure 1**), consisting of two transmembrane helices, a pore loop, and a selectivity filter. Four of these 'pore module' oligomerises represent the minimal structure of a functioning K channel. A multiple sequence alignment of Arabidopsis TPK/KCO channels revealed the existence of some important differences in modular structure between different family members: first, AtKCO3 is missing the N-terminal pore module; second, AtTPK4 has deletions outside of the pore modules in both N- and C-terminal domains that led to the removal of potential regulatory sequences such as the 14-3-3 binding motif and EF-hands; and third, the sequence of AtTPK4 was the most different from the consensus sequence of the entire TPK family (not counting AtKCO3) (Figure S1, Table S1).

Based on a very limited distribution of KCOs in plants and similarity, current evolutionary models assume that KCO3 is the product of a relatively late TPK4 gene duplication event [19][25][34]. To further elucidate phylogenetic relationships within the Arabidopsis TPK/KCO family, we separately analyzed the N- and C-terminal pore modules in terms of the sequence (**Figure 1** and Figure S1), sequence similarity (Table S1), and phylogeny (**Figure 2**). It was found that all N-terminal pore regions were closely related, while the C-terminal copies were more divergent, suggesting ongoing evolutional processes on that domain (**Figure 3**). The C-terminal pore region of AtTPK4 was the most different from that of other TPK isoforms (Table S2). Furthermore, pore domain comparison confirmed our alignment results (Figure S1), showing that AtKCO3 lacks the N-terminal pore domain as was previously shown [34][35].

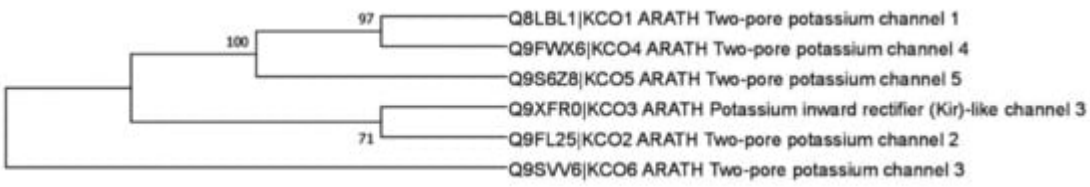

Figure 2. Phylogenetic tree of the TPK and KCO proteins of *Arabidopsis thaliana*. Full-length proteins from Arabidopsis were used for phylogeny reconstruction with the maximum likelihood method and WAG substitution model.

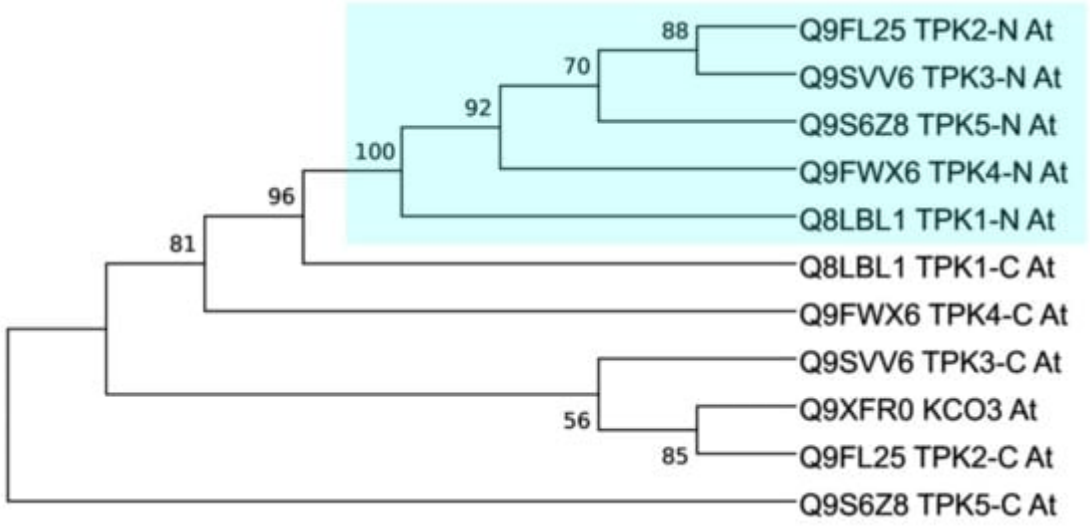

Figure 3. Phylogeny estimation of the *Arabidopsis thaliana* N- and C-terminal pore modules. The maximum likelihood method and LG substitution model were used. N-terminal pores are highlighted.

2.2. Pore Alignment and Analysis of the Voltage-Dependent Potassium Channel Signature

To test whether the observed differences between the first and second pores of Arabidopsis TPK family members are present in other species, the sequences of the TPK proteins from different taxa (photosynthetic bacteria, green and red algae, “low” plants, mono and di-cotyledons, SAR, metazoa, and viruses) were analyzed. Each pore sequence was extracted and examined individually. This showed that some pores of the TPK proteins exhibited significant similarity to the pores of voltage-dependent (PVG) channels such as AKT K^+ channels, represented by the ion transport protein domain PF00520 (Pfam database classification) (Table S3) (E-value (≤0.001). Interestingly, both domains (‘ion channel’ PF07885 and ‘ion transport protein’ PF00520) belong to the voltage-dependent potassium channel superfamily (PLN03192). The similarity was especially evident in the region of the selectivity filter GYGD motif and could point to a common evolutionary origin [36]. However, the studied taxa differed greatly where the presence of such ‘voltage signatures’ (V-signatures) is concerned: Archaea, Bacteria, and SAR, organisms that predominantly have K^+ channels with a single ion channel domain (i.e., only one pore loop) typically show V-signatures. The metazoa (multicellular animals), on the other hand, have mainly two-pore channels, neither of which shows a V-signature. Streptophyta (higher plants) have a distinctly different configuration with a V-signature in the first pore but not the second. Green and red algae have several intermediate patterns, where two-pore domain proteins show none, one, or two V-signatures. Amino acids, matching VDC signatures of the model species (e.g., Arabidopsis, rice, and *Physcomitrella patens*) are highlighted on the alignments (Figures S2–S4). The recent structural and evolutional revision of plant VG K^+ channels suggests that these proteins form a

completely different (from metazoan) channel group and originated from another (not metazoan) procaryotic ancestor [37].

2.3. Variation in the Selectivity Filter Motif in the First and Second Pore

Ion selectivity is defined by the GYGD motif, where Y and D residues are crucial for K^+ selectivity and open state, respectively [38]. We found the exact match to the GYGD motif of TPK sequences from photosynthetic bacteria to high plants (Figures S2–S7). However, for bacteria and algae, we noticed a high degree of motif variation, where Y is often replaced by L or F. According to this observation, it could be suggested that such amino acid substitutions in the GYGD motif reduce the selectivity strength and provide the ability to transport other monovalent cations, most probably Na^+ [38]. The second crucial amino acid in the GYGD motif is D, which is a crucial determinant of open state stability [38]. It was found that the first pore of KCO-like from photosynthetic bacteria KCO-like exhibited the variability of both sites, while for the second pore selectivity filter motif, it looks like GxxD (Figure S5). Unfortunately, for photosynthetic bacteria, there is only a limited number of sequences without VDC available, therefore, we cannot state that this conclusion would be the ultimate. In contrast with photosynthetic bacteria, the TPKs of green and red algae had variations only for Y (to F and L) in the first pore, but for the second pore, we found different variants from conserved GYGD to the complete absence of this motif (Figures S6 and S7).

A rather interesting example of the motif variation was found in low plants and monocotyledons (Figures S3 and S4). While the first pore GYGD was mostly conserved among these taxonomic plant groups, the second pore was conserved, but also not well-aligned/shifted. The functional outcome of such disturbance remains unclear. In the majority of Dicotyledons, in contrast, both pores have conserved a GYGD motif, with the only exception for a *Glycine max* (A0A0R0GFP1_N) for the first pore, and KCO3 with GFGD in *Arabidopsis thaliana* (Figure S2). However, it was reported that one of the Tobacco TPK isoforms may have variations (VHG and GHG instead of the GYG) in the second pore selectivity filter [39].

2.4. TPK/KCO Phylogeny

2.4.1. General TPK Phylogeny (Based on the Alignment of Full-Length Proteins)

An initial phylogenetic analysis was carried out using the ‘ion channel’ domain PF07885, which consists of two transmembrane domains (TMDs) interspersed by a pore loop and henceforth referred to as a ‘pore region’ (**Figure 1**). This analysis showed the presence of TPKs and KCOs in a wide range of taxa across all biological kingdoms, which included many different plant species (**Figure 4**, Table S1). Within plant species, the TPKs/KCOs cluster into two main clades, which can be further sub-divided into sub-clades ‘A’ and ‘B’. Clade I contains the evolutionary most recent TPK members found in Embryophyta and flowering plants (mono- and dicotyledonous). Within this clade, sub-clade A contains the more ‘advanced’ TPKs from higher plants, while sub-clade B contains members from early land colonizers such as *Physcomitrella patens*, together with *Marchantia polymorpha* and *Selaginella moellendorffii*.

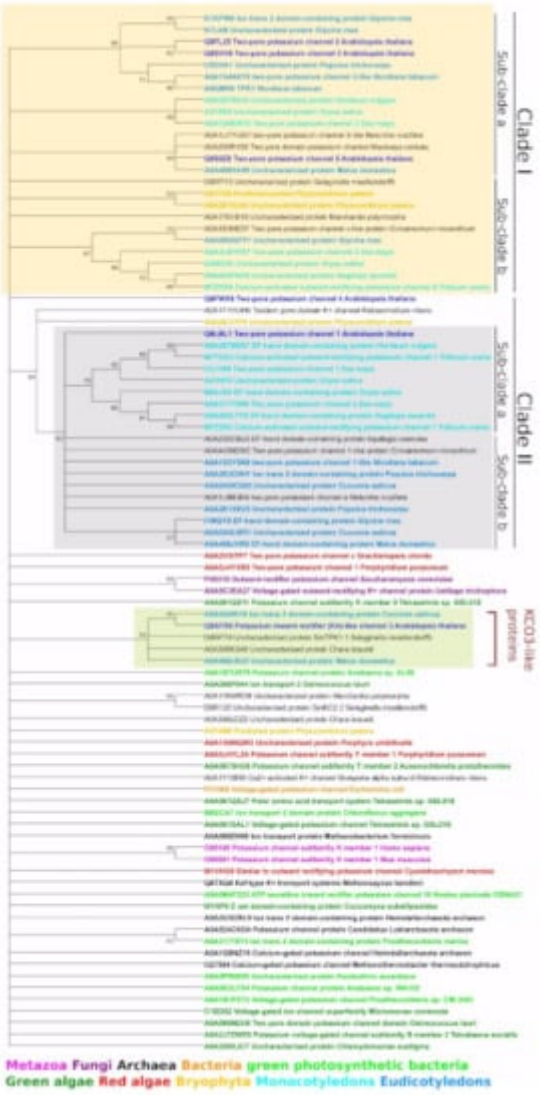

Figure 4. Phylogeny estimation of the TPK proteins. Full-length proteins from selected taxa were used for phylogeny reconstruction with the maximum likelihood method and LG substitution model.

Clade IIA contains TPK members from only monocotyledon species, with high similarity to AtTPK1, whereas clade IIB forms a collection of TPK channels from dicotyledons and more primitive flowering plants such as *Nelumbo nucifera* and *Cinnamomum micranthum*. Interestingly, the branch of the freshwater alga *Klebsormidium nitens* and bryophyte *Physcomitrella patens* acts as a parental form for the entire clade II. The lack of relationship between AtTPK4 and any of the other Arabidopsis TPKs led to this channel being positioned apart from other TPK isoforms, between clade I and II.

Previous work reported the presence of a KCO (AtKCO3) only in *Arabidopsis thaliana* and *A. lyrata* [19][34], positioned near AtTPK2. However, our analysis showed that AtKCO3 is located on a separate branch, together with KCO3-like proteins from the lower plant *Chara braunii*, the lycophyte *Selaginella moellendorffii*, apple (*Malus domestica*) and cucumber (*Cucumis sativus*). Thus, these data not only show that KCO type channels are far more ubiquitous than previously reported, and their presence in the *S. moellendorffii* genome supports the idea that

KCO-like single-domain ion channels could be the basis for all TPK K^+ channels since lycophytes form one of the oldest lineages of extant vascular plants.

2.4.2. Domain-Based TPK Phylogeny (First and Second Pores Taken Separately)

The phylogeny analysis was carried out with the N- and C-terminal ion channel domains PF07885 (**Figure 1**), which were further referred to as the first and second pores, respectively. We made a major observation, that, in general, TPK proteins with a VDC signature (from the AKT K^+ channels, representing ion transport protein domain (PF00520)) and VDC-free proteins (depicted as NV) tend to form separated clusters (Figure S8). We found sub-clusters formed separately by VDC and VDC-free proteins for every taxon: mono- and dicotyledons, green and red algae, viruses, bacteria, and archaea. Some minor exclusions for this rule could be found in viral sub-clades: Clade Ia had one exception, while Clade Ib was represented rather by a mix of VDC and VDC-free proteins. Clades Ic and Id, in contrast, were formed by VDC and VDC-free proteins, respectively. Interestingly, land plants have formed separated sub-clusters: one for VDC-free (Clade IIa) and two for VDC (Clade IIb and IIc) proteins. Other taxa, in contrast, were clustered in many small groups of closely related proteins (red and green algae, archaea, bacteria, metazoa, and SAR).

As a possible site of an ancient gene transfer (or convergent evolution), we noticed several separated branches, where proteins from different taxa were clustered together. Branch 1, where Archaea VDC-free protein A0A133VJR6 was clustered together with proteins from photosynthetic bacteria *Kouleothrix aurantiaca* (VDC-free A0A0P9D655) and *Anabaena* sp. WA102 (VDC-containing A0A0K2LYX4). The second branch contains two VDC proteins: archaeal A0A062V1H8 and bacterial *Bilophila wadsworthia* (E5YBI1). Similarly, the third branch contains proteins from bacteria and archaea: two VDC archaeal proteins (*Heimdallarchaeota archaeon* A0A2U3CBL9 and *Halopelagius longus* A0A1H1DI30) and two bacterial (VDC protein *Vibrio nigripulchritudo* U4KGM9 and VDC-free *Sedimentisphaera salicampi* A0A1W6LMM0).

Previously conducted TPK channel phylogeny analyses have had some limitations. For example, Voelker et al. (2010) [19] only used 12 plant species (“low” plants, mono- and dicotyledons); Gomez-Porras et al. (2012) [34] only used five plant species; and Marcel et al. (2010) [25] used much wider taxa (68 channels in sum) also including protist, fungi, and multicellular animals. However, our analysis (Figure S8) had some advantages: (1) analysis of domains (not full-length sequences) allowed us to also include single-pore channels (KCO3-like proteins) from different species (plants, bacteria, archaea, viruses); (2) separated analysis of first and second pores (“N” stands for N-terminal and “C” for C-terminal) allowed us to show the different origin of first and second pores; (3) channels from all available taxa (metazoa, fungi, archaea, bacteria, virus, green and red algae, bryophyta, mono- and dicotyledons, SAR) allowed us to identify possible points of horizontal gene transfer (Branches 1 to 3) between different taxa; and (4) highlighted the absence of a VDC signature (“NV” stands for “no voltage gate”), which allowed us to trace the evolution pathway of different pores, because in most cases, “NV” is combined with “C”. Details of the used legend and abbreviations used are also presented in Table S3.

In total, these results support our assumption on the different origins of the first and second pore. The close inter-taxa connection was found only between Archaea and bacteria. Land plants have formed separated clusters, while

proteins from other taxa were represented by many small branches of closely related proteins.

3. Current Insights

The results of our structural and phylogenetic analyses of TPK proteins reveals the significant complexity of these channels and their evolution. Our study provides new evolutional and structural insights into plant TPK/KCO3 proteins. Furthermore, comparative and evolutionary analysis of plant TPKs/KCO3 with other taxa revealed substantial structural differences of plant TPKs. Interestingly, we found the presence of VDC signatures only in the first pores for TPKs from higher plants. Further application of bioinformatic structural analysis revealed domination of TPK split forms (first pore with VDC signature and second VDC free) and strict GYGD selectivity filters in higher plants. Perhaps, such structural features of TPKs became beneficial for the higher plants after land colonization, and can be connected with vacuolar specialization and possible involvement in cellular signaling in the form of Ca^{2+} vacuolar content control and potassium homeostasis. In addition, phylogenetic analysis of Arabidopsis KCO3 suggests that KCO-type channels may be an ancestral form to all TPKs. The presence of this type of channel in genomes of some higher plant species could be explained by potential viral transfer from some protozoan species to the ancient algal endosymbiont. Perhaps the KCO3-like channels in higher plants may interact with “modern” TPKs and regulate their activity. Taking together the literature analysis and the results of our study, we can suggest the possibility for an alternative evolutionary pathway of KCO3-like channels in plants that is different to the dominated hypothesis of gene duplication and subsequent pore deletion.

Results of our general phylogenetic analysis indicate that TPKs/KCOs proteins are distributed between two main clades, which can be further sub-divided into sub-clades ‘A’ and ‘B’. Albeit some similarities with previous phylogenetic reports [19][25][34], our new phylogenetic tree comprises the extended number of subclades and a large array of different plant species and forms of TPKs/KCO channels. Thus, the current phylogenetic inventory is the most updated reconstruction of plant TPK phylogeny, supporting the idea of the multiplication and complexation of TPK isoforms during land colonization and evolution in general. Taken together, our results indicate that plant TPK channels are considerably different from their counterparts from other taxa and may have several possible options of evolutional and structural development. Results of our domain-based phylogeny analysis suggest the different origins of the plant TPK first and second pores. The first pore contains the VDC signature (the dominant feature for all taxa, but metazoa); the second pore without a VDC signature, was, most probably transferred from some metazoa species via viruses to the ancestors of the green lineage (**Figure 5**).

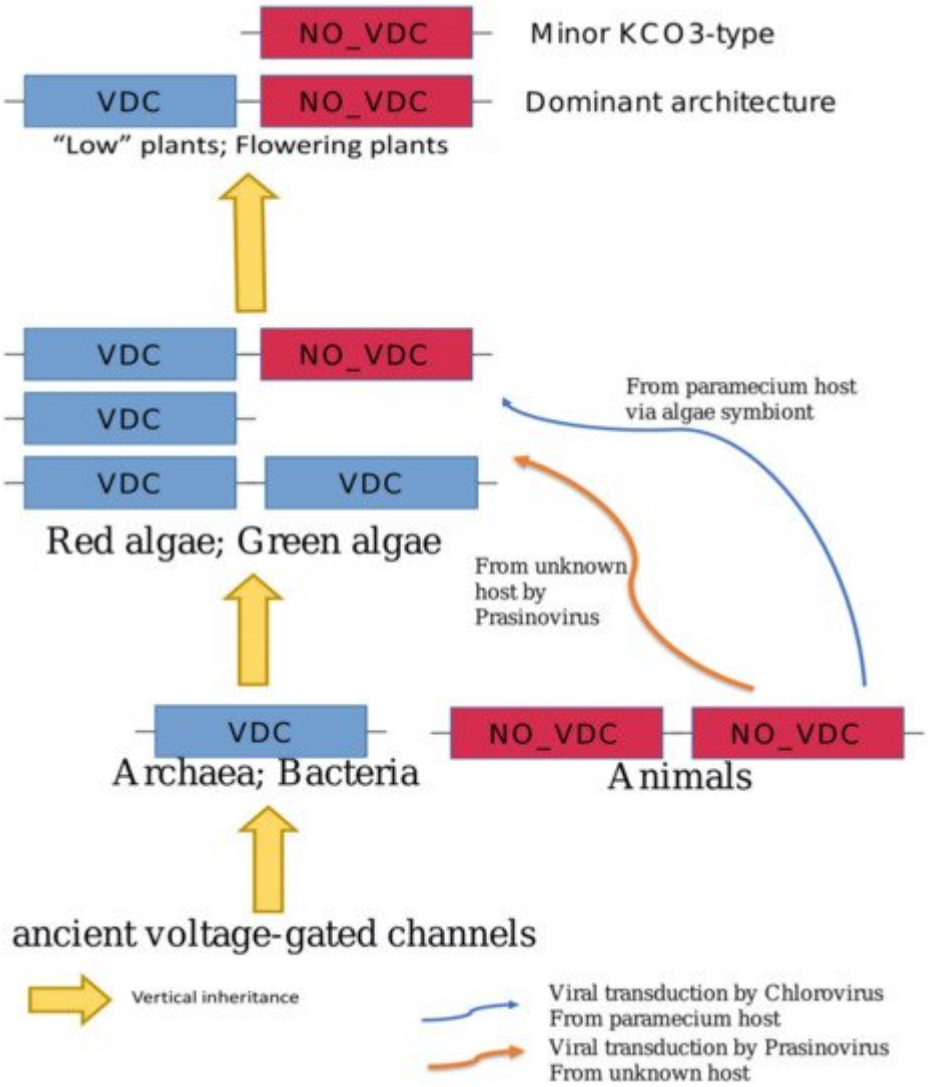

Figure 5. Simplified scheme of possible evolutional pathways of the TPK channels.

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