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Maize plasma membrane aquaporin ZmPIP2;5, but not ZmPIP1;2, facilitates transmembrane diffusion of hydrogen peroxide

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ABSTRACT

Plant aquaporins play important roles in transmembrane water transport processes, but some also facilitate the diffusion of other small uncharged solutes ranging from gases to metalloids. Recent evidence suggests that the transmembrane movement of hydrogen peroxide, an intra- and intercellular multifunctional signaling and defense compound, can be regulated by aquaporins. We addressed the question whether maize aquaporins belonging to the plasma membrane intrinsic protein (PIP) subfamily facilitate hydrogen peroxide diffusion using heterologous expression in the yeast *Saccharomyces cerevisiae*. We showed that ZmPIP proteins belonging to the PIP1 and PIP2 groups were significantly expressed in yeast cells only after codon optimization of their cDNA. In accordance with previous localization studies in oocytes and plants, ZmPIP1;2 was mainly retained in intracellular membranes, while ZmPIP2;5 was localized to the plasma membrane. However, upon co-expression with ZmPIP2;5, ZmPIP1;2 was re-localized to the plasma membrane. Using a non-functional plasma membrane-localized ZmPIP2;5 mutant to deliver ZmPIP1;2 to the plasma membrane, we demonstrated that, in contrast to wild type ZmPIP2;5, ZmPIP1;2 was not permeable to hydrogen peroxide. Our study further highlighted the fact that, when using the yeast system, which is widely employed to study substrates for plant aquaporins and other transporters, although positive transport assay results allow direct conclusions to be drawn regarding solute permeability, negative results require additional control experiments to show that the protein is expressed and localized correctly before concluding on the lack of transport activity.

1. Introduction

Hydrogen peroxide (H_2O_2) is a signaling molecule controlling a wide spectrum of metabolic processes in all organisms [1]. However, it is a reactive oxygen species and can oxidize various cellular targets, thereby causing cell damage or even death. Cellular levels of H_2O_2 must therefore be tightly regulated and one way to do this is to regulate the membrane permeability for H_2O_2. Recently, it was established that aquaporins (AQPs) are important in facilitating the diffusion of H_2O_2 across membranes [2–5].

AQPs belong to the major intrinsic protein superfamily and play important roles in facilitating the passive bi-directional diffusion of water and/or a variety of small and non-charged compounds across membranes in organisms from all kingdoms of life [6]. The substrate specificity of AQPs is isoform-dependent and determined by selectivity filters along the channel. AQPs represent a potentially fast regulatory system for controlling H_2O_2 membrane permeability compared to the rather slow metabolic modification of the plasma membrane by a change in its chemical composition. H_2O_2 shares several physicochemical features with water (e.g. size, electrochemical properties, and ability to form hydrogen bonds), which makes it a good candidate for a typical AQP substrate (reviewed in [5]). A comprehensive screen aimed at discovering H_2O_2-permeable AQPs revealed that Arabidopsis tonoplast intrinsic protein 1 and human AQP8 are highly permeable to H_2O_2 [2]. Later, the ability to facilitate the transmembrane movement of H_2O_2 was demonstrated for a variety of other isoforms from different plant AQP subfamilies, namely plasma membrane intrinsic proteins (PIPs), nodulin26-like intrinsic proteins, and X-intrinsic proteins, when heterologously expressed in yeast [3,7,8]. In addition, molecular dynamic simulation analysis strongly supports the idea that some PIP isoforms show H_2O_2 permeability [3].

While knowledge about the H_2O_2 permeability of various plant AQPs has been obtained in heterologous expression systems and molecular simulation studies, mammalian AQPs have been demonstrated to also facilitate H_2O_2 transport in a homologous system [4]. These authors showed that AQP8 promotes the uptake of H_2O_2 directly into mammalian cells and that the level of endogenous AQP3 expression can modulate intracellular accumulation of H_2O_2. More importantly, permeability of AQP3 to extracellularly generated H_2O_2 was found to be necessary for downstream intracellular signaling by H_2O_2 [4]. Before speculating about the *in planta* functions of certain plant AQP isoforms in H_2O_2

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channeling, it is necessary to determine that these isoforms can transport H_2O_2 across the plasma membrane.

Expression of several *Arabidopsis thaliana* AtPIP2 isoforms increases the sensitivity of yeast cells to H_2O_2, while expression of AtPIP1s does not [3,8,9]. The division into the two sequence-related PIP1 and PIP2 groups is based on phylogenetic analyses. Compared to PIP2s, PIP1s have a longer N-terminal section, a shorter C-terminal section, and a shorter extracellular loop A [10]. PIPs assemble in membranes as tetramers, in which each monomer represents a functional channel. PIP1s and PIP2s exhibit different water channel activities when expressed in *Xenopus laevis* oocytes, with only the PIP2 isoforms inducing a high transmembrane water flow [11]. When co-expressed, *Zea mays* ZmPIP1s and ZmPIP2s physically interact to modify their trafficking to, and/or stability within, the oocyte membrane and synergistically increase the water permeability [11]. Similar results have been obtained with PIP1 and PIP2 isoforms from *Mimosa pudica*, *Nicotiana tabacum*, *Beta vulgaris*, *Vitis vinifera*, *Triticum turgidum*, and *Hordeum vulgare* [12–17]. When expressed singly in maize cells, ZmPIP1s and ZmPIP2s differ in their subcellular localization, ZmPIP1s being retained in the endoplasmic reticulum (ER), whereas ZmPIP2s are targeted to the plasma membrane, and, on co-expression, ZmPIP1s are re-localized to the plasma membrane as a result of their physical interaction with ZmPIP2s [18]. These data indicate that ZmPIP2s, but not ZmPIP1s, possess signals that allow them to be transported to the plasma membrane and that hetero-oligomerization of the two proteins in tetramers is required for trafficking of ZmPIP1s to the plasma membrane [18]. In addition to the impact on subcellular localization and water permeability, hetero-oligomerization of PIP1 and PIP2 isoforms modulates other protein features, such as acid inhibition, mercury sensitivity, and solute selectivity [13,16,19].

No data on the H_2O_2 permeability of PIP1s or PIP2s from monocots are yet available. The aim of this study was therefore to determine whether maize ZmPIP1;2 or ZmPIP2;5 is able to facilitate the movement of H_2O_2. As both isoforms form hetero-oligomers, we also wanted to determine whether hetero-tetramers have a different effect on H_2O_2 permeability compared to PIP1 or PIP2 homo-tetramers. We also investigated whether co-expression of ZmPIP1;2 and ZmPIP2;5 was necessary for the efficient plasma membrane localization of ZmPIP1;2 in *Saccharomyces cerevisiae*, as is the case in oocytes and plant cells. We showed that ZmPIP1;2 and ZmPIP2;5 could only be efficiently immunodetected when codon-optimized sequences were expressed in yeast. ZmPIP1;2 was mainly retained in intracellular membranes, while ZmPIP2;5 was localized to the plasma membrane, but, following co-expression, ZmPIP1;2 was also localized to the plasma membrane. When expressed alone, ZmPIP2;5 increased the sensitivity of yeast to externally applied H_2O_2. To determine whether ZmPIP1;2 was also able to conduct H_2O_2, it was co-expressed with an inactive mutated ZmPIP2;5 which was still able to traffic ZmPIP1;2 to the plasma membrane, and the results strongly suggested that ZmPIP1;2 is not permeable to H_2O_2.

2. Materials and methods

2.1. Cloning and vector construction

ZmPIP1;2 and *ZmPIP2;5* cDNAs were optimized for expression in yeast by adapting their codon usage (performed by GeneArt®, Regensburg, Germany) to obtain *ZmPIP1;2*^{OPT} and *ZmPIP2;5* ^{OPT}. Primers (Table S1) matching the *ZmPIP* sequences were used to PCR amplify the ZmPIP cDNA sequences and generate the respective *ZmPIP* constructs (Table S2). The PCR products were directionally sub-cloned into either the USER-yeast expression vector pYeDP60u [20], pRS425-pTPIu, or pRS426-pTPIu using a uracil excision-based improved high-throughput USER cloning technique [21] or into the yeast expression vector pRS416-pTPI-N-ter-GFP using restriction/ligation cloning (SpeI and XhoI).

2.2. Yeast strains and growth assay

A wild-type *S. cerevisiae* strain (BY4741) and a deletion mutant strain 31019b (*Δmep1–3*) [22] were transformed with either an empty vector (control) or vectors carrying the cDNAs encoding the *AQP* homologues. The yeast cells were grown on synthetic medium containing 2% galactose (SG) (strain31019b) or glucose (SD) (wild-type), 50 mM succinic acid/Tris base, pH 5.5, 0.7% yeast nitrogen base without amino acids (Difco). The medium was supplemented, according to the auxotrophic requirements, with 0.3% methionine (M), 0.3% histidine (H), and 0.3% leucine (L) when BY4741 was transformed with one construct or with M and L when it was transformed with two constructs. Growth of the transformants was tested as described by Bienert et al. [2]. Yeast cells were diluted in sterile water to different A_{600} values (1, 0.01, and 0.0001) and the dilutions spotted on solid medium containing different concentrations of H_2O_2 (SD-HM medium in the co-expression experiments using BY4741 wild type yeast and SG medium in the single expression experiments using the 31019b yeast strain). Growth was examined after incubation for 5–8 days at 28 °C after spotting.

2.3. Membrane protein extraction and Western blotting

S. cerevisiae strain 31019b (*Δmep1–3*) expressing individual AQPs or transformed with the empty vector pYeDP60u were pre-cultured overnight in uracil-free medium, then expression was induced overnight in 1% yeast extract, 2% peptone (both from Difco), and 2% galactose (Sigma) and the cells harvested. All subsequent steps were at 4 °C. The cells were suspended in homogenization buffer [220 mM Tris/HCl pH 7.5, 42 mM EDTA pH 8, 42.5% glycerol, 10 μM ATP, and 2 μg/ml protease inhibitors (leupeptin, aprotinin, antipain, pepstatin and chymostatin)], and broken using acid-washed glass beads (Sigma). Cell debris was removed by two centrifugations (5000 *g* and 6000 *g*, both for 5 min) and the microsomal fraction collected by centrifugation at 14,000 *g* for 1 h. The pellets were resuspended in suspension buffer (20% glycerol, 100 mM Tris/HCl pH 7.5, 1 mM EDTA pH 8, 1 mM DTT, and proteinase inhibitor cocktail) and 10 μg of microsomal membrane proteins (quantified using the bicinchoninic acid assay) separated by SDS-PAGE on 12% gels, transferred onto a polyvinylidene difluoride membrane, and subjected to Western blotting using antibodies raised against ZmPIP1;2 or ZmPIP2;5 [23] and the enhanced bioluminescence method.

2.4. Expression of GFP-PIP fusion proteins and microscopy

Yeast cells expressing the fusion protein alone or together with another non-tagged PIP were grown on SD medium (without uracil or without uracil and leucine). During the exponential growth phase (A_{600} = 1.3 to 2.0), the fluorescence signal from the expressed fusion protein was observed to study its subcellular localization using an epifluorescence microscope (UV excitation: 470–540 nm, emission: 525–550 nm, Leica, Wetzlar, Germany).

3. Results

3.1. Expression of recombinant ZmPIP1;2 and ZmPIP2;5 in yeast requires codon optimization of the open reading frame

When heterologously expressed in *S. cerevisiae*, several Arabidopsis PIP2 isoforms facilitate the transmembrane diffusion of H_2O_2 [3,8]. We therefore tested whether PIPs from monocots have the same property. To do so, *ZmPIP1;2* or *ZmPIP2;5* cDNA was cloned into the yeast vector pYeDP60u under the control of the galactose-inducible promoter and introduced into the *Δmep1–3* yeast strain (see Materials and methods). As shown in Fig. 1, cells transformed with an empty vector (negative control) were not sensitive to the presence of concentrations of H_2O_2 up to at least 1 mM in the external growth medium and similar results were

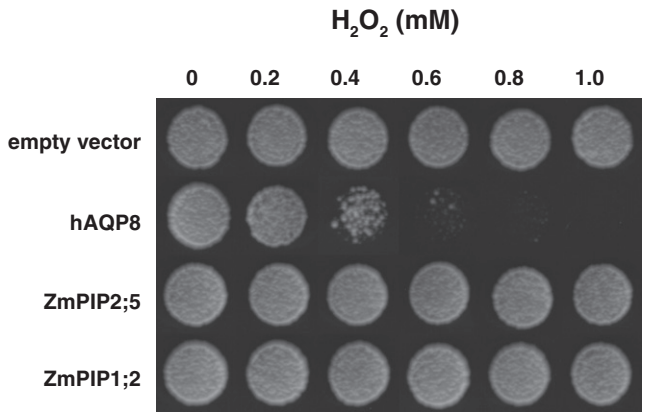

Fig. 1. Hydrogen peroxide sensitivity of yeast cells expressing maize ZmPIP1;2 and ZmPIP2;5. Cultures of Δ*mep1–3* yeast cells transformed with the empty vector pYeDP60u or pYeDP60u carrying the indicated *AQP* cDNA were diluted in sterile distilled water to an A_{600} of 0.01 and spotted on medium containing the indicated concentration of hydrogen peroxide and growth recorded after 8 days at 28 °C.

obtained for cells transformed with the *ZmPIP1;2*- or *ZmPIP2;5*-containing vector after induction by galactose. In contrast, expression of hAQP8, which was shown to conduct H_2O_2 in a previous study [2], resulted in yeast cells showing sensitivity to H_2O_2 at a concentration of 0.4 mM, with cell growth being completely inhibited in the presence of 0.8 mM H_2O_2.

The observation that ZmPIP2;5 did not facilitate the transmembrane diffusion of H_2O_2 in yeast was unexpected, as it contains the same amino acid residues in its selectivity filter as known H_2O_2-permeable Arabidopsis PIPs and shows an overall sequence identity of >75% with these PIPs. It was previously shown that the expression of cDNA sequences of plant AQPs in yeast might fail to result in the production of a functional protein [24–26]. To test whether ZmPIP1;2 and ZmPIP2;5 were expressed, microsomes from *Δmep1–3* yeast cells transfected with an empty vector or the *ZmPIP1;2*- or *ZmPIP2;5*-containing vector were prepared after 14 h of galactose induction and the presence of ZmPIP1;2 or ZmPIP2;5 examined by Western blotting using specific antibodies [23]. Compared to empty vector transformants, no specific signals were detected in the extracts from yeast transformed with the PIP isoforms at the apparent molecular masses of 26 and 55 kDa (Fig. 2) corresponding, respectively, to the monomeric and disulfide-bridged dimeric forms of ZmPIP1;2 and ZmPIP2;5 [19], showing lack of expression of ZmPIP1;2 and ZmPIP2;5.

One possible explanation of this lack of expression was inefficient translation of the mRNAs due to a difference in A/T and G/C nucleotide content and codon use frequency between yeast and maize. Studies on recombinant AQP production in the yeast *Pichia pastoris* have demonstrated the importance of codon optimization for enhanced protein expression [27,28]. The GC nucleotide content and codon usage for *ZmPIP1;2* and *ZmPIP2;5* were adapted to that in yeast, resulting in the constructs *ZmPIP1;2*^{OPT} and *ZmPIP2;5*^{OPT}. The codon adaptation index (CAI) is a measure of how well the codon usage in a coding DNA sequence matches the bias of a certain host. Gene optimization increased the CAI for *ZmPIP1;2* and *ZmPIP2;5* from 0.58 to 0.90 and from 0.48 to 0.93, respectively (Table 1). Values above 0.9 are considered to result in high expression rates. To test whether codon optimization was sufficient for expression of ZmPIP1;2 and ZmPIP2;5, we extracted microsomes from Δ*mep1–3* cells transformed with the *ZmPIP1;2*^{OPT}- or *ZmPIP2;5*^{OPT}-containing vectors 14 h after protein induction and, as shown in Fig. 2A, Western blotting showed the presence of strong signals for ZmPIP1;2 and ZmPIP2;5 at apparent molecular masses of 26, 55, and 110 kDa corresponding, respectively, to the monomeric, dimeric, and tetrameric forms. Since identical amounts of proteins were loaded on the gels (Fig. 2B), these data demonstrated that codon

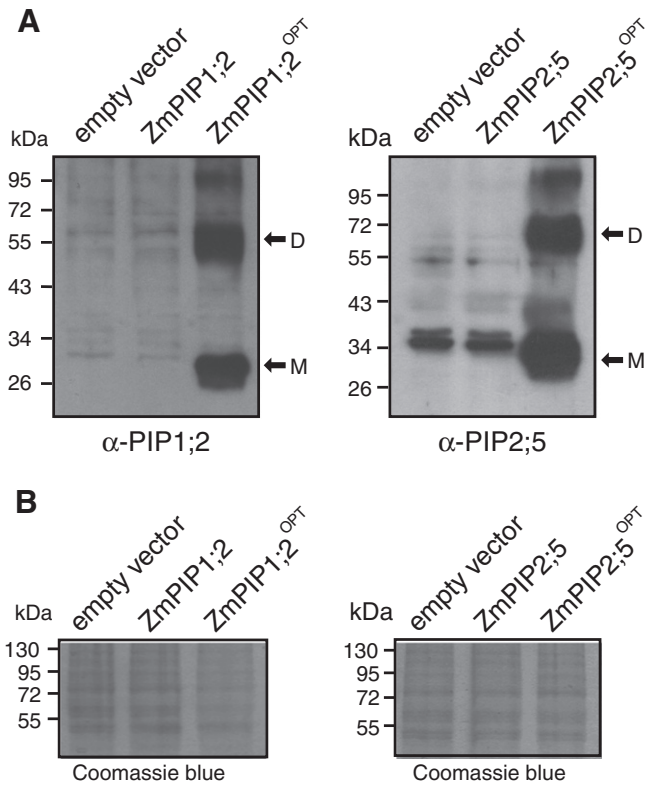

Fig. 2. Heterologous expression of ZmPIP1;2 and ZmPIP2;5 in *S. cerevisiae*. Western blot showing the expression in *S. cerevisiae* of ZmPIP1;2 and ZmPIP2;5 using vectors containing either the native *ZmPIP1;2* or *ZmPIP2;5* cDNAs or the codon-optimized *ZmPIP1;2*^{OPT} or *ZmPIP2;5*^{OPT} cDNAs after induction of the GAL10-CYC1 promoter. Microsomal membrane proteins from Δ*mep1–3* yeast cells transformed with the empty vector pYeDP60u or pYeDP60u carrying the indicated *AQP* homolog were isolated and 10 μg subjected to SDS gel electrophoresis and Western blotting using antibodies against ZmPIP1;2 or ZmPIP2;5 (A). Coomassie blue staining of the corresponding gels are shown (B). The positions of the monomeric (M) and dimeric (D) forms of PIPs and the molecular mass markers are indicated.

optimization of *ZmPIP* cDNAs highly increased the production of the proteins.

3.2. Expression of ZmPIP2;5, but not ZmPIP1;2, increases the hydrogen peroxide sensitivity of yeast cells

As shown in Fig. 3, expression of ZmPIP2;5 using the *ZmPIP2;5*^{OPT} vector increased the sensitivity of yeast cells to H_2O_2. Their growth was inhibited in a dose-dependent manner at H_2O_2 concentrations of 0.4 mM or higher, similar to the results seen with the highly H_2O_2-permeable positive control hAQP8. In contrast, yeast cells transformed with an empty vector or the *ZmPIP1;2*^{OPT} vector were not sensitive to concentrations of H_2O_2 up to at least 1 mM. These results suggest that ZmPIP2;5, but not ZmPIP1;2, facilitates the transport of H_2O_2 across yeast membranes.

Table 1
Codon optimization of *ZmPIP1;2* and *ZmPIP2;5* cDNAs for expression in *S. cerevisiae*.

Aquaporin isoform	Codon adaptation index (CAI)	Average GC content (%)	Sequence identity (%)	Modified codons (%)
ZmPIP2;5	0.48	67		
ZmPIP2;5^{OPT}	0.93	47	74	66
ZmPIP1;2	0.58	60		
ZmPIP1;2^{OPT}	0.90	47	79	49

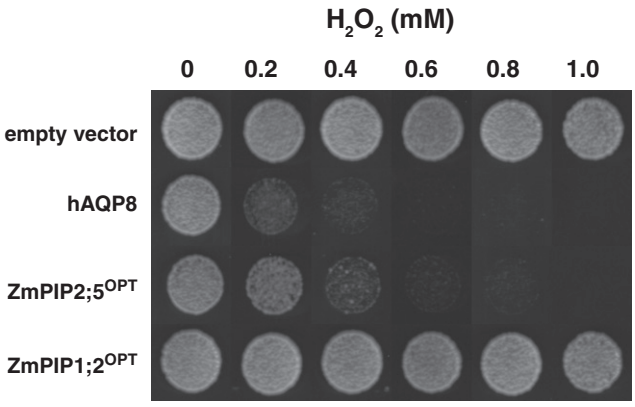

Fig. 3. Hydrogen peroxide sensitivity of yeast cells expressing ZmPIP1;2 or ZmPIP2;5 from the codon-optimized ZmPIP1;2^{OPT} and ZmPIP2;5 ^{OPT} cDNAs. Cultures of Δ*mep1–3* yeast cells transformed with the empty vector pYeDP60u or pYeDP60u carrying the indicated *AQP* cDNA were diluted in sterile distilled water to an A_{600} of 0.01 and spotted on medium containing the indicated concentrations of hydrogen peroxide and growth recorded after 6 days at 28 °C.

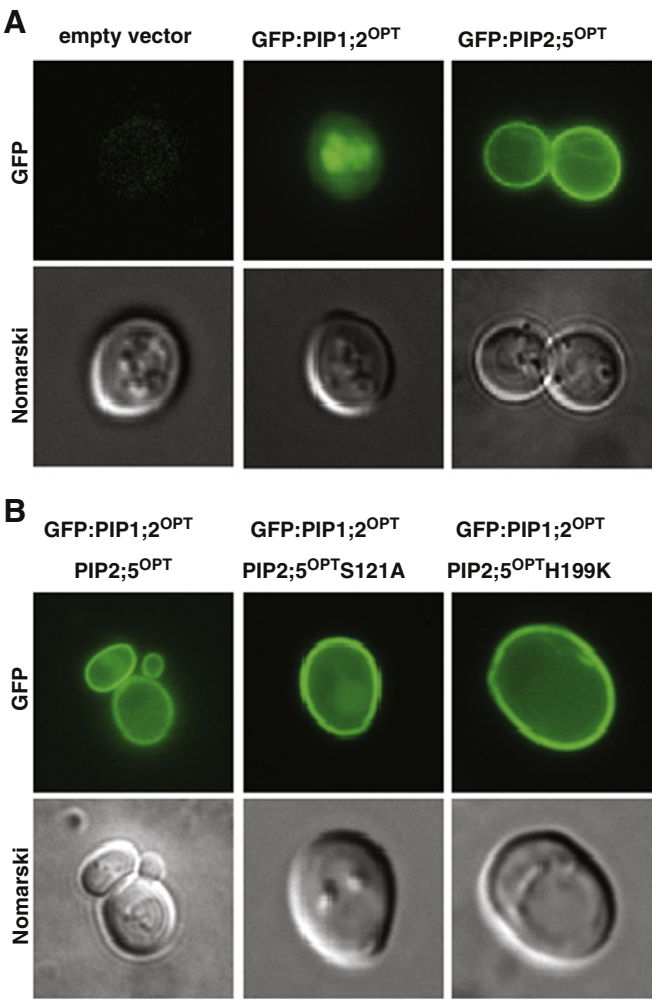

Fig. 4. Localization of GFP-ZmPIP proteins in yeast cells. Wild-type *S. cerevisiae* cells (BY4741) expressing GFP:ZmPIP1;2^{OPT} or GFP:ZmPIP2;5^{OPT} (A) or co-expressing GFP:ZmPIP1;2^{OPT} and ZmPIP2;5^{OPT}, ZmPIP2;5^{OPT}S121A, or ZmPIP2;5^{OPT}H199K (B) were examined by fluorescence microscopy with Nomarski optics.

3.3. Co-expression of ZmPIP2;5 and ZmPIP1;2 results in the re-localization of ZmPIP1;2 to the plasma membrane

To investigate whether the lack of H_2O_2 sensitivity of ZmPIP1;2-expressing cells was due to the failure of ZmPIP1;2 to reach the plasma membrane, we investigated the subcellular localization of each of the ZmPIP isoforms fused to GFP; N-terminal fusion of GFP to ZmPIPs was chosen, as this has been shown not to affect either the activity of ZmPIPs or their ability to interact in Xenopus oocytes or plant cells [11,18,19]. As shown in Fig. 4A, yeast cells expressing GFP:ZmPIP2;5 showed a sharp fluorescent signal around the cell corresponding to the plasma membrane, in addition to some internal signals, while GFP:ZmPIP1;2-expressing cells showed fluorescent signals mainly in internal structures and the cytosol, and not in the plasma membrane.

We previously showed that, in Xenopus oocytes and maize protoplasts, ZmPIP1;2 and ZmPIP2;5 interact to control the amount and/or stability of ZmPIP1;2 in the plasma membrane [11,18]. We therefore investigated the localization of GFP:ZmPIP1;2 in yeast cells when co-expressed with ZmPIP2;5. As shown in Fig. 4B (left panel), a strong and sharp fluorescence signal was detected in the plasma membrane, similar to that seen with GFP:ZmPIP2;5 expressed alone (Fig. 4A), demonstrating that, as observed in oocytes and plant cells, ZmPIP1;2 has to be co-expressed with ZmPIP2;5 in order to be detected in the yeast plasma membrane.

3.4. ZmPIP1;2 does not facilitate the diffusion of hydrogen peroxide across yeast membranes

To investigate whether ZmPIP1;2 was permeable to H_2O_2, we developed a test in which ZmPIP1;2 was delivered to the plasma membrane without its activity being masked by the presence of other active AQPs. As shown above (Fig. 4B), co-expression of ZmPIP1;2 and ZmPIP2;5 resulted in plasma membrane localization of ZmPIP1;2, but, since ZmPIP2;5 is permeable to H_2O_2, it would be difficult to demonstrate any potential H_2O_2 transport activity of ZmPIP1;2 in this system. However, a non-functional ZmPIP2;5 mutant that was still able to bring ZmPIP1;2 to the plasma membrane would allow such a differentiation. Two PIP2 mutants have previously been shown to be non-functional with respect to water and/or H_2O_2 transport in yeast. One of these, *N. tabacum* NtPIP2;1S118A, is a non-functional water channel even though it reaches the plasma membrane [29], but whether NtPIP2;1 is permeable to H_2O_2 is not known. The other, AtPIP2;1H199K, does not conduct H_2O_2 and water through yeast membranes, but whether it localizes to the plasma membrane is not known [3]. We mutated both homologous amino acid residues in ZmPIP2;5 to generate ZmPIP2;5S121A and ZmPIP2;5H199K and tested whether the mutated proteins were still able to deliver ZmPIP1;2 to the plasma membrane. As shown in Fig. 4B, co-expression of ZmPIP2;5S121A (center panel) or ZmPIP2;5H199K (right panel) with GFP:ZmPIP1;2 resulted in strong and sharp fluorescent signals in the cell periphery, demonstrating the plasma membrane localization of GFP:ZmPIP1;2 and the ability of these ZmPIP2;5 mutants to interact with, and deliver, GFP:ZmPIP1;2 to the plasma membrane.

We then tested the H_2O_2 channel activity of ZmPIP2;5S121A and ZmPIP2;5H199K in a toxicity growth assay. As shown in Fig. 5, when expressed alone, ZmPIP2;5S121A or ZmPIP2;5H199K did not increase the sensitivity of yeast to H_2O_2 up to a concentration of at least 1.2 mM, a result similar to that seen with the control or ZmPIP1;2-expressing cells, demonstrating that this system could be used to investigate the ability of ZmPIP1;2 to increase the sensitivity of yeast to H_2O_2 via facilitated transmembrane diffusion. When ZmPIP1;2 or GFP:ZmPIP1;2 was co-expressed with ZmPIP2;5S121A or ZmPIP2;5H199K, the growth of the respective yeast transformants was unaffected compared to the empty vector controls. These results show that plasma membrane-localized ZmPIP1;2 is not permeable to H_2O_2 and that the lack of phenotype is not due to failed expression or inadequate membrane localization.

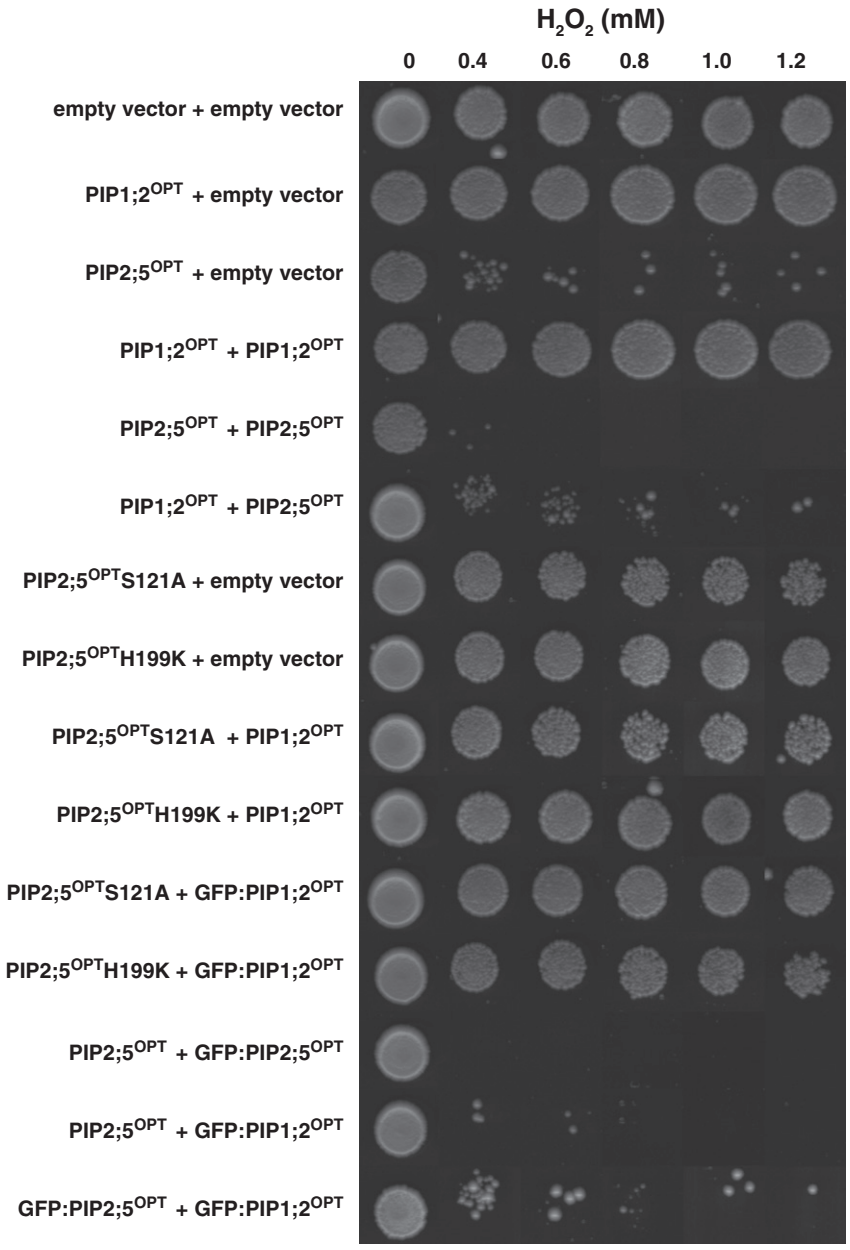

Fig. 5. Hydrogen peroxide sensitivity of yeast cells expressing ZmPIP1;2 in their plasma membrane. Cultures of wild-type *S. cerevisiae* cells (BY4741) co-transformed with the indicated combinations of empty vectors (pRS425-pTPlu or pRS426-pTPlu) and pRS425-pTPlu, pRS426-pTPlu, or pRS416-pTPI-N-ter-GFP carrying the indicated *AQP* cDNA were diluted in sterile distilled water to an A_{600} of 0.01 and spotted on medium containing the indicated concentrations of hydrogen peroxide and growth recorded after 7 days at 28 °C.

To exclude the possibility that the N-terminal tagging with GFP modified the PIP interaction or the functionality of ZmPIP1;2 or ZmPIP2;5, we co-expressed ZmPIP2;5 with either GFP:ZmPIP2;5 or GFP:ZmPIP1;2 and GFP:ZmPIP2;5 with GFP:ZmPIP1;2 and, as shown in Fig. 5, we found that the sensitivity of all co-expression transformants was similar to that of the ZmPIP2;5-expressing yeast cells, demonstrating that GFP-tagging did not affect the behavior of the protein. Finally, we showed that expression of the same ZmPIP isoform from two co-transformed vectors did not change the growth phenotype (Fig. 5).

4. Discussion

4.1. Codon optimization is needed for ZmPIP expression in yeast

The initial yeast growth and toxicity experiments, which resulted in a lack of growth phenotype compared to the positive control, might have suggested that ZmPIP1;2 and ZmPIP2;5 were not permeable to H_2O_2; however, immunodetection showed that the proteins were not expressed. These data demonstrate that the demonstration of protein expression is an essential control experiment when no transport function is observed. However, specific antibodies directed against the proteins of interest are not always available, especially when a huge range of proteins has to be analyzed. One alternative approach for verifying the expression of the proteins of interest is to fuse them to a detectable marker-tag (GFP-tag, HIS-tag, flag-tag, etc.). However, tagging of a protein might also result in a non-functional protein and this can only be tested if a function has already been experimentally demonstrated. If the protein of interest is not expressed, optimization of the construct design becomes necessary. If the sequence of interest comes from an organism with a different codon usage than the host species, codon optimization of the cDNA can successfully improve protein production. Optimization of the coding sequence of diverse AQPs in terms of both

codon composition and AT/GC content resulted in optimal protein production in *P. pastoris* and *S. cerevisiae* [27,28,30]. In our study, expression from codon-optimized *ZmPIP1;2*^{OPT} and *ZmPIP2;5*^{OPT} cDNAs led to greatly enhanced protein production compared to undetectable amounts using the non-optimized sequences. The GC content of coding sequences from monocots, including maize, barley, and rice, is generally much higher than that in eudicot or yeast sequences. The translation of monocot mRNA in the yeast system might require the use of rare codons and therefore result in very low protein production [31]. However, some monocot AQPs have been demonstrated to be functional when expressed in yeast [24,31,32].

4.2. Co-expression of ZmPIP1s and ZmPIP2s is needed for ZmPIP1s to be localized to the plasma membrane

Demonstration of the successful production of the protein in the absence of transport activity is still not sufficient to conclude that it has no transport ability. As discussed below, GFP-tagged ZmPIP1;2 was not detected in the yeast plasma membrane, indicating that no, or only a negligible amount of, ZmPIP1;2 reached the plasma membrane. Many transport assays in yeast rely on a plasma membrane localization of the protein to be investigated. In the case of proteins that are correctly or incorrectly localized to the internal membranes of yeast, re-targeting to the plasma membrane can be achieved by fusing it to a peptide or protein that will deliver it to the plasma membrane or by co-expressing it with a partner that will ensure plasma membrane localization, as is the case for maize PIP proteins [11]. Very low, or no, trafficking of various PIP1 isoforms to the plasma membrane is frequently observed in Xenopus oocytes and plant cells [11,18]. Here, we showed that correct ZmPIP1;2 plasma membrane localization in *S. cerevisiae* relied on the concomitant presence of ZmPIP2;5. Yeast is the third organism in which this interesting observation has been made, the others being the plant and oocyte systems. The observation that, in the absence of PIP2s, PIP1s do not reach the plasma membrane and/or are very unstable in these different cell types suggests that it is due to an intrinsic feature of PIP1 proteins, rather than the cellular, tissue, or organism context. This remarkable observation can be explained by conserved regulation mechanisms and the fact that PIP1s might (i) lack ER export signals, (ii) contain ER retention signals that are hidden after interaction with PIP2 proteins, or (iii) contain proteolytic degradation signals that are no longer accessible after interaction with PIP2 proteins. As expression of a few PIP1 isoforms has been shown to result in some plasma membrane localization and in transport phenotypes in oocytes and yeasts [16,25,33,34], it will be interesting to investigate in detail any sequence differences to try to identify key residues or motifs that are responsible for the detected differences in membrane localization and transport ability.

To date, no threshold level for the amount of AQP required in the plasma membrane to observe a transport phenotype in yeast, Xenopus, or plant cells has been established. Otto et al. [16] estimated the relative amount of NtAQP1 in the yeast plasma membrane as 4.1% of the total NtAQP1 expressed, which is a relatively low amount, but clearly enough to facilitate the membrane diffusion of CO_2. Co-expression of NtAQP1 with NtPIP2;1 results in an increase in total PIP oligomers in the plasma membrane to 14.2% [16]. Quantitative differences in the amount of a PIP in the plasma membrane might account for the conflicting transport ability of certain isoforms reported in different studies, as is the case for the H_2O_2 permeability of AtPIP2;1 [3,8].

4.3. Water and hydrogen peroxide transport by ZmPIPs

We demonstrated that the expression of ZmPIP2;5 increased the sensitivity of yeast to H_2O_2, indicating that at least some monocot PIP2s can facilitate the diffusion of this physiologically important molecule. This result is in agreement with previous studies showing that PIP2s from Arabidopsis are able to conduct H_2O_2 [3,8]. These studies also investigated the permeability of PIP1s, but no transport was observed, although the PIP1 proteins were detected by Western blot analyses and it was therefore speculated that PIP1s are impermeable to H_2O_2. However, our results suggest that the lack of H_2O_2 permeability of PIP1s observed in these studies might be primarily explained by the fact that the tested isoforms were localized in internal membranes (see above). However, efficient re-localization of ZmPIP1;2 to the plasma membrane as a result of co-expression with an inactive ZmPIP2;5 mutant did not result in an increased sensitivity of the yeast transformants to externally supplied H_2O_2. These results were somewhat unexpected, as PIP1s and PIP2s have the same four residues, phenylalanine, histidine, tyrosine, and arginine, in their aromatic arginine (ar/R) selectivity filter that is crucial for substrate selectivity [3,35]. Substitutions at, or near, these residues modulate the specificity of the channel. Molecular analysis and simulations on SoPIP2;1 have suggested that the ar/R region is the most critical determinant for H_2O_2 conduction by PIPs [3]. The energy barriers for H_2O_2 along the channel path were, at most points, a little higher than those for water, but the maximal barriers for both were seen at a similar location. The dipole moment of water or H_2O_2 forces the molecules into a preferential orientation within the channel, especially at the sites of the maximal energy barriers. The residency time for H_2O_2 was maximal in the ar/R selectivity filter which is crucial for substrate selectivity, indicating a major energy barrier for H_2O_2 conduction. Mutations that increased the pore diameter in this region showed increased H_2O_2 conduction/sensitivity [3]. Since all PIP2 and PIP1 isoforms have the same four amino acids in the ar/R region, the difference in H_2O_2 permeability must be due to on other, as yet unknown, selectivity-determining regions in the protein. Previous data from yeast and *in silico* simulation studies suggesting that water and H_2O_2 use the same channel path [2,3] and oocyte experiment results demonstrating that co-expression of ZmPIP2;5 does not negatively impact on the water channel activity of ZmPIP1;2 in hetero-tetramers [11,19] suggest that the H_2O_2 permeability of ZmPIP1;2 is not impaired by co-expression with ZmPIP2;5. Together, these data show that PIP1 proteins are indeed not permeable to H_2O_2.

The observation that water-permeable AQPs are not necessarily H_2O_2 permeable, as it is the case for ZmPIP1;2, has previously been reported. While hAQP8 and AtTIP1;1, which were identified to be highly permeable to H_2O_2 [2], are also good water channels [36,37], some water-permeable PIP2s from Arabidopsis do not facilitate H_2O_2 diffusion when expressed in yeast, despite the fact that they do not differ in the amino acid residue composition of their selectivity filters [8]. Whether the detected differences in H_2O_2 permeability between various PIP2 isoforms are due to difference in protein localization or stability was not analyzed [8]. Similar data have been obtained with the human hAQP1. While a lack of H_2O_2 permeability of hAQP1 was observed in yeast toxicity growth assays and direct uptake studies, hAQP1 was shown to be functionally expressed in the yeast plasma membrane where it highly enhanced the water permeability [2]. Conversely, the expression of *Solanaceae* XIPs in yeast induced a high sensitivity to external supplied H_2O_2, even though these XIPs have no significant water transport ability [7]. These studies demonstrate that water diffusion through an AQP does not necessarily come along with H_2O_2 diffusion and that specific selectivity mechanisms for either substrate must exist.

It will be important to elucidate why PIP1 and PIP2 isoforms have different substrate permeabilities (to H_2O_2, but also to e.g. CO_2), even though they have identical key residues in the ar/R selectivity filter normally determining substrate selectivity. The physiological role in H_2O_2 transport of PIP2s in general and ZmPIP2;5 in particular remains to be elucidated.

5. Conclusions

We demonstrated that, in contrast to the wild type ZmPIP2;5, ZmPIP1;2 is not permeable to hydrogen peroxide. This study showed

that investigation of maize PIP transport properties in *S. cerevisiae* requires (i) codon optimization of their cDNA to be significantly expressed and (ii) verification of their subcellular localization. Moreover, correct ZmPIP1;2 plasma membrane localization in *S. cerevisiae* relied on the concomitant presence of ZmPIP2;5, a mechanism which is conserved across organisms and must depend on protein features which remain to be identified.

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