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TOPICAL REVIEW

Prediction of Aquaporin Function by Integrating Evolutionary and Functional Analyses

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Abstract Aquaporins (AQPs) are a family of channel proteins, which transport water and/or small solutes across cell membranes. AQPs are present in Bacteria, Eukarya, and Archaea. The classical AQP evolution paradigm explains the inconsistent phylogenetic trees by multiple transfer events and emphasizes that the assignment of orthologous AQPs is not possible, making it difficult to integrate functional information. Recently, a novel phylogenetic framework of eukaryotic AQP evolution showed congruence between eukaryotic AQPs and organismal trees identifying 32 orthologous clusters in plants and animals (Soto et al. Gene 503:165–176, 2012). In this article, we discuss in depth the methodological strength, the ability to predict functionality and the AQP community perception about the different paradigms of AQP evolution. Moreover, we show an updated review of AQPs transport functions in association with phylogenetic analyses. Finally, we discuss the possible effect of AQP data integration in the understanding of water and solute transport in eukaryotic cells.

Keywords Aquaporin · Evolution · Function · Integration

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General Background of Aquaporins

Osmolarity and the corresponding water movement represent one of the crucial environmental factors that lead to cellular homeostasis. For many years, water was considered to enter and leave the cells exclusively through the lipid membrane. It was proposed later, the existence of *hydrophilic pores* that would facilitate water and ion transport through the membranes (Stein and Danielli 1956). Those pores were later named aquaporin (AQP) (Preston et al. 1992). Since then more than 7,000 articles have been indexed under the tag "aquaporin" in PubMed resulted in a detailed picture of what a water channel is. AQPs are not only specific water channels but also solute transporters; i.e., most AQPs transport other molecules such as glycerol, urea, and arsenic, among others.

AQPs are transmembrane channels. Thus, the ability of a molecule to cross an AQP channel depends on its own characteristics (size, polarity, charge) and on the features of the AQP involved. As other channels relevant in physiology, AQPs are passive transporters. Water crosses an AQP

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channel or a lipid membrane, and in both cases the driven force for the movement of water molecules is the osmotic gradient. However, the kinetics of water transport depends on the permeability characteristics of each pathway, generally being AQPs more permeable than the lipid bilayer, which implies that AQPs allow a passage of larger flow of water in shorter time intervals.

The Perception of AQPs as a Protein Family

Since the discovery of mammalian AQPs in 1992 and until now much effort has been made to understand the structure and function of AQPs. However, the idea of AQPs as a protein family was formally introduced with a phylogenetic analysis of putative proteins that can transport water and solutes in bacteria and eukaryotes, hypothetically evolutionarily related to previously characterized AQPs (Zardoya and Villalba 2001). In this work, it was showed for the first time a powerful bioinformatic tool, where a putative functional domain was used to automatically identify new AQPs. This superfamily of integral membrane channel proteins was called major intrinsic protein (MIP). Although this work constituted a fundamental step in the understanding of the structure and function of AQPs, it also introduced confusion regarding the definition of AQPs as a family of proteins. This probably occurred because the MIP superfamily is a structural and a functional family with no clear evidence of being a family of homologous genes. As will be discussed later, a phylogenetic analysis using several proteins and showing congruence (same topology) between gene and species trees is a strong evidence for the presence of an evolutionary family; but this type of congruence was not reported for the AQP family. The AQP family could be constituted by non-homologous proteins with similar structure and motifs due to evolutionary convergence.

The lack of precision described above continues to exist. Here, we discuss the consequences of assuming, when reconstructing AQPs' history, that AQPs are a family of monophyletic proteins affecting the classification of AQPs and prediction of their function.

Structure and Motifs in AQPs

As previously mentioned, the AQP denomination is linked to the MIP definition. But what is the MIP superfamily? MIP is considered a conserved domain (cd00333) by The National Center for Biotechnology Information (Marchler-Bauer et al. 2013). According to this definition, the MIP superfamily is divided into two families based on the distinct primary sequences: AQPs and glycerol uptake facilitators (GlpFs) (Zardoya and Villalba 2001). However, there is no clear division between families in terms of functionality; therefore, MIP and AQPs are considered synonymous and are used interchangeably. MIPs have six transmembrane helices (TMH1–6) and two additional membrane-embedded domains (Maurel et al. 1993) (Fig. 1). The monomeric MIP units contain functional pores that can be stably assembled as tetramers (Verbavatz et al. 1993) (Fig. 2). Nevertheless, it was demonstrated that each monomer in the tetramer is a functional unit (Preston et al. 1992).

The sequences of the amino- and carboxy-terminal halves of MIP genes are similar to each other and are arranged as tandem repeats, apparently originated from the duplication of a half-sized gene (Quigley et al. 2002; Zardoya and Villalba 2001). Each half of the molecule bears one hydrophobic loop which includes two highly conserved Asn–Pro–Ala (NPA) motifs (Fig. 1) involved in the primary selectivity of these transporters (Johanson et al. 2001; Zardoya and Villalba 2001). NPA motifs play a critical function in charge and size obstacle (Wallace and Roberts 2004). After the first NPA motif, there is a second motif known as aromatic/arginine (ar/R) constriction (for comprehensive review see Wu and Beitz 2007). This motif is composed of four residues, two from the helices 2 (H2) and 5 (H5) and two from loop E (LE1 and the invariant R; Fig. 1). This filter seems to be the narrow part of the pore, involved in the rejection of large molecules (Fu 2000; Gomes et al. 2009; Sui et al. 2001; Wallace and Roberts 2004). It has been proposed that the selectivity of the ar/R constriction region is related to proton repulsion and to the binding, through hydrogen bonds, to uncharged molecules such as water and glycerol (Wallace and Roberts 2004). One further step forward in the selectivity, i.e., the discrimination between molecules such as water and glycerol, seems to be given by the P1–P5 motif, which is composed of five amino acid residues located in extracellular loop regions and integral membrane domains of AQPs (Fig. 1). Finally, the AEF motif (Ala–Glu–Phe) is located in the TMH1 of AQPs (Fig. 1) (Zardoya and Villalba 2001). Although this motif is conserved in AQP proteins, its function is still unknown. Regarding AQPs gating and/or regulation, some AQP, are regulated by protonation (Tournaire-Roux et al. 2003). It has been described that protonation (but also, phosphorylation and cation binding) directly affects protein conformation, modifying their transport activity. For the plant AQP SoPIP2;1, the mechanism of a transition from an open to a closed state involves the protonation of conserved histidine residues that moves the LD loop to a position that blocks the water pore (Hedfalk et al. 2006). However, the gating mechanisms for other AQPs are not yet clear. It has been described another putative pH sensing motif −μHφφφ (−: acidic residue, μ: hydrophobic residue, H: histidine, φ: polar non-charged residue) that is located in the

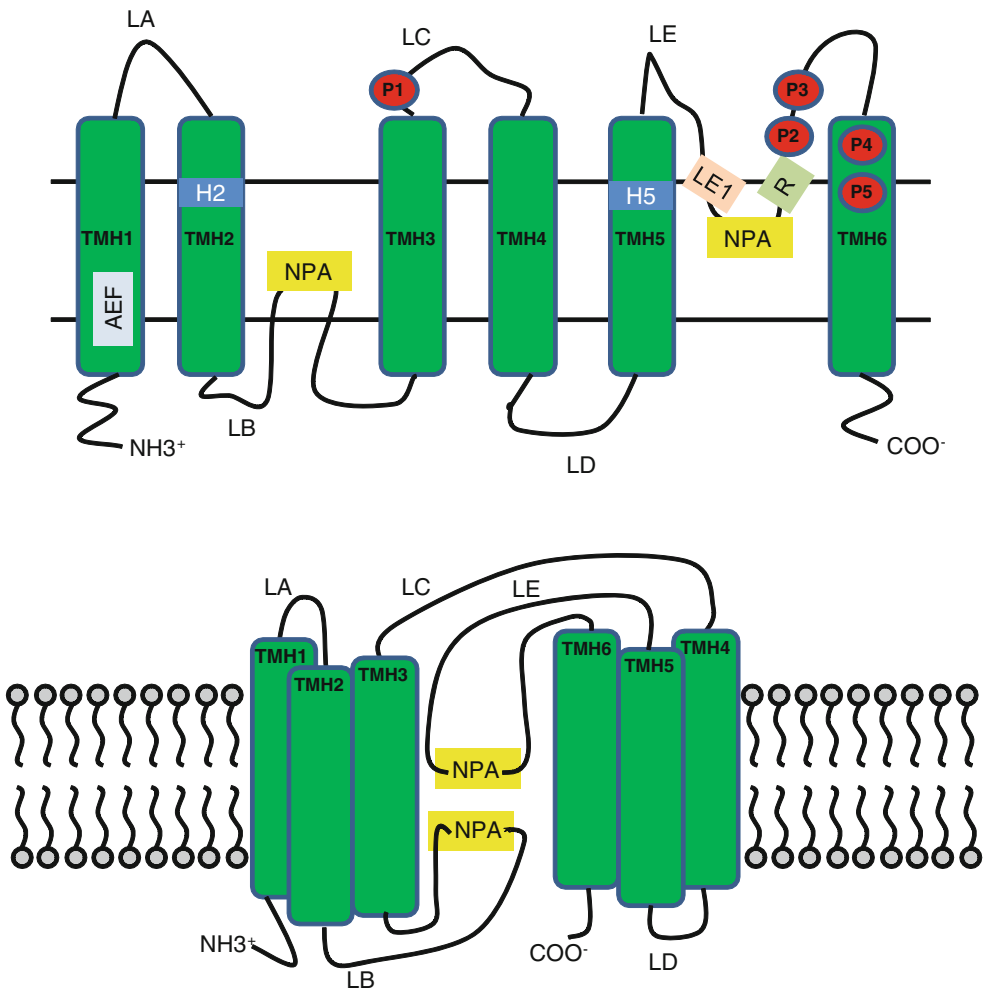

Fig. 1 Schematic representation of the classical structure of AQPs. An AQP monomer showing the six transmembrane helices (TMH1–6) connected by two intracellular (LB and LD) and three extracellular (LA, LC and LE) loops. The conserved residues from the first selective filter (NPA motifs) and the second filter (ar/R constriction) are shown in *red* and *blue*, respectively. The P1–P5 residue positions and the putative pH sensing motif—μHφφφ are shown in *red* and *gray*, respectively (Color figure online)

intracellular LD loop of many plant AQPs and in the extracellular LC loop of *Arabidopsis thaliana* TIP5;1 (Soto et al. 2010). However, the functional validation of this motif in the external and internal sense of pH changes remains to be explored in more detail. AQPs can also form homo- or hetero-oligomers (Jozefkowicz et al. 2013; Neely et al. 1999; Zelazny et al. 2007), but the conditions and/or the motifs involved are not completely understood. Despite the identification of new specific AQPs motifs (e.g. AEF, ar/R, and P1–P5–μHφφφ) described through the last decade, the NPA residues and the six TMHs are still the ones used to identify new classes of putative AQPs.

Classical Clustering of AQPs

AQPs are present in the three domains of life: Bacteria, Eukarya, and Archaea. In Eukarya, the greatest AQP family diversification occurred in vertebrates and plants. While the classification of animal AQPs (AQP0–12) is broadly consistent and reflects their evolutionary relationships, for plant AQPs is different. In plants, AQP subfamilies were initially named and organized considering their putative subcellular localization, but different subcellular localization within each of the subfamilies has already been reported (for comprehensive reviews see Bienert and Chaumont 2013; Hachez et al. 2013; Ishibashi et al. 2011; Wudick et al. 2009). Currently, plant AQPs are classified into seven subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), x intrinsic proteins (XIPs), hybrid intrinsic proteins (HIPs), and GlpF-like intrinsic proteins (GIPs) (Danielson and Johanson 2008; Johanson et al. 2001). However, as previously described for the bacterial and eukaryotic AQPs, there is no work supporting the evolutionary integrity of the seven plant AQPs subfamilies.

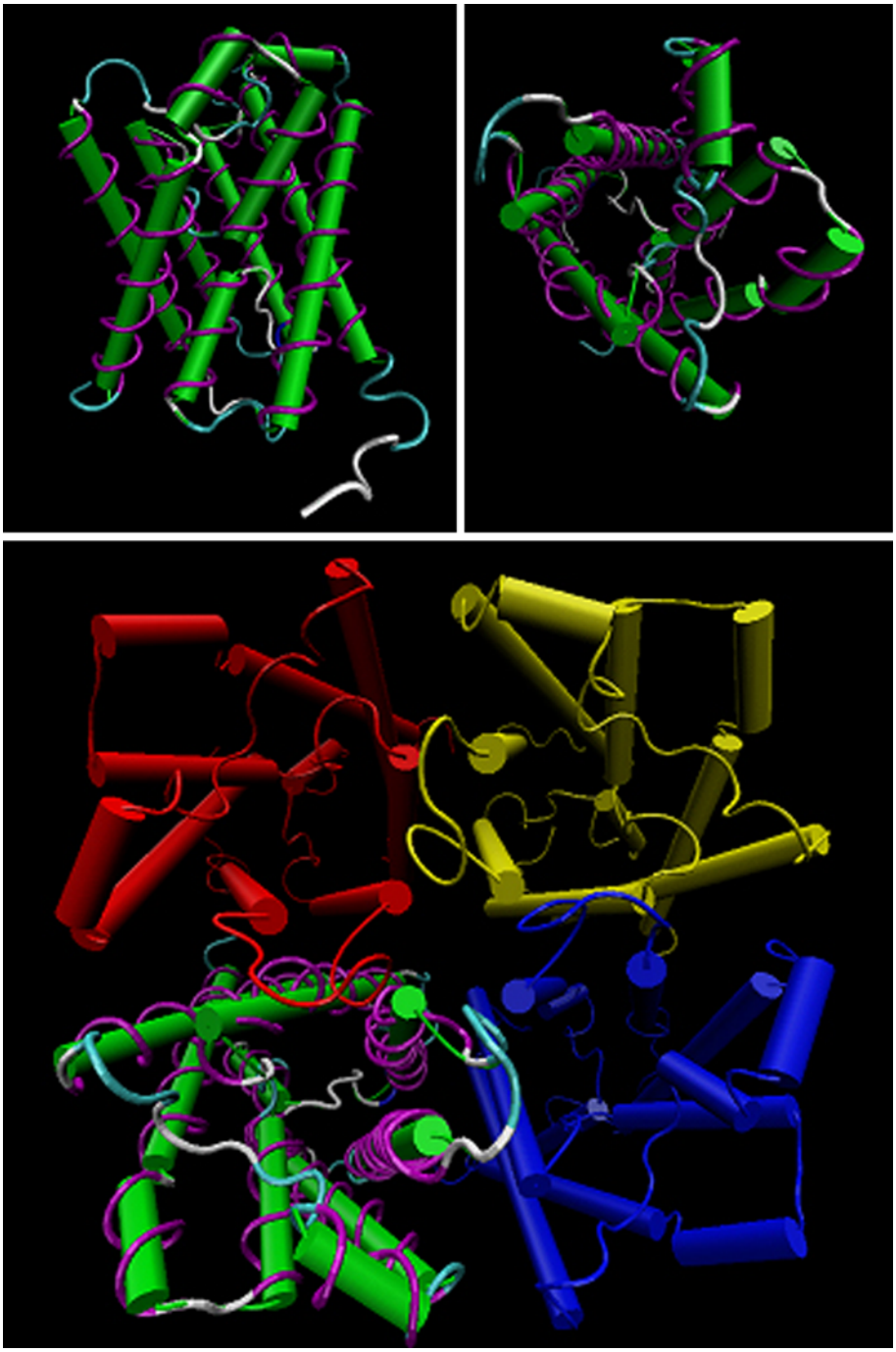

Fig. 2 Cartoon representation of the crystal structure of the spinach aquaporin SoPIP2;1 (Hedfalk et al. 2006) in an open conformation to 3.9 Å resolution (2B5F.PDB from www.pdb.org). Side view (on *top*) and end on view from the extracellular surface of the tetramer (*bottom*), each monomer is indicated in a different *color* (chain A in *yellow*, chain B in *green*, chain C in *blue* and chain D in *red*). MIP tridimensional structure performed by the Cn3D macromolecular structure viewer software (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). The MIP structure consists of six transmembrane helical protein segments lying parallel to the membrane plane (*left*). A view showing the MIP pore oriented nearly perpendicular to the bilayer plane (*right*) (Color figure online)

Figure 3 shows the randomness of plant AQPs classification at different levels such as individual proteins and groups within subfamilies. After the release of new genomic data, classification of novel AQPs was done using different criteria. In some cases, new AQPs were named according to the order of availability of the sequence in the database, or to the amino acid identity regarding the AQPs of *A. thaliana* as taxonomic criteria, while new phylogenetic trees containing reference proteins of Arabidopsis and/or other plant species were built.

The Hypothetical Lateral Transfer of AQPs Between Bacteria and Eukarya

In the recent years, several reports have suggested that all proteins with a MIP functional domain from Bacteria and Eukarya are actually homologous despite their very low amino acid identity (e.g. <5 %). Under this assumption, AQPs from extremely distant taxa (e.g., Bacteria and Eukarya domains) have been included in the same phylogenetic tree (Fig. 4), obtaining incongruence (i.e. different

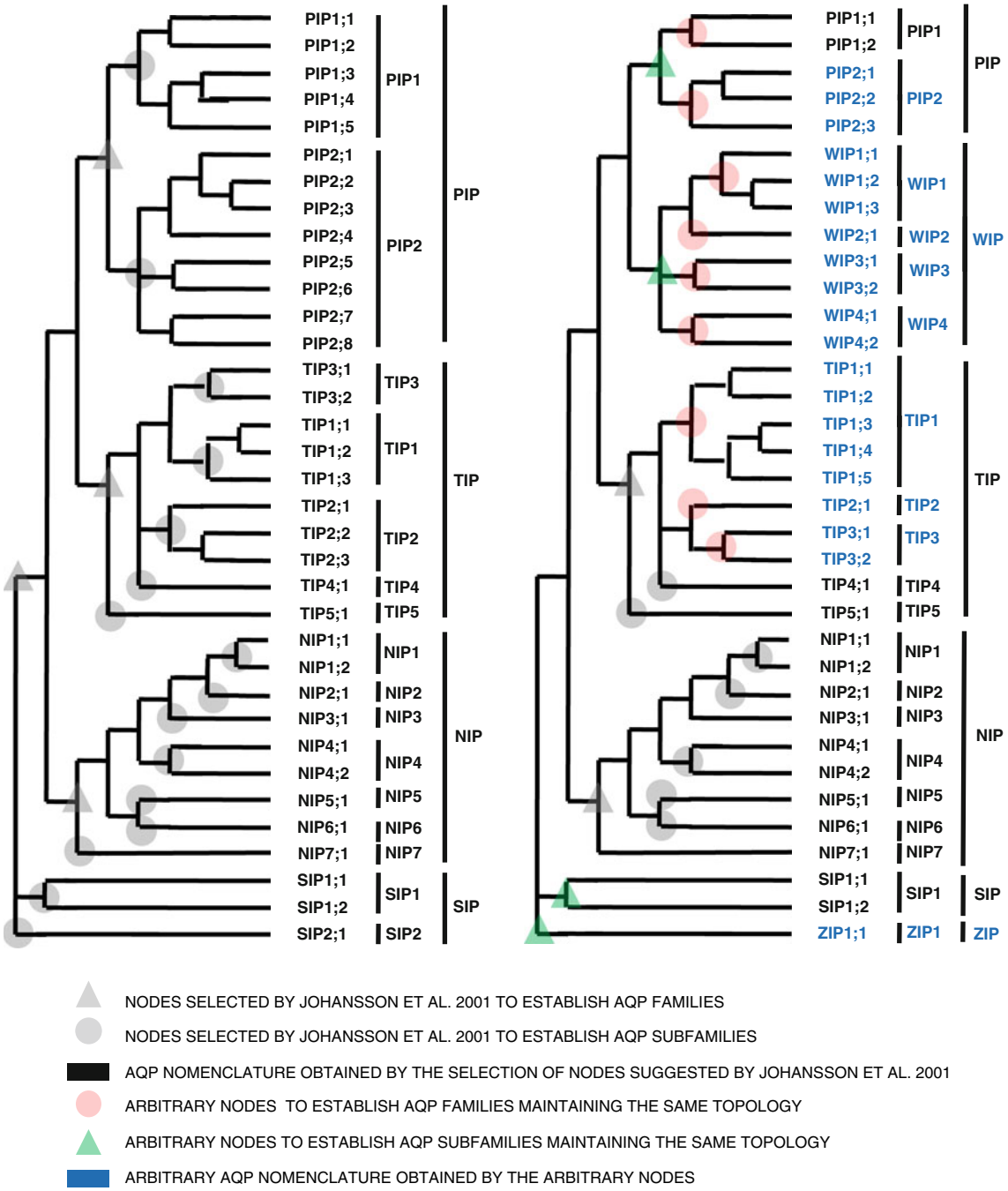

Fig. 3 Nomenclature of plant aquaporins under Johansson et al. framework (2001) and the alternative view of this topology. Both possibilities are arbitrary

topology) between AQPs and organismal trees (Danielson 2010; Danielson and Johanson 2008; Heymann and Engel 1999; Johanson et al. 2001; Quigley et al. 2002; Zardoya 2005; Zardoya et al. 2002; Zardoya and Villalba 2001). It has been suggested that these inconsistent patterns are due to the existence of multiple lateral transfer events, such as genetic exchange of AQP genes between unrelated organisms, such as plants, vertebrates, and bacteria. For example, it has been proposed that the animal AQP3 subfamily and the plant NIP subfamily clusters were acquired by lateral transfer, hypothetically derived from the glycerol facilitator (EcGlpF) from *Escherichia coli* and bacterial NIP-like proteins, respectively (Danielson 2010; Park and Saier 1996; Zardoya et al. 2002). Nevertheless, an unexpected position of a protein within a phylogenetic tree may also be explained by gene duplication, lineage-specific gene loss events, and large amino acid distances (Andersson 2005; Delsuc et al. 2005; Koonin 2003).

The relationships described between AQPs from Bacteria and Eukarya are supported by statistics (high bootstrap values; Danielson 2010; Park and Saier 1996); this information was used to support the lateral transfer hypothesis. However, it is important to point out that obtaining a strongly supported tree does not necessarily indicate that the tree is correct (Delsuc et al. 2005). It is possible to obtain an inaccurate, but statistically supported, phylogenetic tree if the method used does not correctly handle the properties of the data (Delsuc et al. 2005). To avoid overestimation of gene transfer, general congruence with the organismal tree, except for the transfer event, must be observed (Phillips 2006); it is also necessary to find an independent evidence such as localization within genomic islands (Ayub et al. 2007; Yan et al. 2008), or to take advantage of powerful algorithms specifically developed to statistically support gene transfer events (Abby et al. 2010).

The comparative study of genomes from Bacteria and Eukarya indicates that a major fraction of the genes in the prokaryotic genomes have been acquired by horizontal transfer (Koonin et al. 2001). The quantity of horizontal transfer of genes is often associated with the microorganism lifestyle. In addition, the transfer of genes in eukaryotic cells is commonly associated with symbiotic or parasitic relationships with bacteria (Novichkov et al. 2004). Fixation and long-term persistence of horizontally transferred genes

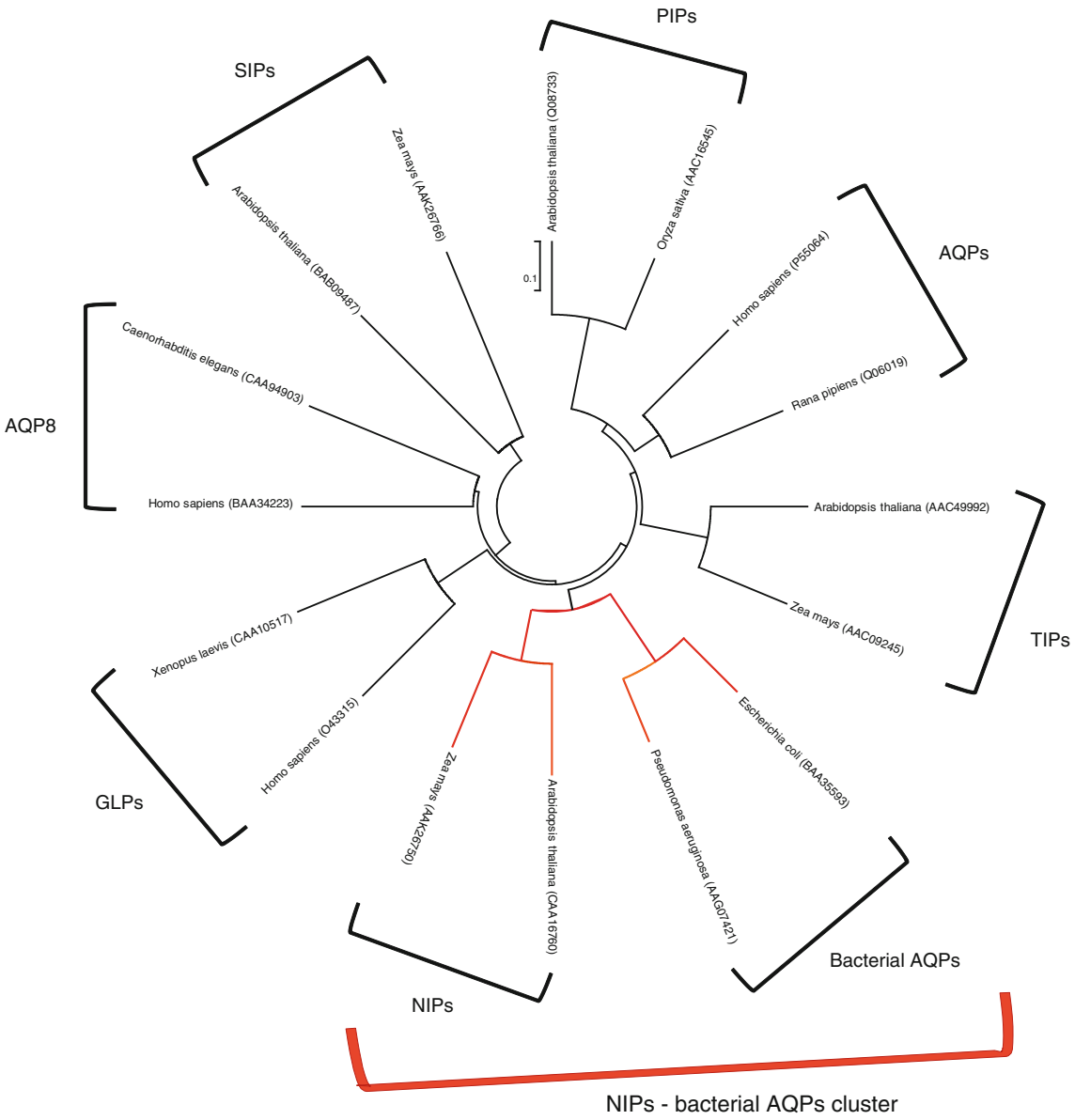

Fig. 4 Schematic representation of evolutionary relationships between eukaryotic and bacterial MIPs based on previously phylogenetic analyses (Zardoya and Villalba 2001)

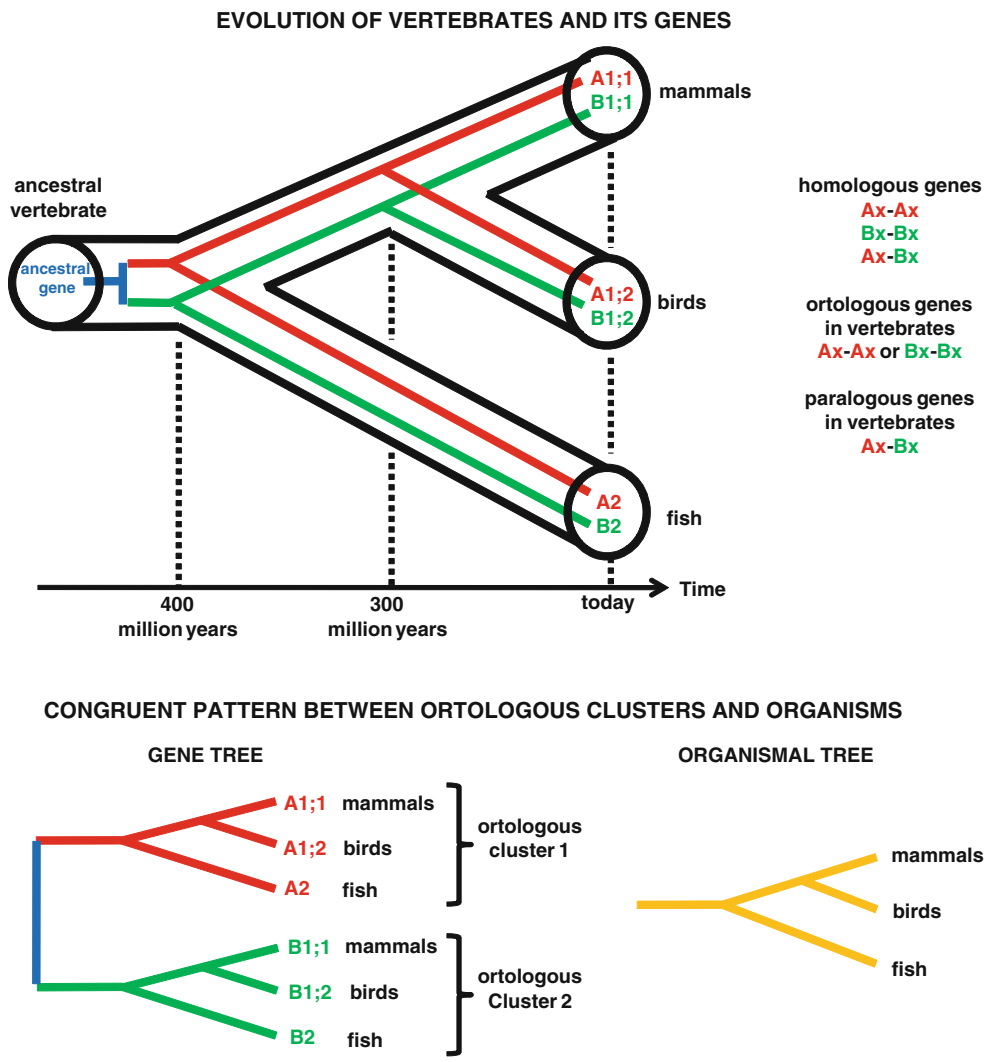

Fig. 5 Schematic representation of homologous, orthologous, and paralogous genes

imply that these genes present a selective advantage on the recipient organism (Phillips 2006). Therefore, works proposing the acquisition of bacterial genes in the eukaryotic host should explore the evolutionary advantage of this hypothetical transfer event. This exploration should consist of empirical evidence predicting functionality.

In conclusion, lateral transfer of AQPs between Bacteria and Eukarya could be an artifact. Thus, the inclusion of these distant or unrelated proteins within the same phylogenetic tree (e.g. used as an out-group) can produce a negative impact on the reconstruction of AQP evolution.

Essential Concepts for a Critical View of Evolutionary Studies of AQPs

The most rigorous way to represent the evolutionary history is through the construction of phylogenetic trees. The main assumption is that the genes analyzed within the tree are homologous genes (Fig. 5). On the other hand, because it is difficult to know which genes are actually homologous, some type of approximation to preselect homologous genes to be included in a phylogenetic tree is needed. The most common approach is to constrain the phylogenetic analysis to proteins that, as a whole, have more than 25 % of amino acid identity (Hughes et al. 2005). But, no sampling criterion (for example >25 % of amino acid identity) is sufficient to ensure that two genes are homologous. It is possible that two genes with high amino acid identity (e.g. 50 %) are non-homologous (by convergent evolution) or genes with low amino acid identity (e.g. 30 %) are homologous (by functional divergence). This type of criteria (e.g. amino acid identity) is used because there is no statistical elements to designate homology, and this occurs because systematics is a historical science with particular epistemological limitations (Cleland 2002). For example,

the information used for the evolutionary reconstruction arises from the observation, and not from the design and execution of experiments. This means that, in many cases, the results of phylogenetic analysis are accepted by the scientific community but with certain precautions.

The divergence of orthologous genes coincides with and is a product of the divergence of the species in which they are included (Fig. 5). Naturally, orthologous genes are evolutionarily more closely related and are therefore expected to have a similar biological and biochemical function. Robust methods for finding orthologs are based on the analysis of phylogenetic trees. For orthologous assignment, the trees have to be congruent (same topology) with the species tree (Fig. 5).

Different phylogenetic reconstruction methods, such as maximum parsimony, minimum evolution, and neighbor-joining, are available within freely accessible evolutionary software packages such as MEGA (Tamura et al. 2011). These methods compare the nucleotide or amino acid sequences using different parameters of genetic distance and parsimony but, in all cases, independent of the reconstruction method used, the number of possible phylogenetic trees increases exponentially with the number of sequences (Li 1997). Since a congruent pattern is only one topology, the probability that a congruent pattern occurs by chance is practically null. For example, when a phylogenetic tree has only 20 sequences, the probability to obtain a congruent pattern by chance (congruent pattern/possible trees) is $1/2 \times 10^{21} \approx 0$ (Fig. 6).

As described previously works on AQP evolution contained numerous inaccuracies in methods and/or interpretation of results, but this does not imply that by correcting these problems would be possible to obtain a consistent phylogeny, especially considering that AQPs constitute a broadly diversified family of genes.

The Novel Paradigm of AQP Evolution: Vertical Transfer

In this complex background, the phylogeny of eukaryotic AQPs has been recently re-evaluated by restricting the analysis to proteins with high amino acid identity (>25 %) and using sequences from well-characterized species of flowering plants and vertebrates (Soto et al. 2012). Since members of the subfamilies PIPs, TIPs, NIPs, and SIPs from flowering plants and AQPs plus aquaglyceroporins from vertebrates met the requirement of amino acid identity (>25 %), were included in the analysis. In contrast, the subfamilies XIPs, GIPs, and HIPs were not analyzed because they did not meet that requirement. As previously explained, this result does not imply that the XIPs, GIPs, and HIPs subfamilies are not homologous to the rest of eukaryotic AQPs, but shows that it was not possible to analyze their evolution according to the strict criterion of selection of proteins included within a same phylogenetic tree.

This strict criterion has shown congruence between AQPs (210 proteins) and organismal (13 species of eukaryotes) trees (Soto et al. 2012). The probability of finding a congruent pattern (vertical transfer) by chance was practically null. The advantage of this new perspective is that its congruence allowed defining clusters of

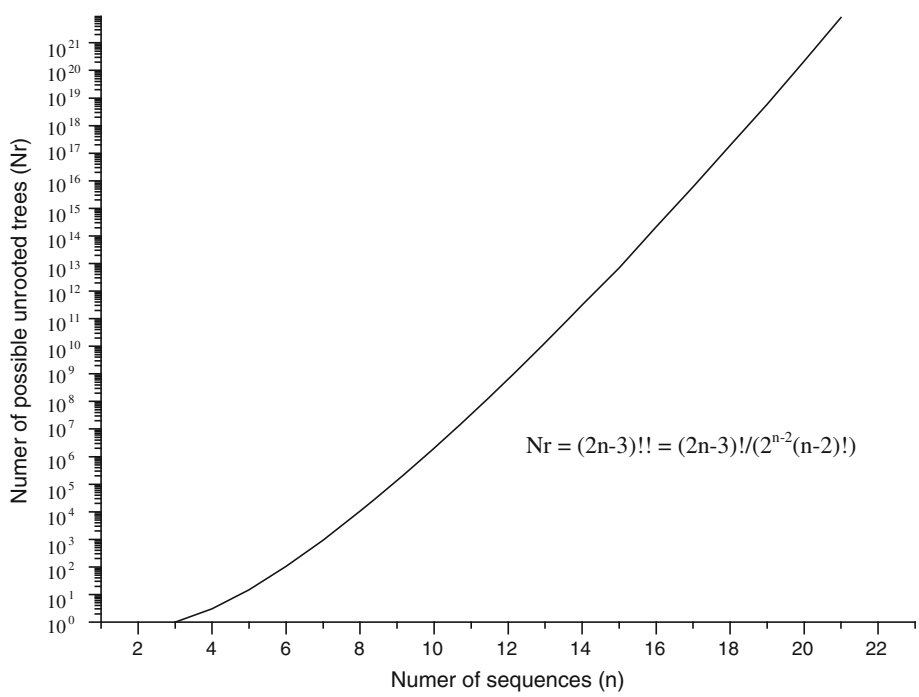

Fig. 6 Exponential function describing the relationship between number of possible unrooted phylogenetic trees and number of genes or proteins analyzed

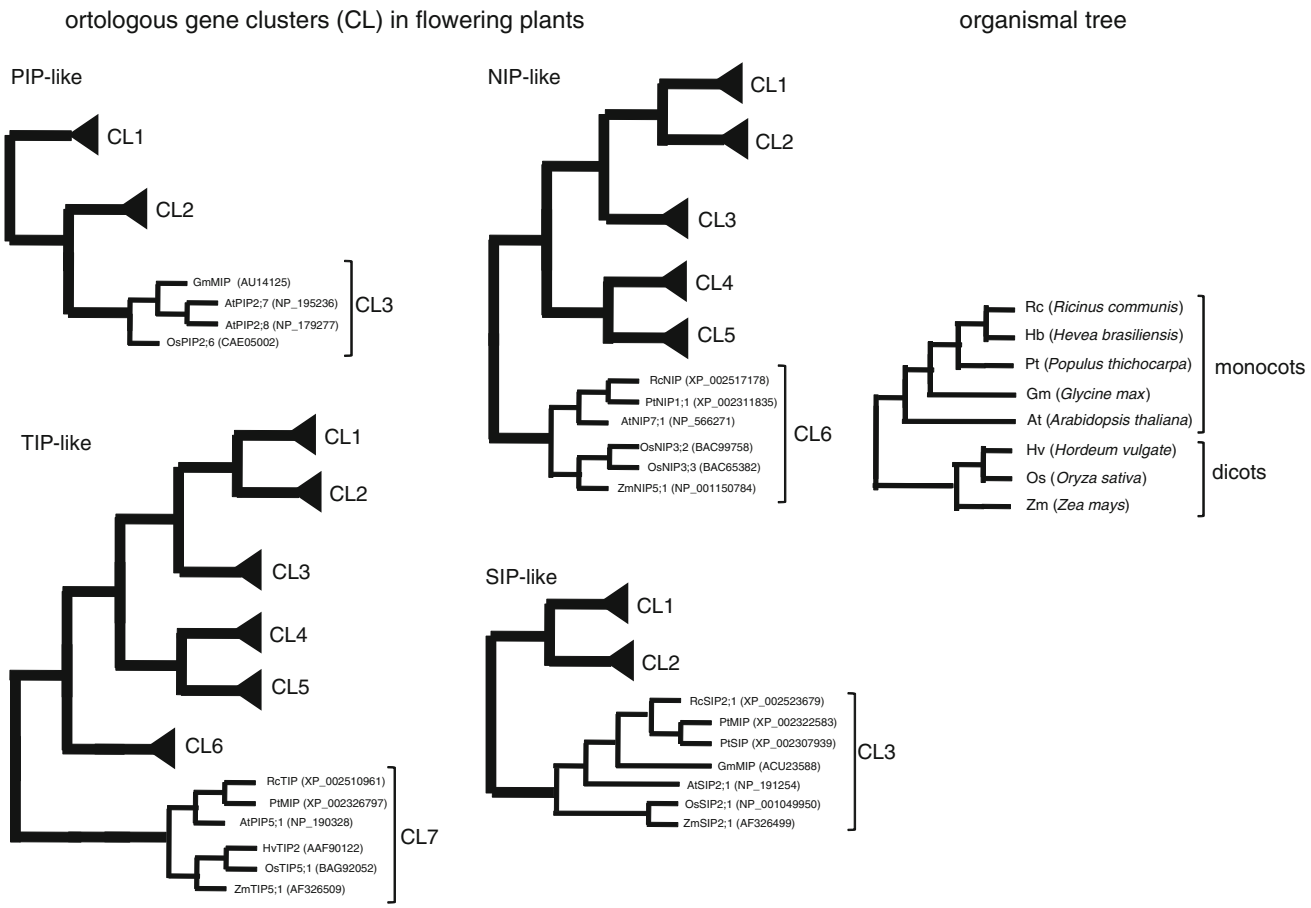

Fig. 7 Schematic representation of orthologous gene clusters in plants based on Soto et al. (2012)

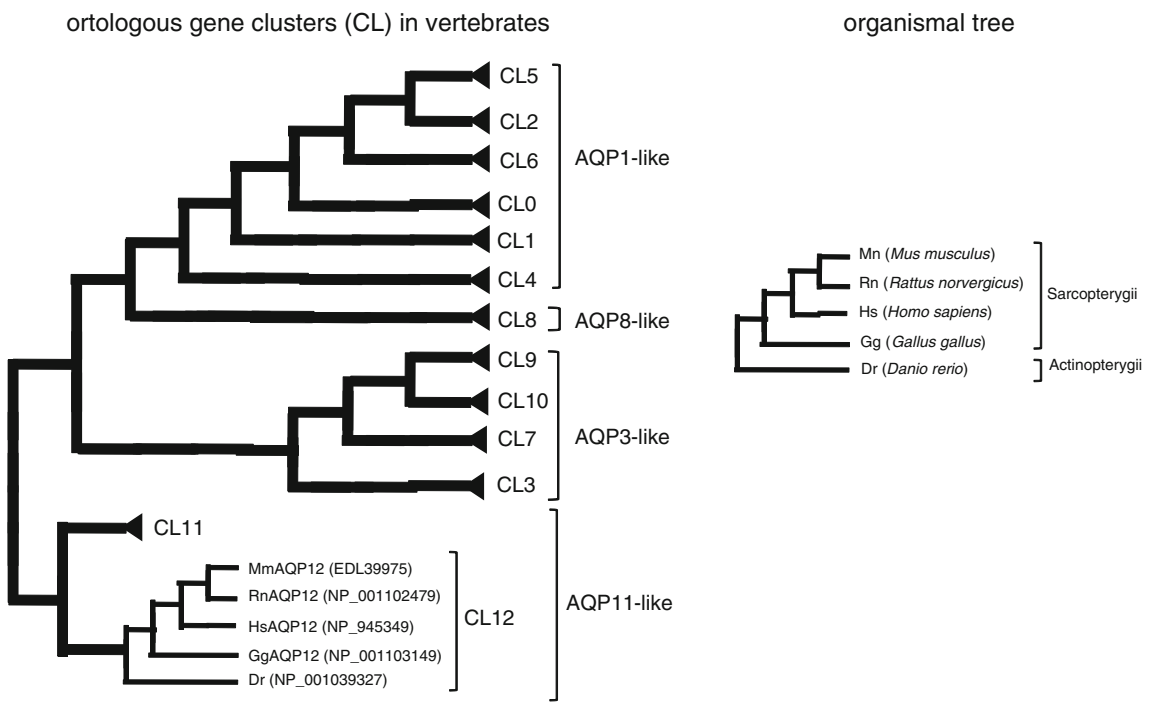

Fig. 8 Schematic representation of orthologous gene clusters in animals according to Soto et al. (2012)

orthologous genes for both flowering plants (19 clusters; Fig. 7) and vertebrates (13 clusters; Fig. 8). This leads to the description of specific conserved motifs for each orthologous cluster as a useful tool for automatic assignment of orthologs (Fig. 9) (Soto et al. 2012). The identification of conserved motifs in each subfamily and in each cluster of orthologous genes offers a framework for studying the possible functional implication of such motifs. This is a powerful tool, and the availability of new genomes of flowering plants and vertebrates could serve to define these motifs more precisely.

This paradigm offers the opportunity to establish a new classification of eukaryotic AQPs based on their evolutionary relationships. In this way, the current nomenclature differs significantly from a nomenclature based on the identification of orthologous genes. Although the nomenclature is important per se, it also has significant influence in the functional field. For example, given the current nomenclature, AtPIP2;6 of Arabidopsis can be considered the equivalent protein (ortholog) of OsPIP2;6 of *Oryza sativa*, suggesting an equivalent role in both plants. However, AtPIP2;7 and AtPIP2;8 are equally related to OsPIP2;6 expecting similar functions for all these proteins (Soto et al. 2012). Another advantage of having clusters of orthologous genes is the possibility of evaluating evolutionary constraints. The fact that PIPs have greater evolutionary constraints than TIPs, NIPs, and SIPs, support the prediction of greater functional constraints for PIPs (Soto et al. 2012). In turn, some of the TIPs and NIPs orthologous gene clusters also showed high evolutionary constraints, suggesting functional constraints.

This AQP phylogenetic framework for flowering plants and vertebrates can be used to predict a putative function of individual AQPs on the basis of orthologous genes from *A. thaliana* and *Homo sapiens*. However, the separate identification of clusters of orthologous genes in plants and vertebrates does not allow extrapolating the function between AQPs belonging to organisms of both kingdoms. As previously suggested by other phylogenetic analyses (Cerda and Finn 2010; Finn and Cerda 2011; Tingaud-Sequeira et al. 2010), our phylogenetic framework revealed that each subfamily of plant AQPs was related to a subfamily of animal AQP, thus showing a pattern of vertical transfer which predicts the presence of at least four families of AQPs in the ancestral eukaryote from which plants and vertebrates derived (Soto et al. 2012). We suggest that the four AQPs subfamilies described in animals (AQP1-, AQP8-, AQP3-, and AQP11-like) and plants (PIP-, TIP-, NIP-, and SIP-like) are derived from four ancestral AQPs subfamilies: A–D, respectively (Fig. 10). Thus, a pattern of vertical transfer in the evolution of AQPs of animals and plants at all levels, i.e., within (Figs. 7, 8) and between kingdoms (Fig. 10), was observed.

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                    PIP-like
            subfamily TGINPARS[FIL]
                  CL1 MEGK
                  CL2 M[AG]KX
                  CL3 MSKE

                    TIP-like
subfamily  [AG][AGS]MNPA[CRSV][ASV]F
     CL1   EFISTLIFVXAGX........NILAGGAFXGASMNPAVXF
     CL2   EFXSMXIFVFAGX........NILXGGAFDGASMNPAVSF
     CL3   EFIATLLFVFAGV........NILAAGPFSGGSMNPARSF
     CL4   EXXXTXXFVFAXE........NXLXGGPFXGAXMNPARXF
     CL5   EFXSTLXFVFAGV........NILXAGPFSGGSMNPARSF
     CL6   EXXXTFLFVFXGV........NXXAGXXXXGASMNPARSF
     CL7   EFXSTFXXVXXXV........XVLAAGXXXGXSMNPAXXF

                    NIP-like
subfamily  [AG]S[LM]NP[AGV]R[ST][ILV]
     CL1   EXXGTY......NVXXAXXXXXASMNPXRXX
     CL2                NVFVAGPXSGASMNPARSX
     CL3   EXXGTF......XSIXAGXXSGGSMNPARTL
     CL4                NILXXGPXXGXSMNPVRXL
     CL5                NIXIAGXXTXASMNPVRTL
     CL6                XXLXXGXXXGXSXNPARXL

                    SIP-like
subfamily  [LM]NPAXXXXWA
     CL1   [FY]NP[TC]
     CL2   [FY]NP[AS]
     CL3   [FY]NPL

Blue are hydrophobic residues: ACFILVWM
Fuchsia are large hydrophobic aminoacids: FIWLM
Green are polar aminoacids: NQST
Pink are negative residues: DE
Red are positive residues: KRHGPY
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Fig. 9 Motif characterization of plant aquaporins. Illustration of amino acid motifs for each subfamily (PIPs, TIPs, NIPs, and SIPs) and each orthologous cluster (CL). The letter *X* represents similar amino acids. *Blue*, *fuchsia*, *green*, *pink*, and *red letters* represents hydrophobic (ACFILVWM), large hydrophobic (FIWLM), polar (NQST), negative (DE) and positive (KRHGPY) amino acids, respectively (Color figure online)

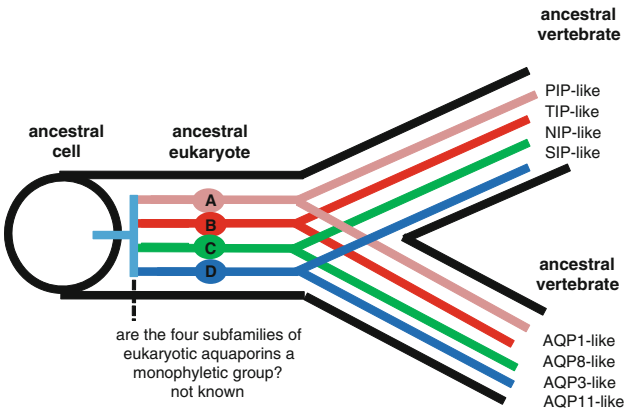

Fig. 10 Evolution of MIP superfamily in plants and animals. The hypothetical ancestral eukaryote has four AQP subfamilies (AD)

Functional Transfer of Eukaryotic AQPs Under the Novel Evolution Paradigm

This new framework allowed the comparison for each individual protein of the evolutionary patterns together with the described functions. We previously showed a correlation between the phylogenetic analysis and the functional information of 47 AQPs (Soto et al. 2012). Tables 1 and 2 describe the evolutionary location (subfamilies A–D), their current nomenclature, and molecules that they transport, the organisms that contain them for 106 AQPs that showed strong correlation between evolutionary and functional data.

AQPs of the subfamily A (PIP- and AQP1-like) transport water and also CO_2 as a common ancestral feature. Afterward, some AQPs of this subfamily acquired the capacity to transport other solutes, like glycerol, urea, and hydrogen peroxide. Some AQPs show poor water transport, however, they are permeable to anions as is the case of AQP6 (Table 2). Among PIPs, PIP2s transport water, whereas for PIP1s there is a great functional divergence: some reports show that PIP1s do not form functional homotetramers in plasma membrane (Fetter et al. 2004; Zelazny et al. 2007), or that it transports CO_2, or that it has low water transport capacity (Table 1). Several reports showed that PIP1 is localized in the plasma membrane only when it is expressed together with PIP2 (Bellati et al. 2010; Fetter et al. 2004); if it is expressed alone, then it is retained in the endoplasmic reticulum (Fetter et al. 2004; Jozefkowicz et al. 2013; Zelazny et al. 2007). In this context, the possibility of PIP1–2 hetero-oligomerization became a new way of regulation of water transport.

AQPs of the subfamily B (TIP- and AQP8-like) transport water and urea. However, many reports have demonstrated that several Arabidopsis TIPs transport not only urea but also ammonia (Table 1). In particular, as it is discussed later, *At*TIP5;1 is an urea transporter that belongs to the most divergent cluster of Arabidopsis TIPs (Soto et al. 2010), suggesting that this function could be ancestral. Furthermore, the ability to transport hydrogen peroxide could also be an ancestral feature, as several members of this subfamily share this capacity. Interestingly, new features appeared after functional divergence because some AQPs of this subfamily transport glycerol (Table 1).

AQPs of the subfamiliy C (NIP- and AQP3-like) are named aquaglyceroporins because almost all members of this group transport water and glycerol (Tables 1, 2). The transport of metalloids may be an ancestral feature as many plant NIPs and also AQP7 and -9 transport arsenite. AQP10 was found to be expressed in the small intestine and transport water, glycerol, and urea (Table 2). The fact that urea and boric acid transport was observed only in the animal and plant members of this subfamily, respectively, suggest that the transport of glycerol and urea might not be an ancestral feature but a putative event of functional divergence. Additionally, it was reported that many NIP-like AQPs transport other compounds, such as formamide and lactic acid (Table 1).

AQPs of the subfamily D (SIP- and AQP11-like) include the most recently identified AQPs. They are unusual because only the second NPA motif located in LE is conserved while the NPA motif in LB loop is modified, such as NPC in AQP11 and AtSIP1;2 or NPT in AQP12 and AtSIP1;1 (Soto et al. 2010). Also, their N-terminal tail is shorter than the N-terminal of the rest of the AQPs, a characteristic that has been assigned to explain their intracellular localization (Maeshima and Ishikawa 2008). Due to this, it has been difficult to establish their transport substrate specificity when expressed in Xenopus oocytes.

In summary, although AQPs from all subfamilies show different pattern of solute transport, all AQPs are permeable to water and so, this can be considered the ancestral feature shared by all the four subfamilies.

The potential of the proposed evolutionary framework in the prediction of functionality of plants and animals AQPs can be illustrated, with the example of the animal AQP8 and plant TIPs both members of the subfamily B (Fig. 7). Within TIPs, the cluster 7, includes AtTIP5;1 of *A. thaliana*, which is the most divergent TIP and therefore the most similar to the ancestral protein that gave rise to all TIPs (Fig. 7). Therefore, the evolutionary framework predicts that AQP8 would have a function equivalent to that of TIP5;1. It was described that TIP5;1 is an urea transporter located in pollen tube mitochondria when overexpressed in the pollen vegetative cell of *A. thaliana* (Soto et al. 2008, 2010). Based on these results, it has been proposed that AtTIP5;1 would be involved in the efflux of urea from mitochondria during the urea cycle (Soto et al. 2010). The urea cycle is well conserved in all living organisms (Goldraij and Polacco 2000; Kojima et al. 2006; Mobley et al. 1995; Pedrozo et al. 1996; Yu et al. 1997): urea is synthesized by a mitochondrial arginase and degraded by a cytosolic urease. Therefore, a mitochondrial transporter that would export urea from the mitochondrion into the cytosol has been predicted for many years discarding the possibility of a passive transport (Rodela et al. 2008). Due to AQP8 is the putative ortholog of AtTIP5;1 (Fig. 10), our phylogenetic framework predicts that AQP8 is involved in the urea cycle in vertebrates as it was suggested previously (Calamita et al. 2006, 2007; Holm et al. 2005; Liu et al. 2006; Soria et al. 2013).

Table 1 Functional characteristics of plants AQPs disaggregated by subfamily

	H_2O	Gly	NH_4	Urea	B	As	H_2O_2	CO_2	I	O	References
SF A											
AtPIP1;1	+						−				Hooijmaijers et al. (2012), Kammerloher et al. (1994)
AtPIP1;2	+						−	+			Heckwolf et al. (2011), Hooijmaijers et al. (2012), Kammerloher et al. (1994), Tournaire-Roux et al. (2003)
AtPIP1;3	+						−				Hooijmaijers et al. (2012), Kammerloher et al. (1994)
AtPIP1;4							−				Hooijmaijers et al. (2012)
AtPIP1;5							−				Hooijmaijers et al. (2012)
NtAQP1	+	+						+	−		Biela et al. (1999), Otto et al. (2010), Uehlein et al. (2003)
ZmPIP1;2							−				Bienert and Chaumont (2013)
ZmPIP1;5	+			+							Gaspar (2003)
SsAQP1	−		+								Moshelion (2002)
AtPIP2;1	+						+/−				Bienert et al. (2007), Dynowski et al. (2008), Hooijmaijers et al. (2012), Kammerloher et al. (1994)
AtPIP2;2	+						+				Hooijmaijers et al. (2012), Kammerloher et al. (1994), Tournaire-Roux et al. (2003)
AtPIP2;4	+						+				Dynowski et al. (2008), Hooijmaijers et al. (2012)
AtPIP2;3	+						−				Daniels et al. (1994), Hooijmaijers et al. (2012)
AtPIP2;5	+						+				Hooijmaijers et al. (2012)
AtPIP2;6							−				Hooijmaijers et al. (2012)
AtPIP2;7							+				Hooijmaijers et al. (2012)
AtPIP2;8							−				Hooijmaijers et al. (2012)
ZmPIP2;1	+										Fetter et al. (2004)
ZmPIP2;4											Fetter et al. (2004)
ZmPIP2;5							+				Bienert and Chaumont (2013), Chaumont et al. (2001), Fetter et al. (2004)
McPIP2;1	+	−		−							Amezcua-Romero et al. (2010)
SoPIP2;1	+										Johansson et al. (1998)
SsAQP2	+		−								Moshelion (2002)
OsPIP2;1	+										Matsumoto et al. (2009), Sakurai et al. (2008)
OsPIP2;2	+										Matsumoto et al. (2009), Sakurai et al. (2008)
OsPIP2;3	+										Matsumoto et al. (2009), Mosa et al. (2012), Sakurai et al. (2008)
OsPIP2;4	+					+					Matsumoto et al. (2009), Sakurai et al. (2008)
OsPIP2;5	+										Matsumoto et al. (2009), Sakurai et al. (2008)
OsPIP2;6	+					+					Matsumoto et al. (2009), Mosa et al. (2012)
OsPIP2;7	+					+					Matsumoto et al. (2009), Mosa et al. (2012)
OsPIP2;8	+										Matsumoto et al. (2009)
NtPIP2;1	+							−			Bots et al. (2005), Otto et al. (2010)
SF B											
AtTIP1;1	+	−		+		+	+				Bienert et al. (2007), Klebl et al. (2003), Liu et al. (2003), Maurel et al. (1993)
TgTIP1;1	+		+	+			+				Azad et al. (2008, 2012)
AtTIP1;2				+			+				Bienert et al. (2007), Liu et al. (2003)
OsTIP1;2	+	+									Li et al. (2008), Sakurai et al. (2008)
TgTIP1;2	+		+	+			+				Azad et al. (2008, 2012)
AtTIP1;3	+	−		+	−						Soto et al. (2008)
AtTIP2;1	+		+	+							Klebl et al. (2003), Liu et al. (2003), Loque et al. (2005), Maurel et al. (1993)
OsTIP2;1	+	+									Li et al. (2008)
TaTIP2;1	+		+	+						+	Holm et al. (2005), Jahn et al. (2004)
TaTIP2;2	+		+								Bertl and Kaldenhoff (2007)

Table 1 continued

	H_2O	Gly	NH_4	Urea	B	As	H_2O_2	CO_2	I	O	References
AtTIP2;3			+	+			+				Dynowski et al. (2008), Loque et al. (2005)
AtTIP3;1	+										Eckert et al. (1999)
OsTIP3;2	–	+									Li et al. (2008)
OsTIP4;1	+	+									Li et al. (2008)
AtTIP5;1	+	–		+	–						Soto et al. (2008)
NtTIPa	+	+		+							Gerbeau et al. (1999)
SF C											
AtNIP1;1	+	+				+	–			+	Dynowski et al. (2008), Kamiya and Fujiwara (2009), Kamiya et al. (2009), Weig and Jakob (2000)
AtNIP1;2		+					+				Dynowski et al. (2008), Weig and Jakob (2000)
AtNIP2;1	+	+					+			+	Choi and Roberts (2007), Mizutani et al. (2006)
AtNIP4;1	+										Soto et al. (2008)
AtNIP5;1	+	+			+					+	Bienert et al. (2008), Mitani-Ueno et al. (2011), Takano et al. (2006)
AtNIP6;1	–	+		+	+	+				+	Bienert et al. (2008), Tanaka et al. (2008), Wallace and Roberts (2005)
AtNIP7;1	+	+		+	+	+				+	Bienert et al. (2008), Li et al. (2011)
CpNIP1				+							Klebl et al. (2003), Liu et al. (2003)
GmNOD26	+	+	+	–	–				–	+	Dean et al. (1999), Hwang et al. (2010), Rivers et al. (1997), Schnurbusch et al. (2010), Wallace et al. (2012)
HvNIP2;1	+			–	+	+				+	Ligaba et al. (2011), Schnurbusch et al. (2010)
LjLIMP2	+	+								+	Guenther and Roberts (2000)
LjNIP5;1						+				+	Bienert et al. (2008)
LjNIP6;1						+				+	Bienert et al. (2008)
OsNIP1;1						+					Ma et al. (2008)
OsNIP2;1	+			+	+	+				+	Mitani-Ueno et al. (2011), Mitani et al. (2008)
OsNIP2;2	+				–	+				+	Bienert et al. (2008), Ma et al. (2006, 2008), Mitani-Ueno et al. (2011), Mitani et al. (2008)
OsNIP3;1						+					Ma et al. (2008)
OsNIP3;2					+					+	Bienert et al. (2008)
PsNIP1;1	+	+									Schuurmans et al. (2003)
PtNIP1;1	+	+									Ciavatta et al. (2001)
TaNIP2;1										+	Montpetit et al. (2012)
ZmNIP2;1				+						+	Gu et al. (2012), Mitani et al. (2009)
ZmNIP2;2										+	Mitani et al. (2009)
ZmNIP2;4				+							Gu et al. (2012)
SF D											
AtSIP1;1	+										Ishikawa et al. (2005)
AtSIP1;2	+										Ishikawa et al. (2005)
AtSIP2;1	–										Ishikawa et al. (2005)

+, presence; −, absence; +/−, controversy between authors. Plants: At, *Arabidopsis thaliana*; Cp, *Cucurbita pepo*; Gm, *Glycine max*; Hv, *Hordeum vulgae*; Lj, *Lotus japonicus*; Mc, *Mesembryanthemum crystallinum*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Ps, *Polygonum sibiricum*; Pt, *Pinus taeda*; So, *Spinacia oleracea*; Ss, *Samanea saman*; Ta, *Triticum aestivum*; Tg, *Tulipa gesneriana*; Zm, *Zea mays*

SF subfamily, *Gly* glycerol, *B* boric acid, *As* arsenic, *I* ions, *O* other compounds (e.g., formamide and lactic acid)

Table 2 Functional characteristics of vertebrates AQPs disaggregated by subfamily

	H_2O	Gly	NH_4	Urea	B	As	H_2O_2	CO_2	I	O	References
SF A											
BtAQP0	+							+			Mulders et al. (1995), Yang and Verkman (1997), Zampighi et al. (1985)
HsAQP0	+								+		Chandy et al. (1997)
DrAQP0	+										Froger et al. (2010)
SaAQP0a	+										Chauvigne et al. (2013)
RnAQP1	+	+		−			+		+	−	Abrami et al. (1995), Li et al. (2011), Ma et al. (1993), Marinelli et al. (1997)
DrAQP1	+	−		−							Tingaud-Sequeira et al. (2010)
HsAQP1	+	+	+					+	+		Abrami et al. (1995), Endeward et al. (2006), Preston et al. (1992), Anthony et al. (2000), Herrera et al. (2006), Musa-Aziz et al. (2009), Nakhoul et al. (1998), Prasad et al. (1998)
SaAQP1aa/ab	+										Chauvigne et al. (2013)
HsAQP2	+	+	−	−				−			Abrami et al. (1995), Fushimi et al. (1993), Geyer et al. (2013), Meinild (1998), Yang and Verkman (1997)
DrAQP4	+										Tingaud-Sequeira et al. (2010)
RnAQP4	+	+/−	−	−				+			Fenton et al. (2010), Geyer et al. (2013), Jung et al. (1994), Meinild (1998), Musa-Aziz et al. (2009), Yang and Verkman (1997)
RnAQP4	+	−	−	−				−			Fenton et al. (2010), Geyer et al. (2013)
RnAQP5	+										Raina et al. (1995), Yang and Verkman (1997)
HsAQP5	+	−	−					+			Meinild (1998), Musa-Aziz et al. (2009)
HsAQP6	+	+		+					+	+	Holm et al. (2004), Liu et al. (2006), Ma et al. (1996)
RnAQP6	−		+					+	+	+	Geyer et al. (2013), Hazama et al. (2002), Ikeda et al. (2002), Liu et al. (2006), Yasui et al. (1999)
SF B											
HsAQP8	+	−	+	−			+	−		+	Bienert et al. (2007), Geyer et al. (2013), Jahn et al. (2004), Liu et al. (2006)
RnAQP8	+	+/−	+	+/−						+	Holm et al. (2005), Ishibashi et al. (1997), Koyama et al. (1997), Liu et al. (2006)
DrAQP8	+	−		+							Tingaud-Sequeira et al. (2010)
SaAQP8b	+			+							Chauvigne et al. (2013)
SF C											
RnAQP3	+	+	+	+			+	−	−	+	Echevarria et al. (1994), Geyer et al. (2013), Hara-Chikuma et al. (2012), Holm et al. (2005), Ishibashi et al. (1994), Meinild (1998), Yang and Verkman (1997), Zeuthen et al. (1997)
DrAQP3	+	+		+						+	Chauvigne et al. (2011), Tingaud-Sequeira et al. (2010)
HsAQP3	+	+				−				+	Chauvigne et al. (2011), Liu et al. (2004)
HsAQP7	+		+			+		−			Geyer et al. (2013), Liu et al. (2004)
MmAQP7	+					+					Liu et al. (2002)
RnAQP7	+	+		+							Ishibashi et al. (1997), Kishida et al. (2000)
DrAQP7	+	+		+						+	Chauvigne et al. (2011), Tingaud-Sequeira et al. (2010)
SaAQP7	+	+		+							Chauvigne et al. (2013)
RnAQP9	+	+	+	+		+		+		+	Geyer et al. (2013), Liu et al. (2002), Tsukaguchi et al. (1999)
DrAQP9	+	+		+						+	Chauvigne et al. (2011), Tingaud-Sequeira et al. (2010)
HsAQP9						+					Liu et al. (2004), McDermott et al. (2010)
SaAQP9b	+	+		+							Chauvigne et al. (2013)
HsAQP10	+	+		+		−					Hatakeyama et al. (2001), Ishibashi et al. (2002), Liu et al. (2004)
DrAQP10	+	+		+							Tingaud-Sequeira et al. (2010)
SaAQP10b	+	+		+							Chauvigne et al. (2013)

Table 2 continued

	H_2O	Gly	NH_4	Urea	B	As	H_2O_2	CO_2	I	O	References
SF D											
MmAQP11	+										Yakata et al. (2007, 2011)
HsAQP11	+										Ikeda et al. (2011)

+, presence; −, absence; +/−, controversy between authors. Animals: Ac, *Anomala cuprea*; Bg, *Blattella germanica*; Bt, *Bos taurus*; Ba, *Bemisia tabaci*; Dr, *Danio rerio*; Gg, *Gallus gallus*; Hc, *Hyla chrysoscelis*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Sa, *Sparus aurata*

SF subfamily, *Gly* glycerol, *B* boric acid, *As* arsenic, *I* ions, *O* other compounds (e.g. formamide and lactic acid)

Conclusions and Prospects

The AQP vertical transfer hypothesis makes predictions that are testable and refutable, as we have demonstrated throughout the text so far. This new paradigm of evolution of plant and animal AQPs offers a novel framework to integrate functional information. It allows two distant groups, the plant and animal AQPs, to work together and support each other, especially in understanding water and solute transport. Furthermore, the availability of new clusters of orthologous genes and specific motifs associated with such clusters offers a starting point for an in-depth understanding of the consensuses and tridimensional structures associated with the functional diversity of AQPs in the specificity of transport, interaction among AQPs and with other molecules, including regulation and subcellular localization. In this context, it is expected that the new consistent evolutionary framework of eukaryotic AQPs increases the ability to properly predict biochemical and biological functions of AQPs. Functional information of individual AQPs by empirical studies is expected to grow and more sequenced genomes of plants and animals are expected to be available, positively influencing the definition and precision of motifs and functions of each cluster of orthologous genes and each AQP subfamily. However, it is necessary to point out that the extrapolation of functionality has the intrinsic restriction of the biochemical and biological diversification processes. For example, the extrapolation of the biochemical and biological functions of the ancestor of flowering plants is not always possible because each of the clusters of orthologous genes in monocotyledonous and dicotyledonous plants evolved independently, incorporating and eliminating various functions related to AQPs. Similarly, although certain features of cell functionality may have been conserved in all vertebrates, it is not expected that the AQPs of fishes and mammals have exactly the same biochemical and biological function, especially when they are exposed to different environments that would potentiate their functional divergence. Finally, the future of the experimental study of AQPs seems to have evolutionary guidance, which, despite its limitations constitutes a solid road toward a better understanding of AQPs.

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