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To cite this version:

Marek Dynowski, Gabriel Schaaf, Dominique Loque, Oscar Moran, Uwe Ludewig. Plant plasma membrane water channels conduct the signaling molecule H2O2. Biochemical Journal, 2008, 414 (1), pp.53-61. 10.1042/BJ20080287 . hal-00478969

HAL Id: hal-00478969

https://hal.science/hal-00478969

Submitted on 30 Apr 2010

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Biochemical Journal Immediate Publication. Published on 08 May 2008 as manuscript BJ20080287

Plant plasma membrane water channels conduct the signaling molecule H_2O_2

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Running title: Hydrogen peroxide permeation in PIP2;1
Keywords: Hydrogen peroxide, reactive oxygen species (ROS), aquaglyceroporins, aquaporin

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Synopsis

Hydrogen peroxide (H_2O_2) is a relatively long-lived reactive oxygen species that signals between cells and organisms. H_2O_2 signaling in plants is essential for response to stress, defense against pathogens, and the regulation of programmed cell death. Although H_2O_2 diffusion across membranes is often considered as a passive property of lipid bilayers, native membranes represent significant barriers for hydrogen peroxide. We addressed the question of whether channels might facilitate H_2O_2 conduction across plasma membranes. The expression of several plant plasma membrane aquaporins in yeast, including PIP2;1 from Arabidopsis, enhanced the toxicity of H_2O_2 and increased the fluorescence of dye-loaded yeast when exposed to H_2O_2. The sensitivity of aquaporin-expressing yeast to hydrogen peroxide was altered by mutations that alter gating and the selectivity of the aquaporins. The conduction of water, H_2O_2 and urea was compared, using molecular dynamics simulations based on the crystal structure of SoPIP2;1 from spinach. The calculations identify differences in the conduction between the substrates and reveal channel residues critically involved in H_2O_2 conduction. The results of the calculations on tetramers and monomers are in agreement with the biochemical data. Taken together, the data strongly postulate that plasma membrane aquaporin pores determine the efficiency of H_2O_2-signaling between cells. Aquaporins are present in most species and their capacity to facilitate the diffusion of H_2O_2 may be of physiological significance in many organisms and particularly in communication between different species.

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Introduction

Water channels -aquaporins- are identified in most organisms, including plants, and have specific physiological functions related to water facilitation in many tissues [1-3]. Aquaporins are tetramers and each subunit forms a pore. Many plant aquaporin homologs are believed to specifically conduct water, while several homologs also conduct other small neutral solutes, such as glycerol, and are, therefore, called aquaglyceroporins [1-3]. The aromatic/arginine (ar/R) region and the highly conserved NPA region (for Asn, Pro, Ala) have been implicated in the selectivity of aquaporins. In the model plant *Arabidopsis thaliana*, 35 aquaporin homologs are encoded in the genome, and several of them conduct urea or ammonia in addition to water [4, 5]. For simplicity, we will designate the proteins of this family, which are also called major intrinsic proteins, as aquaporins throughout the article, despite the ability of several homologs to conduct other solutes.

In several organisms, including mammals and plants, the expression of water channels is highly regulated at the transcriptional level. In addition, the conductance of several aquaporins can be post-transcriptionally regulated by different stimuli. Regulation by phosphorylation [6, 7], calcium [8], extracellular pH [9] or intracellular pH [10] has been reported. Crystal structures have unambiguously proven that the plant plasma membrane aquaporin SoPIP2;1 exists in two different, phosphorylation dependent conformational states, an open and a closed state [11]. These conformations mostly differ in their cytoplasmic pore openings, but the size and geometry of the narrowest part of the pore, the aromatic/arginine (ar/R) region, is nearly identical.

H_2O_2 is a relatively long-lived reactive oxygen species and can function as a signaling molecule in eukaryotes, leading to specific downstream responses [12, 13]. H_2O_2 is a small neutral solute and membranes appear to play an important role in protecting cells against its toxicity, which may derive from H_2O_2 itself or from the more toxic hydroxyl radical derived from H_2O_2. This implies that native membranes restrict free H_2O_2 diffusion and that significant H_2O_2 gradients across membranes exist. Cellular H_2O_2 redox chemistry is mainly restricted to compartments surrounded by membranes such as peroxisomes and mitochondria and may also be found in other endomembrane compartments [14].

In plants, H_2O_2 is generated and released upon various stresses, e.g. during nutrient starvation [15]. H_2O_2 is a signal that controls plant programmed cell death [16]. Specific H_2O_2 generation in plants often involves plasma membrane NADPH oxidases that produce the superoxide anion, by enabling the transfer of electrons from the cytosolic NADPH across the membrane to external molecular oxygen. The spontaneous dismutation of the short-lived superoxide anion (that is very effective at the low apoplastic pH) will produce external hydrogen peroxide. This is followed by H_2O_2 influx and the subsequent activation of Ca^{2+}-channels [17]. Such NADPH-dependent processes have been identified in the plant defense against attack by pathogens ('oxidative burst'), root hair development [18] and the regulation of abscisic acid signaling and stomatal movements [12, 17]. A recent study has shown that the human AQP8 and the plant tonoplast intrinsic proteins AtTIP1;1 and AtTIP1;2 conduct hydrogen peroxide when heterologously expressed in yeast [19]. Earlier work had suggested that algae plasma membranes may contain pores for hydrogen peroxide [22]. In that work, theoretical calculations combined with measurements of the membrane permeability at very high concentrations (up to 350 mM) and the relative unspecific aquaporin inhibitor $HgCl_2$ were used [20].

In this study, we present evidence that several plant plasma membrane aquaporin homologs conduct the signaling- and defense-related small neutral solute H_2O_2. Furthermore, molecular simulations identify channel residues critically involved in H_2O_2 conduction. The conservation of these residues suggests that many plasma membrane aquaporins from plants facilitate the diffusion of hydrogen peroxide. Although many aquaporins have a well-defined physiological function in water conduction, aquaporins may thus also participate in regulating and controlling H_2O_2-signaling.

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Experimental

Yeast strains, media and growth conditions

The *Saccharomyces cerevisiae* strains BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) (Euroscarf) and *Δdur3 (MATα, dur3Δ, ura3Δ)* and 23344c *(MATα, ura3Δ)* [21] were employed for growth tests. Uptake studies were performed in the *yca1* yeast mutant (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, yor197w::kanMX4*) (Euroscarf, Y02453). Yor197w encodes a putative cysteine protease that is similar to mammalian caspases and is involved in the regulation of apoptosis upon hydrogen peroxide treatment. For growth tests cells were grown up to stationary phase in liquid YNB medium (1.7 g / L yeast nitrogen base, 3.0% glucose, 1% arginine, supplemented with histidine, leucine, methionine when appropriate) at 28°C and 160 rpm. The cells were spotted as 5-fold dilution series on freshly prepared plates containing H_2O_2 ranging from 0.25 mM up to 2.5 mM. The pictures were taken after 96 h.

Plasmid constructs for yeast expression

The open reading frames of AtPIP1;1 (*At3g61430*), AtPIP2;1 (*At3g53420*), AtPIP2;4 (*At5g60660*), AtNIP1;1 (*At4g19030*) and AtNIP1;2 (*At4g18910*) were amplified from an *Arabidopsis thaliana* (*Col-0)* cDNA library, cloned into the pGEM-T Easy vector (Promega) and then subcloned into pDR195. AtTIP2:3 was described earlier [5]. The point mutations were inserted by using the Quickchange kit from Stratagene. All constructs were verified by sequencing at GATC (Konstanz, Germany). The sequence encoding the N-terminal c-myc epitope was generated by PCR. SDS-PAGE analysis and western blotting followed standard protocols using a monoclonal anti-c-myc antibody (Sigma). The yeast oxysterol binding protein Kes1 was used as a normalization control and visualized with a monoclonal antibody (a kind gift of Vytas Bankaitis).

Fluorescence assays

To reduce endogenous production of H_2O_2, the *yca1* yeast mutant was employed. This mutant is partially defective in H_2O_2 induced apoptosis (21). Transformants were grown in selective media to midlog phase, and successively supplied with the oxidant-sensitive fluorescent dye 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes) to a final concentration of 20 µM and incubated at 28 °C and 550 rpm for 45 minutes to allow uptake of the dye. Cells were then washed in water and resuspended in an experimental solution consisting of 40 mM Na-phosphate pH 5.8 containing 3% glucose. The fluorescence was measured before and after addition of H_2O_2 (final concentration 2 mM or 10 mM). The fluorescence was measured in 10 min. steps using a TECAN 96-well spectrofluorimeter, set at an excitation wavelength of 493 nm and an emission wavelength of 524 nm.

Structure of the open SoPIP2;1 aquaporin

A model of the "open conformation" SoPIP2;1 aquaporin was obtained by combining the open and closed pore crystal structures of SoPIP2;1. The closed structure (PDB accession number 1Z98; ALA24 – VAL179 and PRO201 – SER274) and the open structure (PDB accession 2B5F; PHE180 - LEU200) were combined [11] and a structural alignment was done using VMD (Suppl. Fig. 2). Serines 115 and 274 were exchanged by phosphoserines to keep the channel in an open state [11]. To avoid an artificial negative charge at the C-terminus (as in the partially resolved C-terminus in the structure), a phenylalanine residue, which is not resolved in the crystal, was added to this critical region. For the simulations, this "open conformation" SoPIP2;1 was embedded into a pre-equilibrated 70 x 70 Å (as a monomer, Suppl. Fig. 3) or 130 x 130 Å (as a tetramer) spanning 16:0/18:1c9-palmitoyloleyl-phosphatidyl-ethanolamin (POPE) lipid bilayer.

The system was hydrated with TIP3P water molecules and counter ions were added to obtain a neutral net charge, using the module LEAP from the AMBER8 suite. The total tetrameric system included approximately 111.200 atoms. One substrate molecule was placed above each monomer (>-30 Å in z-direction from the Cα of valine 206, Fig. 6) and a fifth above the central cavity of the tetramer (Fig. 5). Within a tetramer, the starting position of the substrates differed by ± 3 Å in lateral direction from the central axis of each monomer.

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These four different starting positions were identical for all substrates (i.e. for the center of mass of each substrate).

Molecular dynamics simulations

Molecular dynamics (MD) was carried out using the NAMD2 program [22] using the Amber03 force field. Force field parameters for hydrogen peroxide and the POPE lipid bilayer were estimated using the module ANTECHAMBER and the general AMBER force field (GAFF) of AMBER8. The bond angles, dipole moment and other properties matched the well-known properties of hydrogen peroxide [13]. The force field parameters for urea were provided with AMBER8 and the force field parameters for phosphoserine was obtained from the AMBER Parameter Database (pharmacy.man.ac.uk/amber/) [23]. The system was initially minimized for 1000 steps with the protein atoms fixed. Then another 1000 minimization steps were applied without any atoms fixed. The system was then equilibrated for 2 ns using a timestep of 2 fs with periodic boundary conditions (PBC). A pressure of 1 atm (101,3 KPa) and a temperature of 310 K were applied during equilibration and were kept constant using the Langevin Piston method. Non-bounded interactions were calculated with a cutoff of 12 Å, and the long range electrostatic forces where calculated using the Particle Mesh Ewald method (PME). To keep the protein in position, the Cα atoms of the protein backbone were constrained with a harmonic force of 0.5 kcal x mol^{-1} x $Å^{-2}$.

For monomeric simulations, periodic boundary conditions were used, but the individual MD simulations on tetramers were carried out without PBC, PME and Langevin dynamics. To simulate the permeation across the aquaporin channels, the system was confined by applying a harmonic constraint of 200 kcal x mol^{-1} x $Å^{-2}$ to the 3.5 Å outer layer of water. A second water layer of the same thickness below was constrained with 5 kcal x mol^{-1} x $Å^{-2}$. To keep simulation times reasonable, a constant force vector in z-direction was applied to the permeant molecule, either water, hydrogen peroxide or urea [24]. In initial trials we found that the constant force of ~41 pN was required that solutes entered the channel entrance within a few ns. The trajectories along the z-axis were calculated relative to the Cα atom of valine 206 as reference. The entrance point into the channel along the z-coordinate was defined as Cα of lysine 144 (on average -20.5 Å ± 1.5 Å from valine) and the internal channel exit was defined as Cα of proline 33 (19.2 Å ± 1.3 Å from the valine 206).

Residency time and energy profiles

To construct the overall histogram of the time at each position along the reference axis, the residence time measured for 0.2 Å intervals of each simulation was accumulated in a weighted sum, such that the biased potentials due to the applied force were discounted. The energy profiles were then estimated in 1 Å intervals along the channel axis from transition state theory as [25]:

$$\Delta G^{\ddagger} = -RT \ln\left(\frac{1}{\tau}\frac{h}{Tk_b}\right)$$

where τ is the residence time, $\Delta G^{\ddagger}$ is the Gibbs free energy of activation, T is the absolute temperature, R is the universal gas constant, k_b is Boltzmann's constant, and h is Planck's constant.

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Results

Expression of AtPIP2;1 increased H_2O_2 sensitivity and H_2O_2 accumulation in yeast

We considered the possibility that aquaporins could mediate the conduction of H_2O_2 across membranes, since some channel homologs have been shown to conduct several low molecular weight molecules. Initial studies focused on the Arabidopsis protein AtPIP2;1. When the Arabidopsis AtPIP2;1 was heterologously expressed in yeast, growth and doubling times were unaffected in standard liquid culture or solid media. However, expression of AtPIP2;1 increased sensitivity of yeast cells to H_2O_2 (Fig. 1A). Growth of cells expressing AtPIP2;1 was affected in a dose dependent manner and impaired at 400 µM H_2O_2, while non-expressing controls, transformed with the vector pDR195 without the aquaporin coding insert, were not sensitive up to 1.6 mM H_2O_2 (Fig. 1A). In a similar growth assay, the plant tonoplast aquaporin TIP1;1 was recently shown to conduct hydrogen peroxide [13, 19]. In principle, reduced or impaired growth may be due to factors, which perturb the metabolism in an unknown way.

To further test whether the increased sensitivity of cells expressing AtPIP2;1 was due to increased uptake of H_2O_2, a carboxylated derivative of the oxidant sensitive probe 2',7'-dichlorofluorescein (DCF) was employed. When cells are incubated with a di-ester derivative of the probe, it enters the cytosol by passive diffusion. Once inside the cell, the esters are cleaved by endogenous esterases, rendering the probe impermeable to the membrane. The DCF can be oxidized by reactive oxygen species such as H_2O_2 resulting in a fluorescence increase [26]. To reduce the production of internally generated reactive oxygen species (ROS) by H_2O_2-stimulated apoptosis, we used the yeast *yca1* mutant that is partially defective in undergoing apoptosis [27]. Upon exposure to H_2O_2, the overall intracellular accumulation of reactive oxygen species that was measured with the fluorescent dye, was higher in AtPIP2;1-expressing yeast cells compared to cells transformed with empty vector alone. Consistent with these data, the c-myc epitope tagged AtPIP2;1 was stable in yeast and detected as a monomeric protein of ~30 kDa (Fig. 2A) and as an oligomeric protein (*data not shown*).

Sensitivity of yeast to H_2O_2 is altered by a mutation that reduces PIP2 water conductance

A conserved cytosolic histidine residue has been shown to be involved in PIP2 regulation of water flux by cytosolic pH, a phenomenon that was initially demonstrated using the homolog AtPIP2;2 [10]. Replacement of this histidine by lysine in AtPIP2;2 reduced water conduction and mimicked a closed channel [10]. The relevant histidine at position 199 in AtPIP2;1 (Fig. 2B) was replaced by lysine (H199K) by site directed mutagenesis. As expected, the sensitivity of H199K mutant expressing yeast to grow on H_2O_2 was similar to vector transformed controls (Fig. 2C), although a similar protein level was detected from this mutant in Western analysis (Fig. 2A).

Sensitivity of yeast to H_2O_2 is altered by pore mutations that alter selectivity

Residues in the constriction region of the pores can affect the selectivity properties of aquaporins [28, 29]. Alignments and homology models indicate that the constriction region of all PIP-type aquaporins from Arabidopsis is identical. In AtPIP2;1, the constriction (ar/R) region is composed of the four residues Phe 87, His 216, Thr 225 and Arg 231. In contrast, the "selectivity filter" residues in NIP-type major intrinsic proteins differ, which leads to a larger pore diameter [28]. A mutant, AtPIP2;1$^{NIP1;2}$, in which the ar/R residues in AtPIP2;1 were exchanged to the corresponding residues in AtNIP1;2 (F87W, H216V, T225A) also conducted H_2O_2, as was deduced from its ability to confer increased sensitivity to H_2O_2 when expressed in yeast (Fig. 3C). The ar/R constriction region in this mutant is wider, as can be easily deduced from homology models of this mutant (Suppl. Fig. 1). The residues of the ar/R region of AtPIP2;1 were also exchanged to the corresponding residues of AtNIP5;1, which conducts hydrophobic, non-charged solutes as large as boric acid, but not water, at a high rate [30]. By introducing the three relevant mutations in AtPIP2;1 (F87A, H216I, T225G), the ar/R constriction region was even wider in this mutant channel (designated AtPIP2;1$^{NIP5;1}$) (Suppl. Fig. 1).

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Yeast growth is sensitive to high concentrations of toxic boron. AtPIP2;1 expression affected neither the boric acid sensitivity of yeast, nor enhanced urea uptake on selective plates (Fig. 3B;D). The NIP1;2-like aquaporin mutant (AtPIP2;1$^{NIP1;2}$) did not exhibit novel properties compared to AtPIP2;1. In contrast, the expression of AtPIP2;1$^{NIP5;1}$ enhanced its growth sensitivity to boric acid, indicating boron uptake (Fig. 3B). This mutant form of AtPIP2;1 dramatically increased the sensitivity of yeast growth to hydrogen peroxide beyond that of wild type AtPIP2;1 (Fig. 3C). When expressed in a yeast mutant that lacks endogenous urea transporters (Δdur3; [21]) this construct enabled yeast to grow on urea as a sole nitrogen source, indicating urea conduction across the plasma membrane (Fig. 3D). Thus, an AtPIP2;1 aquaporin with selectivity filter as in AtNIP5;1 apparently facilitates the conduction of larger solutes, including hydrogen peroxide. These observations are in agreement with earlier studies [28] that showed that residues in the ar/R region are critical for substrate selectivity.

H_2O_2 conduction by some, but not all aquaporin homologs

Representatives of individual sub-groups of the plant aquaporins were tested for their capacity to impair yeast growth on plates containing hydrogen peroxide. While cells expressing either AtPIP1;1 or AtNIP1;1 were similar to controls, the yeast expressing AtNIP1;2 or the tonoplast-intrinsic AtTIP2;3 exhibited an intermediate phenotype (Fig. 4). It is interesting to note that AtNIP1;1 had been found to conduct glycerol across the plasma membrane when expressed in yeast [31]. Despite strong expression of the c-myc tagged AtNIP1;1 in yeast (Fig. 2A), it did not increase the sensitivity to H_2O_2 (Fig. 4). We conclude that hydrogen peroxide conduction is not supported by NIP1;1, at least when heterologously expressed in yeast. The AtPIP2;4-expressing cells were the most sensitive to H_2O_2 (Fig. 4). Whether these differences in hydrogen peroxide sensitivity are due to differences in the open probability of the channel, (mis-)targeting of the membrane proteins to other membranes in the heterologous system or due to protein stability, remains unclear at this point. It is, however, notable that AtPIP1;1, AtPIP2;1 and AtPIP2;4 have identical residues at the selectivity filter, which differ in other family members such as AtNIP5;1. It has been repeatedly noted that PIP1-type aquaporins are not active in heterologous systems [3]. This may explain why AtPIP1;1 expression did not alter sensitivity to H_2O_2 (Fig. 4). The tonoplast intrinsic AtTIP2;3, which is known to facilitate ammonia and urea conduction when heterologously expressed in yeast [5] also conducts H_2O_2. This homolog is known to facilitate solute fluxes across the plasma membrane of yeast, despite its intracellular localization *in planta* [5]. Other TIP-type aquaporins, with further different selectivity filter residues in the ar/R region, have been shown to conduct hydrogen peroxide [19].

Molecular simulation of conduction through PIP2;1

The plasma membrane localized aquaporin AtPIP2;1 from Arabidopsis is highly similar to other PIP2-type aquaporins, such as the crystallized SoPIP2;1 channel from spinach. The closest Arabidopsis homolog of SoPIP2;1 is AtPIP2;7, but all PIP2-type aquaporins share a very high conservation within the pore and have the identical selectivity filter residues. Open and closed conformations of the SoPIP2;1 structure are available [11]. We performed molecular dynamics studies on the open SoPIP2;1 channel to compare H_2O and H_2O_2 conduction through plant plasma membrane aquaporins. We initially observed that the closed pores of the tetrameric SoPIP2;1 were highly stable during minimization, equilibration and further simulation, when embedded in a lipid bilayer. In addition, the pores readily filled with a single-file of water during equilibration, but water conduction was impaired by loop D, which closes the pore from the cytosolic side. In contrast, the structures of the lower-resolution open monomers, or homology modeled AtPIP2;1, were less stable during similar simulations and no continuous, stable water line formed, as described in previous simulations [29, 32]. This was probably mainly due to the lower structural resolution *(data not shown)*.

We therefore constructed open pore monomers based on the high-resolution structure of the closed pore, where the backbone came from the closed pore, but loop D was taken from the open conformation *(see methods,* Suppl. Fig. 2*)*. Molecular dynamics (MD) simulations on a

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modified 'open' model of the closed pore structures have already been published [11]. The 'open' pore tetramer embedded in a POPE lipid bilayer is shown in Fig. 5A.

We first tested whether steered molecular dynamics [24] can reproduce the well-established features of H_2O conduction in aquaporins from other species [29, 32]. In accordance with previous reports, a stable water line formed in this 'high-resolution open' monomer structure during a MD simulation (Fig. 5B). Furthermore, the dipole orientation of water molecules reversed at the NPA region, similar as in aquaporins from other species [29, 32]. To increase the probability that a specific water molecule enters a pore, it was positioned close to the external pore vestibule and a constant force was applied to steer the solute into the z-direction (see Fig. 5A) [24]. Since solutes might also pass through the tetramer center, rather than the four channel pores [33], we positioned a fifth water on top of the tetramer center (Fig. 5A, Suppl. Fig. 3).

The trajectories from several independent simulations showed how H_2O molecules traverse the pores (Fig. 6A). We observed coordinated water conduction, similar to what has been described in earlier simulations on other aquaporins [29, 32, 34, 35]. After 4 ns, 7 of 11 (64%) water molecules had crossed the pore entirely (Fig. 6A). Individual water molecules showed two preferred residency positions close to the ar/R region and the NPA region (Fig. 6A). Only one single water molecule that had entered the outer channel vestibule did not cross the entire pore during the full simulation time of 16 ns. This molecule moved away from the central pore axis and got stuck in a cavity close to glutamate 168 in transmembrane helix 4. Furthermore, the continuous water line was broken in some cases at the ar/R region, a feature that was also observed when pressure was applied to accelerate the transit of solutes [36]. In particular cases, the labeled waters did not enter a pore. The trajectories of these waters were excluded from further analysis.

The dwell-time estimates derived from the water trajectories indicated that the major energy barriers within the plant aquaporin SoPIP2;1 pore are the ar/R and the NPA region (Fig. 6A,D), which are critical for selective water flux, as had been concluded earlier [29, 32].

H_2O_2 conduction in PIP2;1

Similar simulations were done with five H_2O_2 molecules replacing the waters at identical starting positions. Hydrogen peroxide competed with water for hydrogen bonds, but it was evident from the trajectories that the entrance of H_2O_2, as well as the conductance, was slightly less favorable compared to water (Fig. 6B). After 4 ns, only two full conduction events were observed (15%), but all H_2O_2 molecules finally crossed the pore during the 16 ns simulations. The major residency positions were similar to those of water, but further sites of increased residency close to the internal pore vestibule were detected (Fig. 6B). A closer look at individual simulations showed that the residency at these positions resulted from the exact position of the flexible cytoplasmic loop D that is responsible for closing the pore. The maximal residency time for H_2O_2 in the ar/R region (indicating a major energy barrier for conduction) is in line with our finding that increasing the pore diameter in this region increases H_2O_2 conduction/sensitivity (Fig. 3).

Comparison of solute conduction simulations in SoPIP2;1

Similar simulations were also done with urea, a solute that was not conducted by AtPIP2;1, as indicated by our growth assays (Fig. 3D). The trajectories for urea indicated that this solute was also not conducted in simulations. No full conduction event was observed within 16 ns. Only one single urea molecule had traversed most of a pore (Fig. 6C). The trajectories identify the ar/R region as insuperable barrier for urea conduction (Fig. 6C,D). This result is strongly supported by the growth assays in Fig. 3, which indicate that mutations in the ar/R region can convert PIP2;1 into a urea conducting channel. Mutations in the ar/R region result in a wider pore diameter, which agrees with the conduction of urea in these mutants [37].

The energy profile of each substrate along the z-axis was calculated from the trajectories and further illustrates the differences in the conduction properties (Fig. 6D). The energy barriers for H_2O_2 along the pore axis was at most positions slightly higher than for water, but the maximal barrier was of similar hight. All three tested substrates have a dipole moment,

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which was preferentially oriented within the pore. This is well observed at the sites of longer residency, where snapshots of H_2O_2 and urea in their preferential positions and orientation are given in Fig. 6E,F.

Neither hydrogen peroxide nor urea permeated through the tetramer center; the solutes did not enter this central cavity. In contrast, the solutes initially positioned above the tetramer center nearly always entered and permeated a pore. For this reason, we also tested whether smaller simulations with monomers give a similar outcome. Such smaller simulations are advantageous, since they saved 39 % of resource time. The monomer was again embedded into a small patch of lipid bilayer (Suppl. Fig. 4). Interestingly, these simulations yielded essentially the same results (Suppl. Fig. 5). The trajectories of water, H_2O_2 and urea resembled those from tetramer simulations. This suggests that monomers are sufficiently rigid to computationally simulate conduction in isolated, monomeric pores.

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Discussion

The plasma membrane is an important protective barrier against extracellular H_2O_2. Yeast strains with impaired ergosterol biosynthesis and, thus, higher membrane permeability to lipophilic substances, were more sensitive to H_2O_2 [38]. At low millimolar concentrations, hydrogen peroxide induces apoptosis in yeast [27, 39]. Gradients of H_2O_2 have been reported across the membranes from mammalian cell lines [40], bacteria [41] and yeast [38], suggesting that native biological membranes generally prevent the free diffusion of H_2O_2. Very recently, tonoplast intrinsic proteins (TIPs) from plants have been shown to specifically conduct H_2O_2 by different assays, including growth sensitivity on hydrogen peroxide containing plates [19]. However, the physiological role of hydrogen peroxide permeability across the tonoplast remains somewhat unclear. We confirmed H_2O_2 conduction by a tonoplast intrinsic protein (Fig. 4). Tonoplast intrinsic proteins (TIPs) have a relatively weak selectivity and conduct many solutes, including urea, ammonium and water. Our analysis indicates that not only intracellular aquaporins, such as TIPs, but also plasma membrane PIP2-type aquaporins, which were previously believed to be highly selective channels for water, conduct hydrogen peroxide.

The *Saccharomyces* genome encodes four aquaporin genes, two of which are highly homologous to specific water channels. Many laboratory yeast strains, however, contain function-abolishing mutations in these aquaporins [42]. Therefore two different laboratory strains were tested for their H_2O_2 growth sensitivity, but only minor differences were observed and all were unable to grow at $\geq$ 3 mM H_2O_2 *(data not shown)*. In both strains, the sensitivity to H_2O_2 increased several-fold with the heterologous expression of AtPIP2;1 aquaporins.

The permeability of the plasma membrane to nonionic substances is generally determined by the membrane fluidity that depends on the content of e.g. sterols [43]. Higher plant plasma membranes are known for their high sterol content, which suggests that native plant membranes are relatively impermeable to non-electrolytes [44]. In the simulations we used a simple POPE-lipid membrane without sterols, which does not reflect the complex properties of a native plant membrane. This simple membrane is expected to confer only a relatively weak diffusion barrier for solutes. In agreement with this, we occasionally observed full conduction events in simulations with water (three full events from all simulations) and hydrogen peroxide (two full events) across the synthetic lipid membrane.

Here we identified a novel substrate of channels by a combination of functional assays and computer simulations. The combined data suggest that PIP2-type plasma membrane aquaporins are efficient hydrogen peroxide channels (Fig. 6). The aquaporin-mediated conduction of H_2O_2 may explain previous observations that algal membranes exhibit permeability to H_2O_2 at high concentrations [20]. The molecular analysis presented here suggests that the ar/R region is critically involved in selectivity towards urea, boron and hydrogen peroxide. Widening the pore allows larger solutes to pass through the channel (Fig. 3B,D), which is in agreement with previous reports [28, 37]. The simulations and functional data suggest that the ar/R selectivity filter region is the most critical determinant of hydrogen peroxide conduction in aquaporins. Since all plasma membrane PIP2 aquaporins from plants have the identical residues at these positions, it appears reasonable to predict that all eight PIP2-type aquaporins from Arabidopsis conduct hydrogen peroxide. The yeast growth tests further imply that some aquaporins, with a different selectivity filter region, also conduct this solute (Fig. 3, Fig. 4).

Interestingly, resource-saving simulations on monomeric SoPIP2;1 yielded an overall result similar to full simulations, although the transit time along the channel seems to be reduced for monomeric simulations. This may be exploited in the future to reduce calculation times and costs. Indeed, simulations on monomers have already been used to probe the conduction properties of aquaporins [45]. Computer simulations on channels also appear to be valuable in predicting novel substrates; this may be used in the future to predict

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physiologically important solutes that cannot be easily identified or measured by functional assays.

Although the pores were readily filled with a line of waters, the ar/R region was only partially occupied in several independent simulations and the flexible side chain of the arginine (R224) bent briefly into the pore. The flexibility of this arginine had been observed in earlier simulations on other aquaporins [34, 35], and was therefore fixed in some simulations [35]. However, the flexibility appears to reflect free arginine rotamers, since this arginine also adopted different conformations within subunits of the AqpZ tetramer [46]. No restrains were applied to any of the side chains during our simulations and the arginine behaved similarly in simulations with water or hydrogen peroxide.

When expressed in yeast, a high permeability of aquaporins to hydrogen peroxide results in susceptibility to H_2O_2, indicating the putative physiological importance of the hydrogen peroxide conduction by aquaporins. It is known that developmental processes, such as root hair growth and stomatal movements, require NADPH oxidase activity and probably subsequent H_2O_2 influx to activate Ca^{2+}-channels [17].

Whether hydrogen peroxide conduction across plasma membranes is altered in Arabidopsis mutants that lack individual aquaporins remains an unanswered question. However, the large number of plasma membrane aquaporins and their partially overlapping expression pattern and redundant function may require plants in which multiple PIP2s are knocked-out [1]. Arabidopsis mutants with strongly reduced water conduction are not yet available [1]. In line with this, we did not find hydrogen peroxide-related phenotypes with plant lines harboring single T-DNA insertions in PIP2 genes in preliminary experiments (*data not shown*).

It appears likely that plasma membrane aquaporins may be far more than just mediators of water conduction. Recent observations suggest that other small solutes, such as CO_2, are also channeled by at least some aquaporins [1, 2]. That excess H_2O_2 influx kills yeast is supported by the growth tests (Fig. 1) and mutant yeast strains [38]. Aquaporins belong to a multi-gene family in plants and it remains a future challenge to identify the physiological contribution of each individual member to hydrogen peroxide conduction.

The ability of pathogens to resist oxidative stress is crucial for their infectiousness and pathogenesis in the host. Aquaporins have also been identified in microbes and pathogens. Pathogens might increase virulence by minimizing the influx of the hydrogen peroxide generated by the plant. Protection against influx of H_2O_2 may be mediated by pore closure, e.g. by de-phosphorylation, transcriptional down-regulation during infection, or even genomic loss of aquaporins.

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Acknowledgements: We thank Junpei Takano for plasmids and discussions and Vytas Bankaitis for providing reagents. This work was supported in part by a grant of the Landesstiftung Baden Württemberg to U.L. Simulations were carried out at the HPC-center of the University of Tübingen, the Höchstleistungsrechenzentrum of the University of Stuttgart (HLRS) and the Scientific Supercomputing Center (SSC) Karlsruhe, Germany.

References

1 Maurel, C. (2007) Plant aquaporins: novel functions and regulation properties. FEBS Lett **581**, 2227-2236
2 Kaldenhoff, R. and Fischer, M. (2006) Functional aquaporin diversity in plants. Biochim Biophys Acta **1758**, 1134-1141
3 Chaumont, F., Moshelion, M. and Daniels, M. J. (2005) Regulation of plant aquaporin activity. Biol Cell **97**, 749-764
4 Liu, L. H., Ludewig, U., Gassert, B., Frommer, W. B. and Von Wiren, N. (2003) Urea Transport by Nitrogen-Regulated Tonoplast Intrinsic Proteins in Arabidopsis. Plant Physiol. **133**, 1220-1228
5 Loqué, D., Ludewig, U., Yuan, L. and von Wiren, N. (2005) Tonoplast aquaporins AtTIP2;1 and AtTIP2;3 facilitate NH3 transport into the vacuole. Plant Physiol. **137**, 671-680
6 Johansson, I., Karlsson, M., Shukla, V. K., Chrispeels, M. J., Larsson, C. and Kjellbom, P. (1998) Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. Plant Cell **10**, 451-459
7 Lee, J. W., Zhang, Y., Weaver, C. D., Shomer, N. H., Louis, C. F. and Roberts, D. M. (1995) Phosphorylation of nodulin 26 on serine 262 affects its voltage-sensitive channel activity in planar lipid bilayers. J. Biol. Chem. **270**, 27051-27057
8 Gerbeau, P., Amodeo, G., Henzler, T., Santoni, V., Ripoche, P. and Maurel, C. (2002) The water permeability of Arabidopsis plasma membrane is regulated by divalent cations and pH. Plant J. **30**, 71-81
9 Zeuthen, T. and Klaerke, D. A. (1999) Transport of water and glycerol in aquaporin 3 is gated by H(+). J. Biol. Chem. **274**, 21631-21636
10 Tournaire-Roux, C., Sutka, M., Javot, H., Gout, E., Gerbeau, P., Luu, D. T., Bligny, R. and Maurel, C. (2003) Cytosolic pH regulates root water transport during anoxic stress through gating of aquaporins. Nature **425**, 393-397
11 Tornroth-Horsefield, S., Wang, Y., Hedfalk, K., Johanson, U., Karlsson, M., Tajkhorshid, E., Neutze, R. and Kjellbom, P. (2006) Structural mechanism of plant aquaporin gating. Nature **439**, 688-694
12 Hancock, J. T., Desikan, R. and Neill, S. J. (2001) Role of reactive oxygen species in cell signalling pathways. Biochem. Soc. Trans. **29**, 345-350
13 Bienert, G. P., Schjoerring, J. K. and Jahn, T. P. (2006) Membrane transport of hydrogen peroxide. Biochim Biophys Acta **1758**, 994-1003
14 Huckelhoven, R., Fodor, J., Preis, C. and Kogel, K. H. (1999) Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. Plant Physiol. **119**, 1251-1260
15 Shin, R. and Schachtman, D. P. (2004) Hydrogen peroxide mediates plant root cell response to nutrient deprivation. Proc. Natl. Acad. Sci. U S A **101**, 8827-8832
16 Gechev, T. S. and Hille, J. (2005) Hydrogen peroxide as a signal controlling plant programmed cell death. J Cell Biol **168**, 17-20
17 Mori, I. C. and Schroeder, J. I. (2004) Reactive oxygen species activation of plant Ca2+ channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. Plant Physiol. **135**, 702-708

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18 Carol, R. J., Takeda, S., Linstead, P., Durrant, M. C., Kakesova, H., Derbyshire, P., Drea, S., Zarsky, V. and Dolan, L. (2005) A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. Nature **438**, 1013-1016
19 Bienert, G. P., Moller, A. L., Kristiansen, K. A., Schulz, A., Moller, I. M., Schjoerring, J. K. and Jahn, T. P. (2007) Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J Biol Chem **282**, 1183-1192
20 Henzler, T. and Steudle, E. (2000) Transport and metabolic degradation of hydrogen peroxide in Chara corallina: model calculations and measurements with the pressure probe suggest transport of H_2O_2 across water channels. J. Exp. Bot. **51**, 2053-2066
21 Liu, L., Ludewig, U., Frommer, W. B. and von Wiren, N. (2003) AtDUR3 encodes a new type of high-affinity urea/H^+ symporter in Arabidopsis thaliana. Plant Cell **15**, 790-800
22 Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kale, L. and Schulten, K. (2005) Scalable molecular dynamics with NAMD. J Comput Chem **26**, 1781-1802
23 Craft, J. W., Jr. and Legge, G. B. (2005) An AMBER/DYANA/MOLMOL phosphorylated amino acid library set and incorporation into NMR structure calculations. J Biomol NMR **33**, 15-24
24 Jensen, M. O., Park, S., Tajkhorshid, E. and Schulten, K. (2002) Energetics of glycerol conduction through aquaglyceroporin GlpF. Proc. Natl. Acad. Sci. U S A **99**, 6731-6736
25 Johnson, F. H., Eyring, H. and Stover, B. J. (1974) The Theory of Rate Processes in Biology and Medicine. (Wiley, New York)
26 Yurkow, E. J. and McKenzie, M. A. (1993) Characterization of hypoxia-dependent peroxide production in cultures of Saccharomyces cerevisiae using flow cytometry: a model for ischemic tissue destruction. Cytometry **14**, 287-293
27 Madeo, F., Herker, E., Maldener, C., Wissing, S., Lachelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J., Wesselborg, S. and Frohlich, K. U. (2002) A caspase-related protease regulates apoptosis in yeast. Mol. Cell **9**, 911-917
28 Wallace, I. S. and Roberts, D. M. (2005) Distinct transport selectivity of two structural subclasses of the nodulin-like intrinsic protein family of plant aquaglyceroporin channels. Biochemistry **44**, 16826-16834
29 Tajkhorshid, E., Nollert, P., Jensen, M. O., Miercke, L. J., O'Connell, J., Stroud, R. M. and Schulten, K. (2002) Control of the selectivity of the aquaporin water channel family by global orientational tuning. Science **296**, 525-530
30 Takano, J., Wada, M., Ludewig, U., Schaaf, G., von Wiren, N. and Fujiwara, T. (2006) The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell **18**, 1498-1509
31 Weig, A. R. and Jakob, C. (2000) Functional identification of the glycerol permease activity of Arabidopsis thaliana NLM1 and NLM2 proteins by heterologous expression in Saccharomyces cerevisiae. FEBS Lett. **481**, 293-298
32 de Groot, B. L. and Grubmuller, H. (2001) Water permeation across biological membranes: mechanism and dynamics of aquaporin-1 and GlpF. Science **294**, 2353-2357
33 Wang, Y., Cohen, J., Boron, W. F., Schulten, K. and Tajkhorshid, E. (2007) Exploring gas permeability of cellular membranes and membrane channels with molecular dynamics. J Struct Biol **157**, 534-544
34 Han, B. G., Guliaev, A. B., Walian, P. J. and Jap, B. K. (2006) Water transport in AQP0 aquaporin: molecular dynamics studies. J Mol Biol **360**, 285-296
35 Zhu, F., Tajkhorshid, E. and Schulten, K. (2004) Theory and simulation of water permeation in aquaporin-1. Biophys. J. **86**, 50-57
36 Zhu, F., Tajkhorshid, E. and Schulten, K. (2002) Pressure-induced water transport in membrane channels studied by molecular dynamics. Biophys. J. **83**, 154-160
37 Dynowski, M. and Ludewig, U. (2006) TrpAQP: Computer simulations to determine the selectivity of Aquaporins. High Performance Computing in Science and Engineering '06, Nagel, W.E., Jager, W. & Resch, M. (Eds.), 187-197

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38 Branco, M. R., Marinho, H. S., Cyrne, L. and Antunes, F. (2004) Decrease of H_2O_2 plasma membrane permeability during adaptation to H_2O_2 in Saccharomyces cerevisiae. J. Biol. Chem. **279**, 6501-6506
39 Jamieson, D. J. (1998) Oxidative stress responses of the yeast Saccharomyces cerevisiae. Yeast **14**, 1511-1527
40 Antunes, F. and Cadenas, E. (2000) Estimation of H_2O_2 gradients across biomembranes. FEBS Lett. **475**, 121-126
41 Seaver, L. C. and Imlay, J. A. (2001) Hydrogen Peroxide Fluxes and Compartmentalization inside Growing Escherichia coli. J. Bacteriol. **183**, 7182-7189
42 Carbrey, J. M., Bonhivers, M., Boeke, J. D. and Agre, P. (2001) Aquaporins in Saccharomyces: Characterization of a second functional water channel protein. Proc. Natl. Acad. Sci. U S A **98**, 1000-1005
43 Walter, A. and Gutknecht, J. (1986) Permeability of small nonelectrolytes through lipid bilayer membranes. J. Membr. Biol. **90**, 207-217
44 Uemura, M., Joseph, R. A. and Steponkus, P. L. (1995) Cold Acclimation of Arabidopsis thaliana (Effect on Plasma Membrane Lipid Composition and Freeze-Induced Lesions). Plant Physiol **109**, 15-30
45 Law, R. J. and Sansom, M. S. (2004) Homology modelling and molecular dynamics simulations: comparative studies of human aquaporin-1. Eur. Biophys. J. **33**, 477-489
46 Savage, D. F., Egea, P. F., Robles-Colmenares, Y., Iii, J. D. and Stroud, R. M. (2003) Architecture and selectivity in aquaporins: 2.5 a x-ray structure of aquaporin z. PLoS Biol. **1**, E72

Figure legends

Fig. 1. Growth of *Saccharomyces cerevisiae* is sensitive to H_2O_2 and sensitivity is increased by aquaporin expression. (A) Saturated cultures of 23344c cells transformed with pDR195 alone or pDR195-AtPIP2;1 (of same optical density) were spotted in 5 fold dilutions on medium not supplemented with hydrogen peroxide (upper), or supplemented with 0.4 mM H_2O_2 (middle), or 1.6 mM H_2O_2 (lower panel). (B) Increased H_2O_2 levels in *Saccharomyces cerevisiae* cells expressing the plant plasma membrane aquaporin AtPIP2;1. Yeast *yca1* cells transformed with either empty pDR195 or pDR195-*AtPIP2;1* were grown to midlog phase and pre-incubated in 2'7'-dichloro-dihydrofluorescein diacetate. Normalized fluorescence (by cell density) detected 30 min after application of 2 mM or 10 mM H_2O_2 is shown.

Fig. 2. Mutations that affect water conduction also affect sensitivity to H_2O_2. (A) Western blot of c-myc tagged AtPIP2;1, the AtPIP2;1 mutant H199K and AtNIP1;1. The monomeric protein band is shown in all cases, Kes1p an oxysterol binding protein from yeast, served as a loading control. (B) Side view of a homology structure of a monomeric AtPIP2;1 pore in ribbon representation. The His199 residue is highlighted by the arrow. (C) Saturated cultures of BY4741 yeast cells transformed with pDR195 alone, pDR195 harboring AtPIP2;1 or the regulatory mutant H199K were spotted in 5-fold serial dilutions. Control plates (no H_2O_2 added) left upper panel and plates containing the indicated hydrogen peroxide concentrations.

Fig. 3. Pore mutations alter sensitivity to H_2O_2 and various other substrates
(A) Expression of pore mutants in the selectivity filter region (the aromatic/arginine region) does not affect growth on standard media. The yeast strain used in this figure was Δ*dur3*. (B) The AtPIP2;1$^{NIP5;1}$ mutant affects yeast sensitivity to boron (B) and H_2O_2 (C). AtPIP2;1$^{NIP5;1}$ rescues growth on urea in absence of an alternative nitrogen source (D). AtPIP2;1$^{NIP1;2}$ is a mutant construct with residues as in AtNIP1;2 (F87W, H216V, T225A); the respective residues in the "selectivity filter" of AtNIP1;2 are Trp, Val, Ala and Arg. AtPIP2;1$^{NIP5;1}$ has

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residues F87A, H216I, T225G; the respective residues in the "selectivity filter" of AtNIP5;1 are Ala, Ile, Gly and Arg.

Fig. 4. Several plant aquaporins increase H_2O_2 sensitivity. Spotted dilutions of yeast BY4741 cells transformed with either empty plasmid pDR195 or plant major intrinsic proteins in pDR195 onto media not supplemented with hydrogen peroxide show equal growth (left panel). Spotted 5-fold dilutions at 1 mM, 2 mM and 2.5 mM hydrogen peroxide (right panels).

Fig. 5. Setup of the molecular dynamics simulations on tetrameric open SoPIP2;1. (A) Side view of the tetramer embedded in a lipid bilayer with the individual starting positions of the waters (or H_2O_2, urea) explicitly shown (arrows). (B) Snapshot of the water-filled structure of an open pore monomer. Residues of the NPA region (residues 101-103 and 222-224) are explicitly shown in yellow. The external pore exit was defined as Cα of lysine 144 (orange) and the internal exit as Cα of proline 33 (orange). Note the coordinated water dipole orientation within the pore.

Fig. 6. Molecular dynamics simulations of H_2O conduction by SoPIP2;1. Trajectories of water (A), hydrogen peroxide (B) and urea (C) across the open SoPIP2;1 pores from individual simulations (colored lines in each panel). The trajectories were calculated relative to Cα of valine 206. Note the starting position of solutes at -35 Å. The average residence time for each molecule is shown at the right of each panel (water: blue, hydrogen peroxide: red, urea: green). (D) The energy profile deduced from the residence time for water (blue), hydrogen peroxide (red), and urea (green) across the SoPIP2;1 pore. The variance along the z-axis of the entrance, selectivity filter (SF), NPA-region and exit is also shown. Open monomers with preferred sites and orientation of H_2O_2 (E) and urea (F). The residues of the constriction site (ar/R, blue) and the NPA region (yellow) are explicitly shown. Note the flexible cytoplasmic loop D that is involved in closing of the pore (shown in green).

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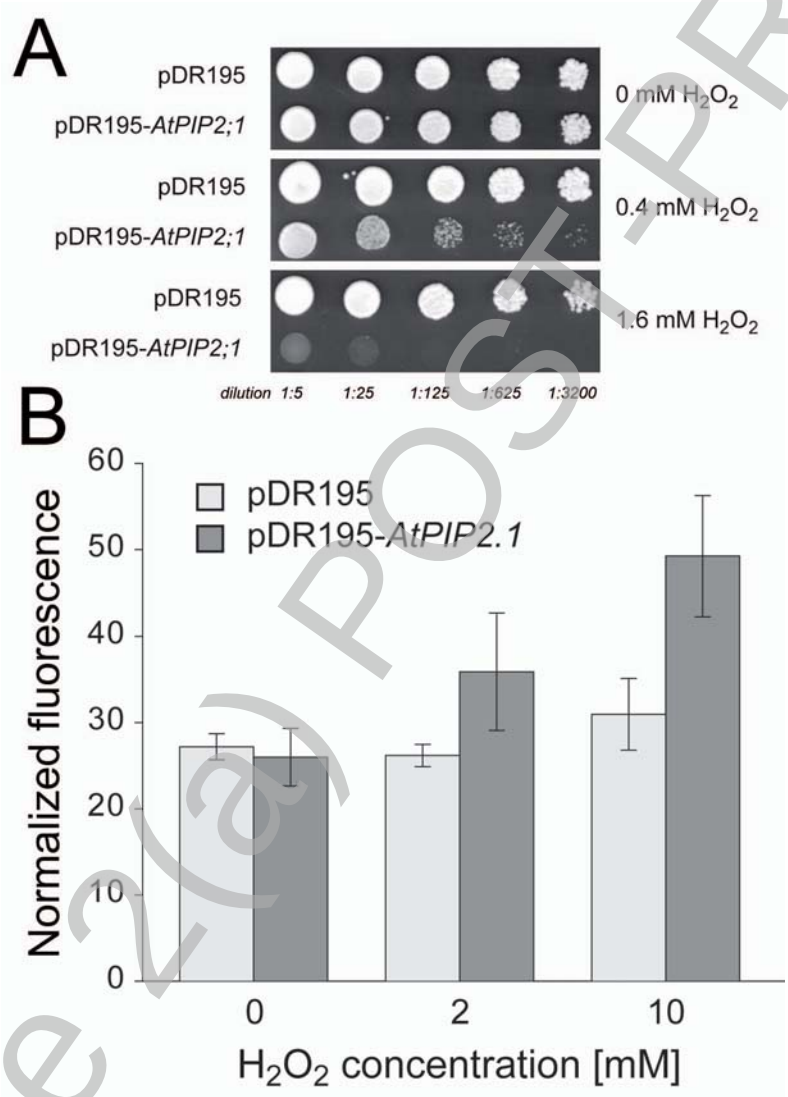

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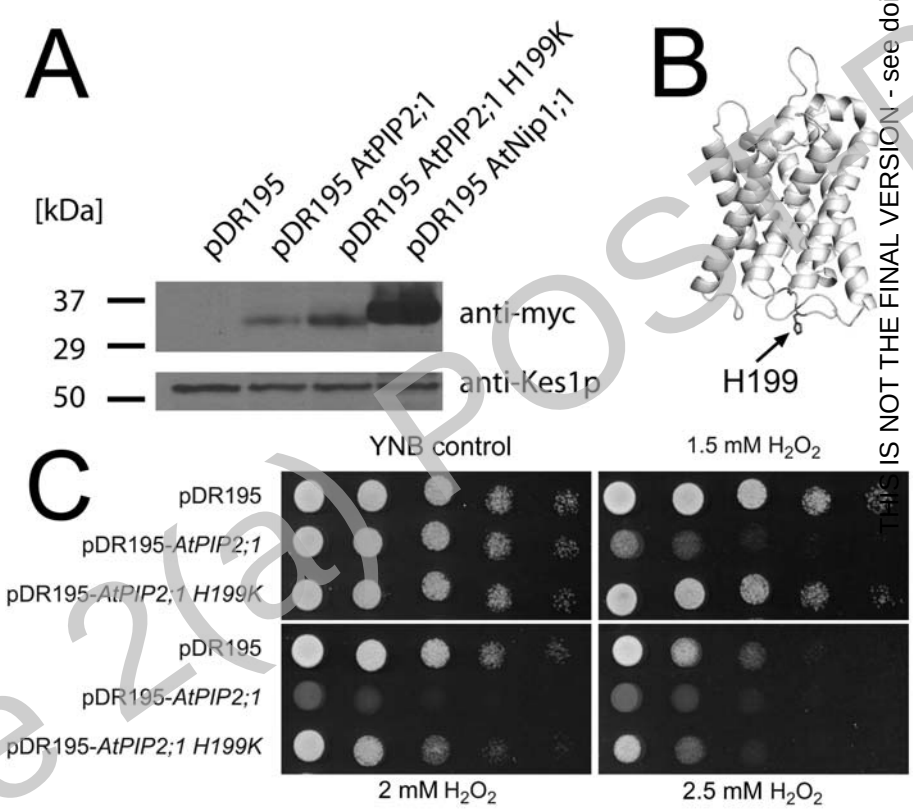

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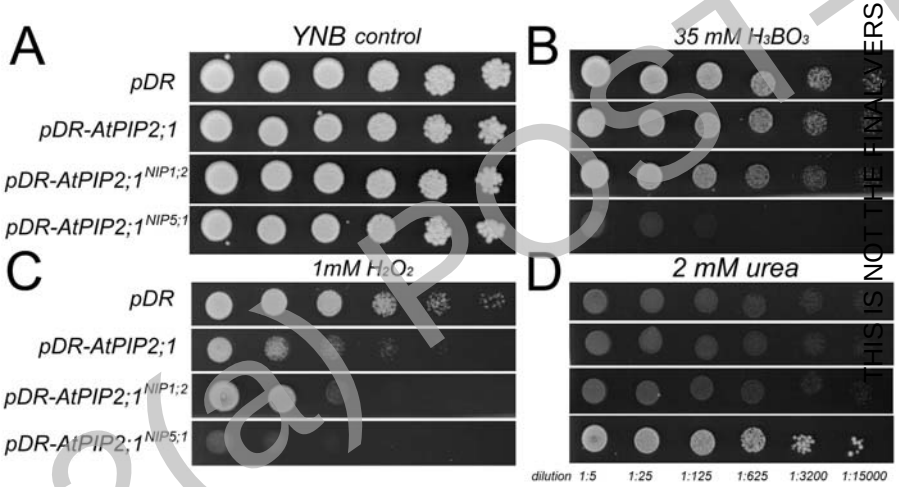

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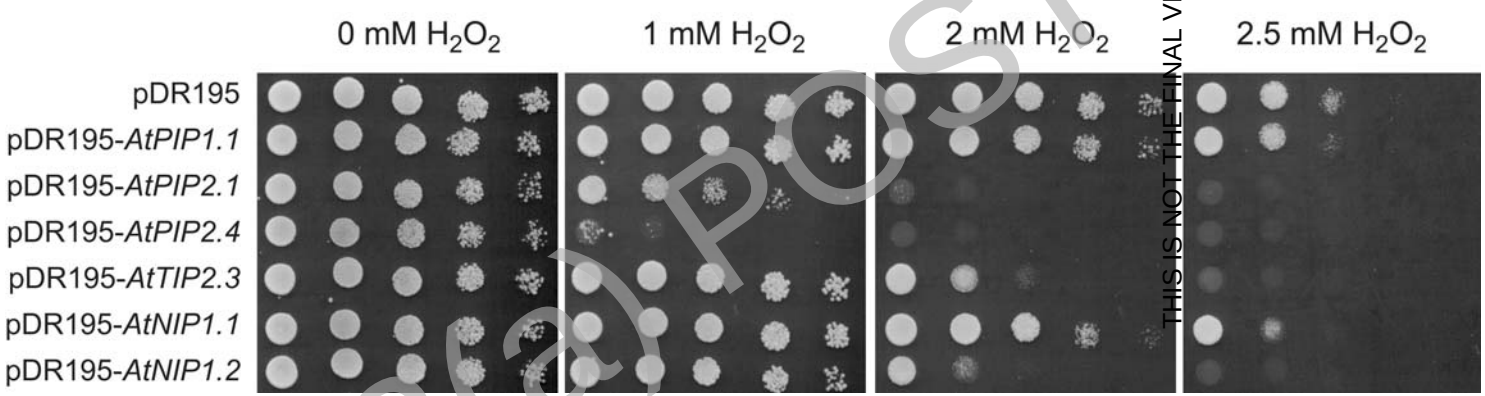

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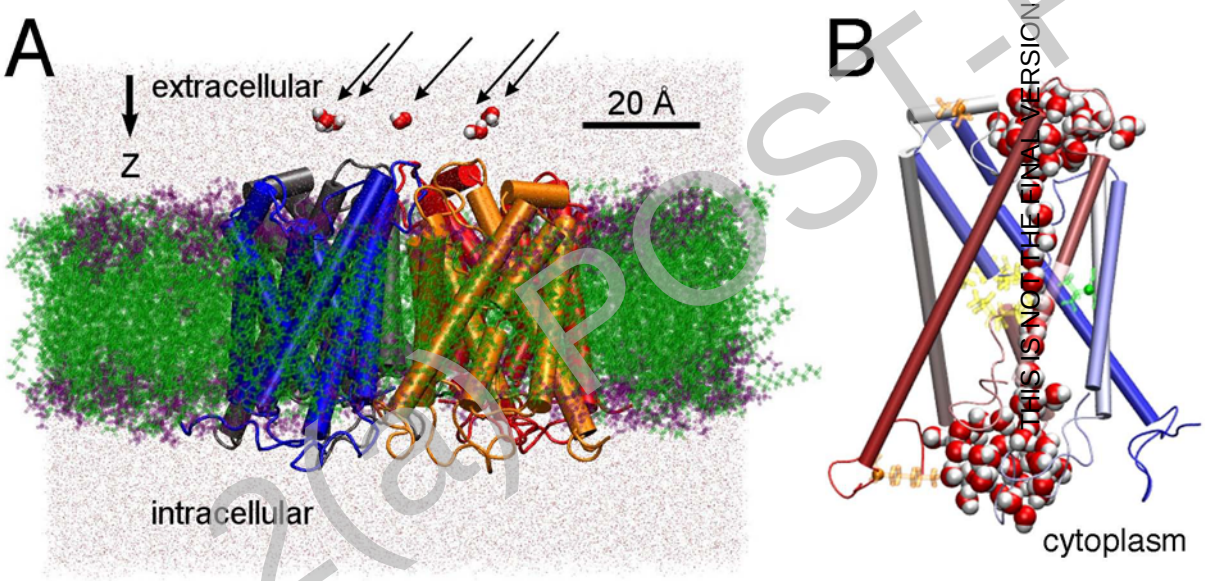

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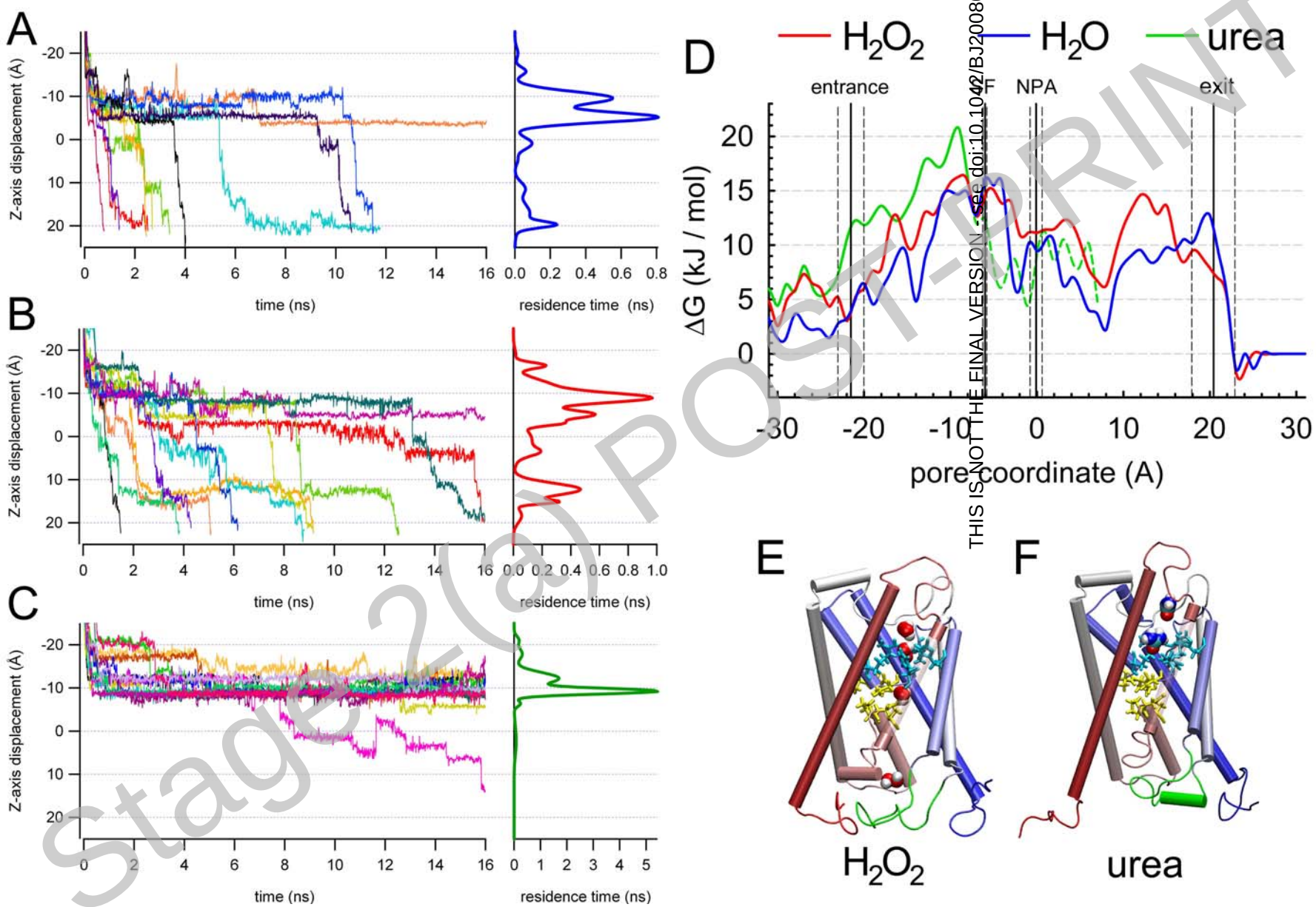

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