H_2O_2 and H_2O transport by *Medicago truncatula* PIP2 aquaporins

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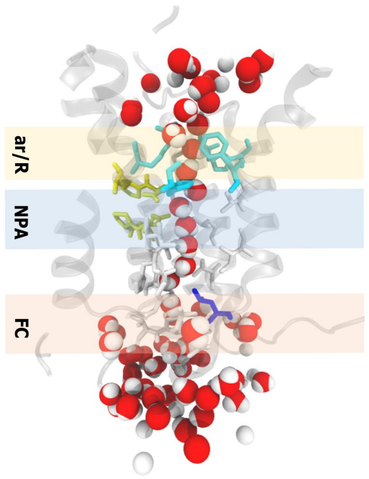
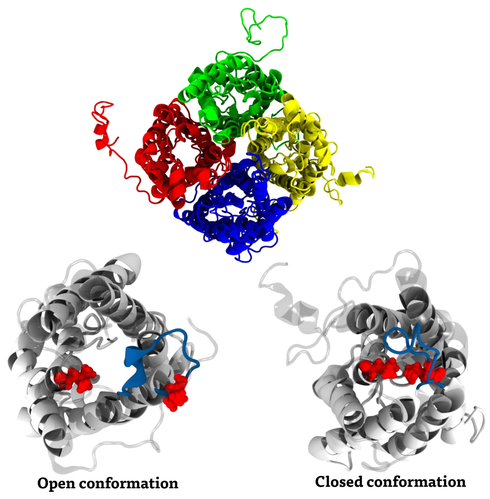
Background

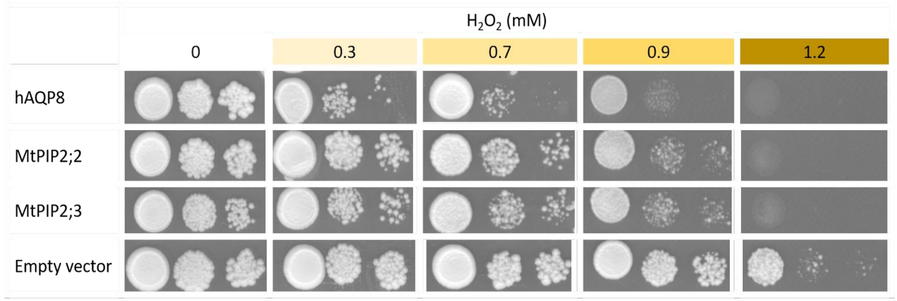
- Aquaporin (AQP) channels are permeable to water. Some AQP can also transport other low molecular mass solutes.
- PIP is a subfamily of the AQP family presented in plants. These channels transport H_2O and H_2O_2.
- The transport mechanism of H_2O_2 remain unknown.



Aquaporin pore constrictions.
Lateral view of a protomer with water molecules, pore constrictions are highlighted. Ar/r: Aromatic/Arginine selectivity filter. NPA: Asparagine-Proline-Alanine proton exclusion motif. FC: Cytoplasmatic filter.

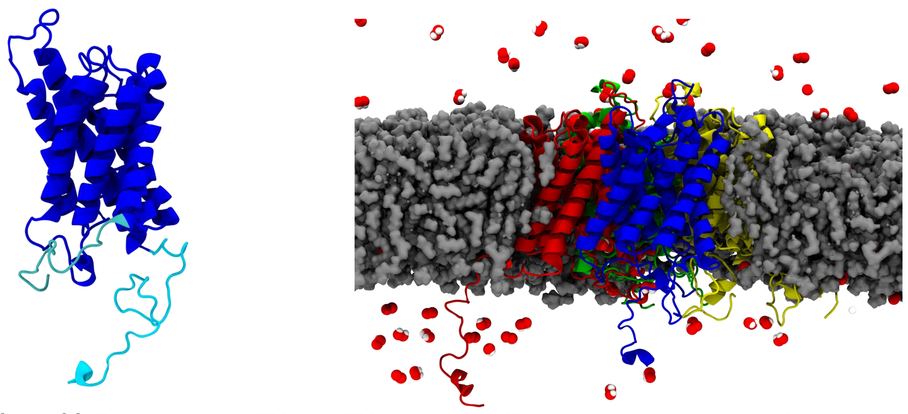


PIP structure. Upper panel: tetrameric conformation of PIP channels, colored by each chain (A: blue, B: red, C: yellow, D: green). Lower panel: Downside view of protomers showing the conformational changes of loopD (blue) in open and closed states. In red: leucine residues used as open/closed channel reference.

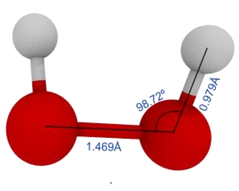


Yeast toxicit assay. Our group described two PIP of *Medicago truncatula* (*Mt*PIP2;2 and *Mt*PIP2;3) as H_2O_2 transporters. *Vitali et al.* In preparation. Thanks to Jozefkowicz, C. and Biernet, G.

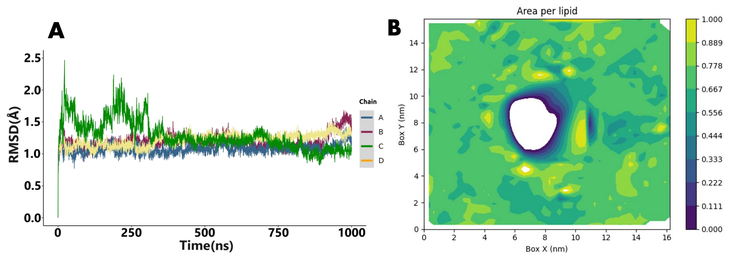
H_2O_2 Molecular dynamics systems



Complete models. Homology models of *Mt*PIP2;2 and *Mt*PIP2;3 with the addition of N and C terminals modeled separately (left) . Modelled tetramers were inserted in a POPC bilayer (right), 0.15mM NaCl, with explicit solvent box with TIP3 water model (ions and water not shown) and different concentrations of H_2O_2.



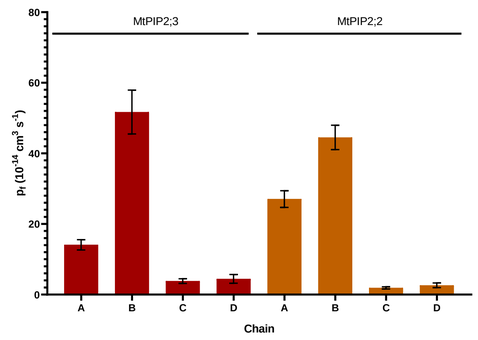
H_2O_2 parameters. Charges of H_2O_2 molecules were calculated with RESP , angles and forces constants by Amber GAFF .



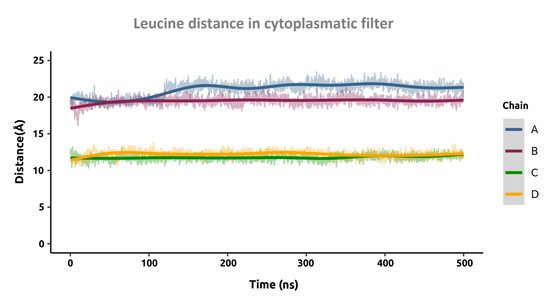
System stability measurements. RMSD (A) of least mobile atoms (protein structural core) or lipid area (B) were measured in models with and whitout N and C terminals, and in abscense of presence of H_2O_2 to test protein and systems stability.

- We created homology models of the PIPs of *Medicago truncatula* *Mt*PIP2;2 and *Mt*PIP2;3, with the addition of N and C terminals.
- Homotetrameric systems of the modeled structures were simulated by equilibrium molecular dynamics for at least 500ns, with ~300mM or ~3M H_2O_2.
- Simulation parameters as RMSD or area per lipid were measured to rule out effects of N- and C-terminals and H_2O_2 on protein structure or membrane integrity.

*Mt*PI2;2 and *Mt*PIP2;3 Homology models: Opening and Closed states



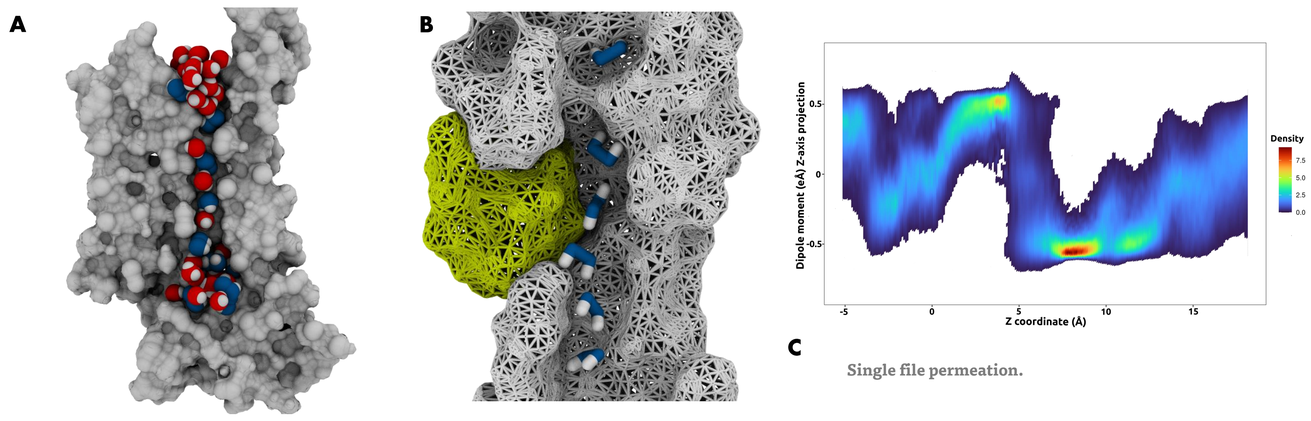
Water permeability of simulated systems. Equilibrium intrinsic permeability (p_f) of *Mt*PIP2;3 and *Mt*PIP2;2 simulations for each chain in open state tetramer.



Channel openning . Distance between Cα of L203 and L114 in *Mt*PIP2;3 system, relevant for channel gating. These residues are further apart in the A and B chains than in the case of C and D, which are presumably closed. Similar results were obtained in the case of *Mt*PIP2;2.

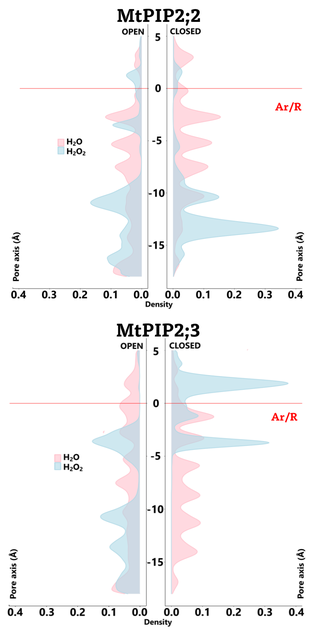
- Measurements of the intrinsic permeability of the channels (p_f), together with opening parameters such as the leucine position of the cytoplasmic filter (see Background: PIP structure image), indicate that the tetramers of *Mt*PIP2;2 and *Mt*PIP2;3 modeled by homology using the open structure of SoPIP2;1 (PDB 2B5F) would have two of their monomers in the open state (A and B) and two in the closed state (C and D).
- Chain A and C were chosen for this study. Chain A was preferred over B as open stated since it has loop D completely resolved, which is of great importance for open/closed conformations.

Mechanism of transport: Behavior of H_2O_2 and H_2O in the pore of PIPs

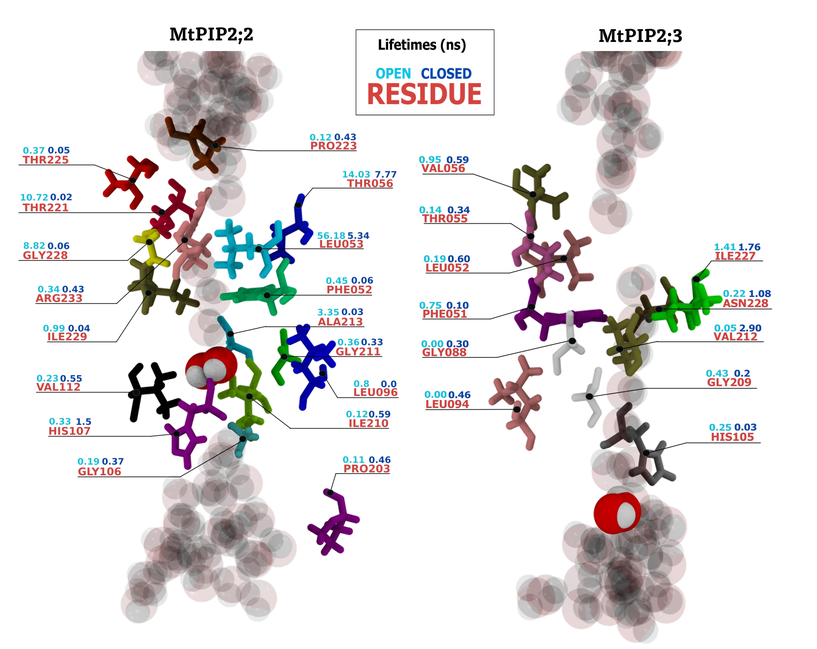


H_2O_2 pore permeation. A: H_2O (red) and H_2O_2 (blue) molecules can cross aquaporin pore in a coordinated way. B: When crossing the NPA zone (yellow highlighted) the H_2O_2 molecules show a change in orientation, similar to that described for water. C: The change in the orientation of H_2O_2 molecules can be measured through the projection of their dipole moment on the Z axis (main axis of the pore).

- Throughout the 500 ns simulations, it was observed that H_2O_2 passes through the pore of aquaporins, both in single file (observed in dynamics with 3M H_2O_2) and in a coordinated way with water molecules (observed both in 3M and 300mM H_2O_2 systems).
- In its passage through the pore, H_2O_2 behaves similarly to water, producing a change in orientation of the molecule when passing through the NPA zone.
- These systems could be useful to analyze the transport mechanism and interactions of H_2O_2 through the pore without the need to force the passage of molecules with biasing potentials.



Probability density profiles. Probability density estimate of finding a molecule of H_2O (pink) or H_2O_2 (light blue) along the pore, during the entire simulation.



H_2O_2 lifetimes. Mean lifetimes of the interactions between the H_2O_2 molecules and the different pore residues, measured in nanoseconds, in the open (light blue) and closed (dark blue) chains of the PIPs under study.

- The probability density profile within the pore, shows different behaviour patterns, indicating that in their passage through the aquaporins, the H_2O, and the H_2O_2 would have different interactions with the amino acid residues.
- When studying the interaction lifetimes of H_2O_2 interactions with each of the pore residues, we found that in the open channel it tends to have longer interactions with residues on the extracellular side, close to Ar/r, while in closed channel these lifetimes decrease in favor of contacts with residues on the cytoplasmic side.

Methods

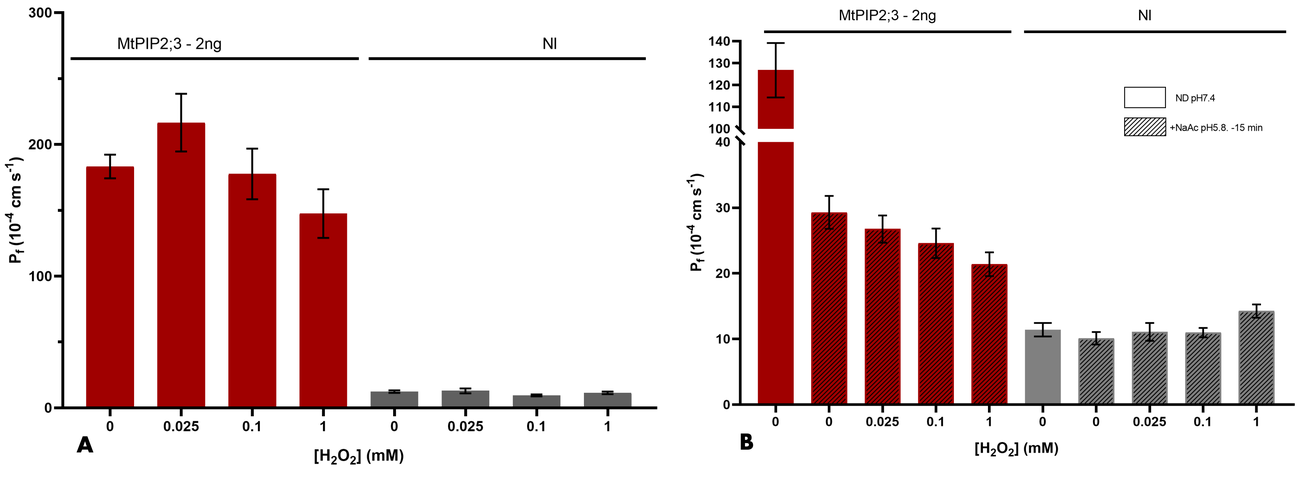
Molecular Dynamics simulations
Molecular dynamics simulations were performed for homotetrameric assemblies of *Mt*PIP2:3 or *Mt*PIP2;2 homology models created with SWISS-MODEL online server, using SoPIP2;1 open state crystal 3D structure (2B5F) as template. The missing Carboxy and Amino terminal ends were modelled using MODELLER software and the assemblies were embedded in a fully hydrated POPC bilayer, with explicit solvent (TIP3P water) and ions (NaCl 0.15M) using the membrane builder tool provided in the CHARMM-GUI website. ~300mM or ~3M H_2O_2 was added to the systems by replacement of water molecules, and 500ns unbiased molecular dynamics simulations were run with in an NPT ensemble with full periodic boundary conditions, performed with AMBER18 MD package, using hydrogen mass repartitioning (HMR) and parameters from AMBER14SB and LIPID17 force fields.

Density probability calculation
The density probability function was calculated by extracting the XYZ positions of the geometric center of the H_2O_2 or H_2O molecules throughout the simulation time with the cpptraj program of the AmberTools package. The origin of the Z coordinate of the system was centered in each simulation frame towards the geometric center formed by the Ar/R residues and the probability density along the pore was calculated by Kernel Density Estimation (KDE) using the software R.

Residence and Lifetimes
Native and non-native contacts between H_2O_2 to each other of monomeric pore residues were calculated using the cpptraj program from the AmberTools package. From these data series, analysis of residence times and lifetimes was carried out using Python and R tools.

Water transport assays
Xenopus laevis oocytes were microinjected with cRNA coding for MtPIP2;3 and incubated for 3 or 4 days in ND96 buffer (96 mM NaCl, 2 mM Kcl, 1mM MgCl2, 1.8 mM CaCl2, and 5 mM HEPES pH 7.5) at 18°C prior to performing the experiments. The osmotic water permeability (Pf) of oocytes injected or noninjected (NI) with cRNA was determined by measuring the rate of oocyte swelling induced in response to ⅓ dilution of the ND96 buffer with 0, 0.025, 0.1 or 1mM H_2O_2 in milliQ water. In experiments performed on the closed states channels, prior to exposure to osmotic shock, the internal pH of oocytes was acidified by preincubating them for 15 min in different pH solutions (NaAc solution: 50 mM NaAc and 20 mM MES for the 5.8–6.8 pH interval or HEPES for the 7.0–7.4 pH interval), supplemented with 1M mannitol until desired osmolarity (~200 mOsmol kg^{-1} H_2O). Oocytes area changes was measured by videomicroscopy and P_f calculated by formula $P_f=V_o[d(V/V_o)/d_t]/[S\ V_w(Osm_{in}-Osm_{out})]$, where V_o is initial oocyte volume, V/V_o is the relative volume, S is the surface area of the oocyte (0.045 cm^2), V_w the partial molecular volume of water (18 cm^3mol^{-1}) and (Osm_{in} - Osm_{out}) the osmotic driving force.

H_2O and H_2O_2 swelling assays



Swelling assays. A. Osmotic water permeability (P_f) of oocytes expressing *Mt*PIP2;3 was determined by measuring the rate of oocyte swelling, induced in response to threefold dilution of ND96 solutions with distilled water with incremental H_2O_2 concentrations, and videomicroscopy of their area. B. Incubation with NaAc pH 5.8 was performed to induce closed states in *Mt*PIP2;3 aquaporins. Noninjected oocytes (NI) as negative controls.

Our results show that in the open state of the channel, the different concentrations of H_2O_2 would not affect the transport of H_2O, or at least not in an observable way. When carrying out this same study using pH solutions to acidify the internal media and induce the closed state of the channel, we observe that although not statistically significative, there is a tendency to obtain less water transport in those conditions exposed to higher concentrations of H_2O_2.

Our results suggest that H_2O_2 can be transported by PIP aquaporins in mixed single files with H_2O molecules. The H_2O_2 passage did not significantly alter H_2O transport rates for any of the two tested *Mt*PIP2. MDS shows that both molecules can be transported in a coordinated way carrying out different interactions inside the pore.

We conclude that there is a kind of mimic between H_2O_2, and H_2O transport trough PIPs pore but with differences in the interaction with pore lining residues

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