Decoding AQPX Channels: Understanding selective transport and localization of *Trypanosoma cruzi* aquaporins

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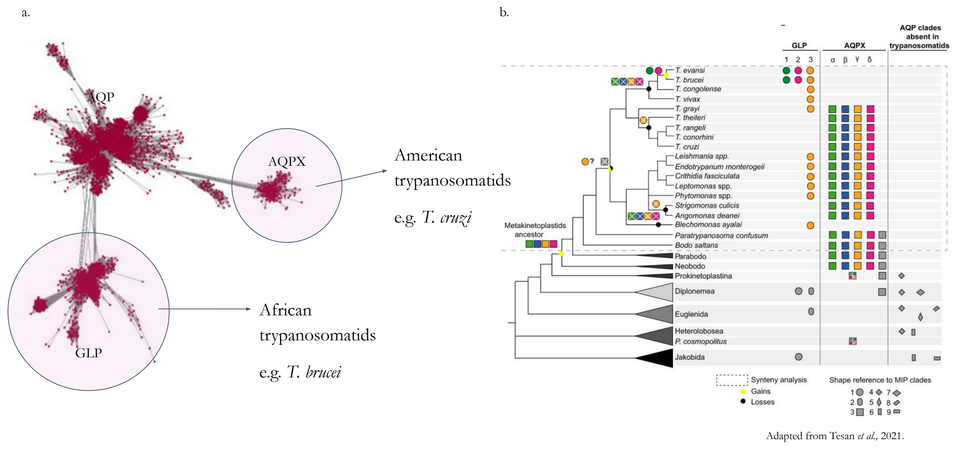
AQPX subfamily – A closer look to *T. cruzi* aquaporins

Aquaporins are channels that can transport water but also other kind of solutes, such as glycerol or urea.

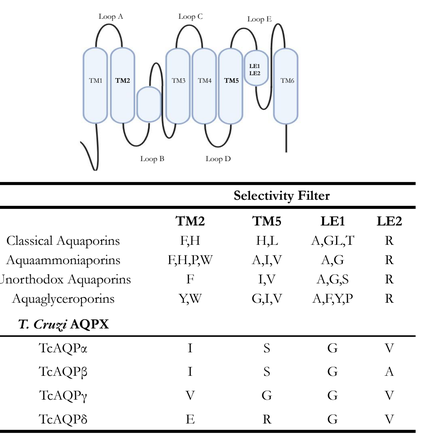
There is an interest in exploiting aquaporins as cellular entry pathways for molecules with pharmacological functions, highlighting their potential in parasites as therapeutic targets.

Aquaporins form tetramers and each protomer has six transmembrane (TM) helices and five loops (A,B,C,D,E). Loops B and E re-enter the membrane, forming a seventh pseudo-transmembrane helix.



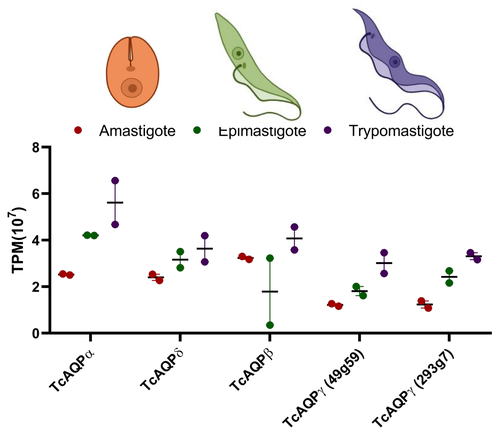
Adapted from Tesan *et al.*, 2021.

Sequence Similarity Network of Cluster 1 MIP superfamily. a. Edge length is a measure of the relative dissimilarity of each pair of sequences. The MIP superfamily can be organized in clusters, the main cluster (cluster 1) can be divided into three subclusters: AQPs, GLPs, and AQPXs[1]. Kinetoplastids MIPs are distributed between GLP and AQPX subclusters. b. There is an asymmetric distribution of MIP repertoire among kinetoplastids. Interestingly, AQPX are only found in american trypanosomes and in GLP subcluster, only a small number of african trypanosomatids aquaporins are found. *T. cruzi* has four AQPs and all of them are AQPX (TcAQPα, TcAQPβ, TcAQPγ, TcAQPδ). Only TcAQP1/α was studied for its localization and water transport[2].



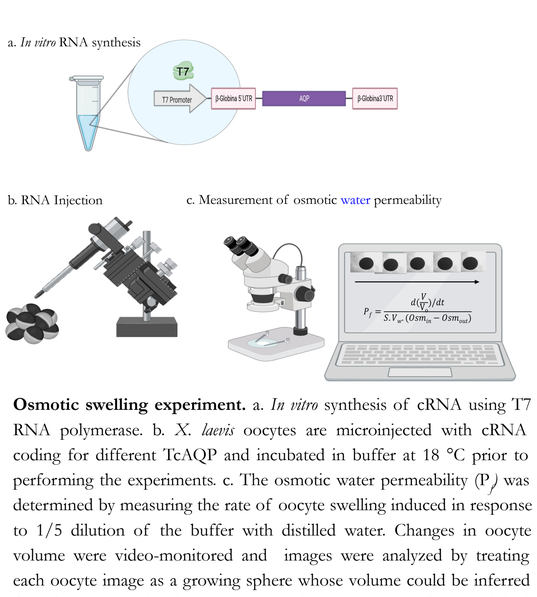
	Selectivity Filter			
	TM2	**TM5**	**LE1**	**LE2**
Classical Aquaporins	F,H	H,L	A,GL,T	R
Aquaammoniaporins	F,H,P,W	A,I,V	A,G	R
Unorthodox Aquaporins	F	I,V	A,G,S	R
Aquaglyceroporins	Y,W	G,I,V	A,F,Y,P	R
***T. Cruzi* AQPX**				
TcAQPα	I	S	G	V
TcAQPβ	I	S	G	A
TcAQPγ	V	G	G	V
TcAQPδ	E	R	G	V

AQPXs present a Selectivity Filter (SF) different from SF in other studied MIPs. SF residues are key structural elements that control transport MIP channels. They are present in TM2, TM5 and loop E (LE1 and LE2). Compared with the SF of classical water channels, AQPXs SF do not keep the R in Loop E (LE2), nor the aromatic amino acids in TM2, having, instead, aliphatic residues. That may result to more hydrophobic and broader filters.

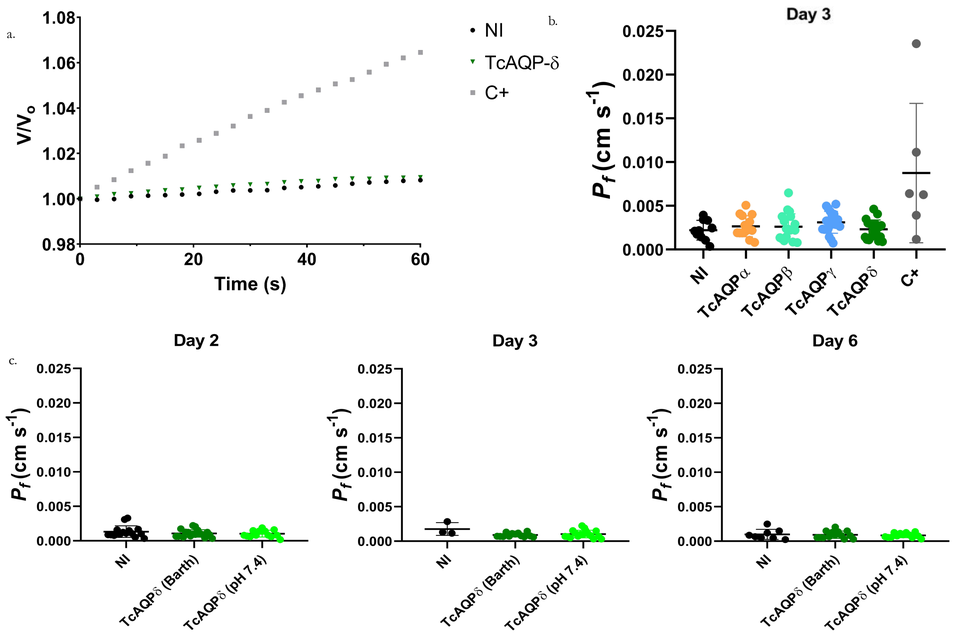


***T. cruzi* aquaporins (Dm28C strain) are expressed across the three stages of development.** Based on RNAseq analysis recently performed[3], the transcripts TcAQP genes were significantly detected. The expression of each transcript was quantified in transcript per million (TPM) units. RNA levels along the *T. cruzi* development stages (Amastigote, Epimastigote, Trypomastigote), were classified into three groups based on their relative abundance: high, medium, and low expression genes. TcAQPα epimastigote and trypomastigote show a relatively high level of expression while the others a medium level.

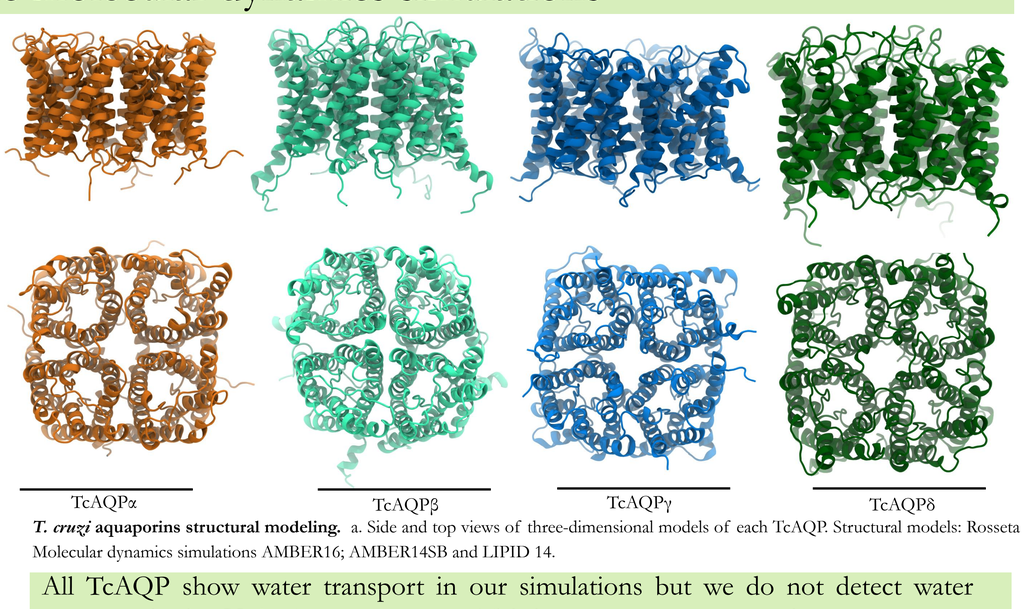
TcAQP water transport - Heterologous expression in *X. laevis* oocytes and atomistic molecular dynamics simulations



Osmotic swelling experiment. a. *In vitro* synthesis of cRNA using T7 RNA polymerase. b. *X. laevis* oocytes are microinjected with cRNA coding for different TcAQP and incubated in buffer at 18 °C prior to performing the experiments. c. The osmotic water permeability (P_f) was determined by measuring the rate of oocyte swelling induced in response to 1/5 dilution of the buffer with distilled water. Changes in oocyte volume were video-monitored and images were analyzed by treating each oocyte image as a growing sphere whose volume could be inferred from its cross-sectional area. P_f: osmotic water permeability, V_o: initial oocyte volume (9×10^{-4} cm^3), V/V_o: relative volume, S: surface area of the oocyte (0.045 cm^2), Vw: water molar volume (18 $cm^3 \cdot mol^{-1}$), $Osm_{in} - Osm_{out}$ the osmotic driving force.



Water transport assay. a. Time course evolution of the relative volume change in a single oocyte injected with cRNA b. The four TcAQP in ND buffer c. Different times of expression and two different buffers.



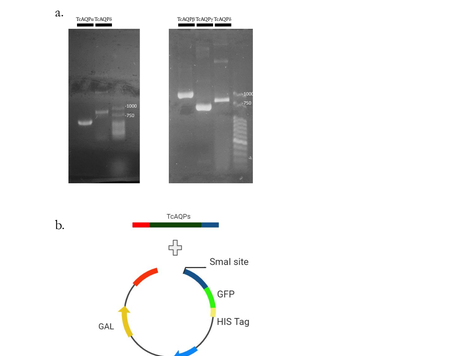
***T. cruzi* aquaporins structural modeling.** a. Side and top views of three-dimensional models of each TcAQP. Structural models: Rosseta Molecular dynamics simulations AMBER16; AMBER14SB and LIPID 14.

All TcAQP show water transport in our simulations but we do not detect water transport in the *X. laevis* oocyte system. This leaves some open questions:

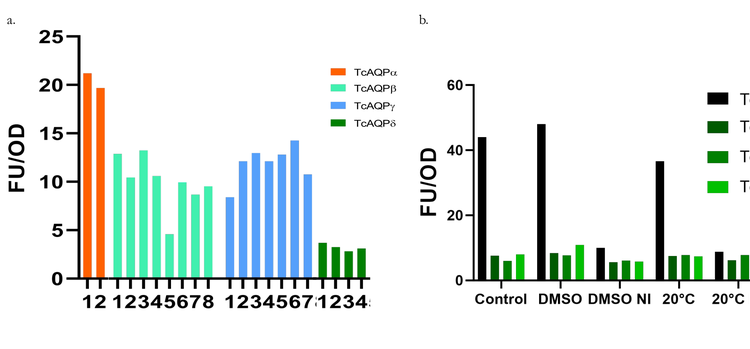
I. Are *T. cruzi* aquaporins water channels?

II. Are *T. cruzi* aquaporins reaching oocyte´s plasma membrane?

TcAQP protein expression - Heterologous expression in *S. cerevisiae* and cellular localization

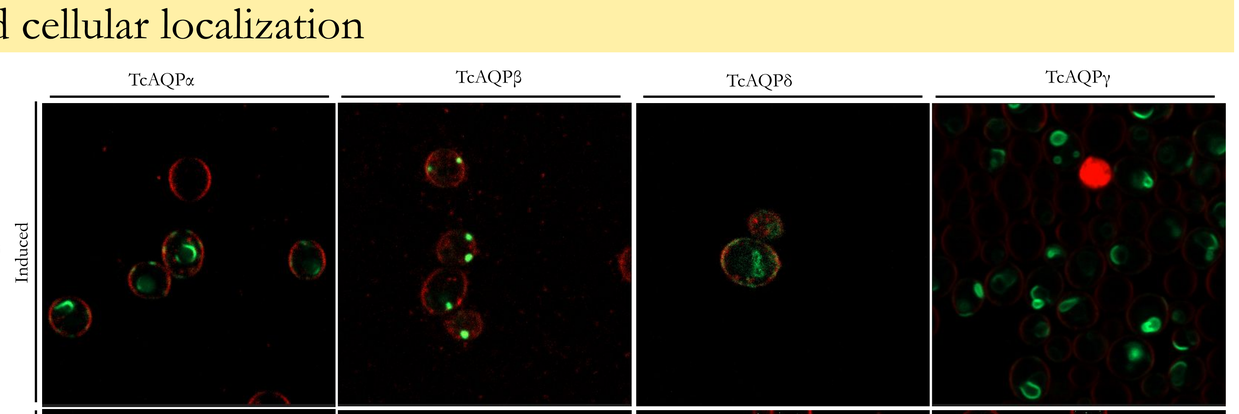


Cloning through homologous recombination in *S. cerevisiae*. a. TcAQP genes were obtained by PCR amplification. The primers used to amplify the genes contains overhangs (35 bp) complementing the homologous recombination arms (blue and red) in this expression vector. b. Schematic representation of the implemented cloning strategy. The vector contains a C-terminal octa-His tag sequence, a TEV protease site (GFP-8His), upstream of GFP-8His sequence a SmaI site, GAL1 promoter sequence and URA selection marker. c. Colonies harbouring the expression vector were confirmed by colony PCR. *pep4* deletion yeast strain FGY217 was used.



Screening of membrane protein expression and optimization. a. UF/OD signal from transformed yeast clones. b. TcAQPδ protein expression optimization Control: standard growth condition (T°:29 °C; YPD buffer, 0.1% glucose, 2% galactose for induction) DMSO: 2,5% v/v DMSO added at induction time.(T°:29 °C; YPD buffer, 0.1% glucose, 2% galactose for induction). 20°C: Different temperature of incubation. (T°:20 °C; YPD buffer, 0.1% glucose, 2% galactose for induction). NI: Not induced. c. In-gel fluorescence of membranes from selected clones. Membranes were evaluated for protein expression by In-gel fluorescence. C+: *Mt*PIP2;3 aquaporin membranes. CN: yeast membrane without a expression plasmid. GFP: purified GFP protein.

Clones harboring the inserted gene are cultured in a selective medium. Assessment of culture growth is performed via spectrophotometry measuring optical density at 600nm following a 16-hour inoculation period. After a culture dilution an induction with 2%P/V galactose takes place when the culture's optical density reaches an OD of 0.6. GFP fluorescence evaluation for each culture is conducted 18 hours post-induction. After selecting the colonies that exhibit the highest fluorescence unitis/optical density (FU/OD), membranes can be harvested through disruption and differential fractionation.



TcAQP-GFP confocal laser scanning microscopy. Induced clones: adding 2% P/V galactose. Not Induced: adding 2% P/V glucose. Confocal microscopy images was obtained with live cells (OD: 0.5). FM-64 (0,16mM, red) was employed as a plasma membrane marker .

The four TcAQPs were expressed in *S. cerevisiae*. Only 3 (TcAQPα , TcAQPβ, TcAQPγ) out of the 4 genes exhibited adequate protein expression when the fluorescence screening was performed. A very low expression level was found for TcAQPδ. Membranes extracted from yeast cultures of each clone displayed a banding pattern consistent with the molecular weight of all four TcAQPs. TcAQPα, TcAQPβ and TcAQPγ display an intracellular localization pattern in yeast. Notwithstanding, even TcAQPδ exhibits very low expression, appears to be expressed in both, intracellular and plasma membrane on the yeats.

Conclusion and Perspectives

T. cruzi aquaporins are AQPX, they exhibit a selectivity filter distinct from classical aquaporins, and it raises the question about their permeation properties.

Initial experiments involved assessing water transport in the *X. laevis* heterologous expression system, where all four *T. cruzi* AQPs were examined. Surprisingly, none displayed significant differences in osmotic water permeability compared to the control (NI). TcAQPα and TcAQPδ were evaluated under multiple conditions, yielding consistent results. Nevertheless, molecular dynamics simulations suggest the capability of these AQPs to transport water.

A secondary heterologous expression system in *S. cerevisiae* was used, resulting in a successful cloning and expression of three AQPs. TcAQPδ, however, remained challenging to efficiently express.

Confocal microscopy revealed intracellular localization in three AQPs (TcAQPα , TcAQPβ, TcAQPγ) and both intracellular and membrane localization for TcAQPδ.

Further investigations will focus on confirming membrane localization in *X. laevis* and *in vivo*, assessing transport profiles in different expression systems and completing molecular dynamics studies.

References

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3. Díaz-Viraqué, F., Chiribao, M. L., Libisch, G., & Robello, C. (2023). 3D genome organization drives gene expression in trypanosomes. Cold Spring Harbor Laboratory. https://doi.org/10.1101/2023.04.01.535209