

Supplemental Information

A high-throughput yeast approach to characterize Aquaporin permeabilities: profiling the Arabidopsis PIP aquaporin sub-family.

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Supplemental Note 1

Examination of the growth curve transformation for correction of non-linearity of optical density measurements of yeast population growth

To examine the effects of growth-curve transformation on data interpretation and conclusions drawn, we took two distinct datasets presented in the result section and re-processed them with and without curve transformation (Supplemental Figures S12 and S13). The first of these datasets was the PIP2-PIP1 co-expression for freeze-thaw treatment (i.e. water permeability) presented in Figure 4D. Freeze-thaw treatment affects λ but not μ or κ (Supplemental Figure S4A). The second dataset was the boric acid treatment featured in Figure 6 and Supplemental Figure S8. BA treatment mainly affects μ and κ (Figure 6A) and therefore contrasts the freeze-thaw treatment. Collectively these two treatments provide an opportunity to assess the effect of curve transformation in scenarios where each key characteristic of the growth curve is changing in response to treatment.

Plotting untransformed versus transformed data showed that the measuring point ϕ and, importantly, ΔAUC , are unaltered by curve transformation (Supplemental Figures S12 and S13). However, as expected, the individual growth characteristics of λ , μ , and κ , were affected with λ being slightly and μ and κ greatly under-estimated when simply using raw 'recorded' OD reads (Supplemental Figures S12 and S13). Plots of untransformed versus transformed data tightly fit linear correlations for each of the key measured parameters used to assess yeast growth and the effect of treatments. This indicates that growth-curve transformation does not introduce artifacts that may affect the interpretation of the growth curves. Rather, transformation provides a more realistic representation of the health/growth of the cultures and provides a standardization for comparing results generated using different plate-readers across different labs; an important factor for broader adoption of this AQP analytical framework.

Supplemental Note 2

AtPIP effects on yeast growth are specific to increased membrane permeability

Heterologous expression of AtPIPs can alter the inherent growth characteristics of the host yeast strain independent of substrate treatment, with some AtPIPs affecting lag phase (λ), growth rate (μ), and carrying capacity (κ) more than others (Supplemental Table S1). These changes may influence the sensitivity of the yeast to treatment and confound our conclusions. However, no correlations were observed between treated ΔAUC values when compared against values for λ , μ , and κ from untreated cultures (Supplemental Figure S11). Therefore, the differential growth responses of certain *AtPIP* yeast lines to specific treatments appears the direct result of AtPIP enhancement of membrane permeability to the tested substrate and not due to indirect effects through subtle changes to inherent growth characteristics.

Supplemental Materials and Methods

Yeast strain genotypes

Wild type strain: 10560-6B (WT AQY1 AQY2; background Σ 1278b; genotype: Mat α ; leu2::hisG; trp1::hisG, his3::hisG; ura352). Provided by Peter Dahl of the S. Hohmann lab.

aqy1 aqy2: YKF 1771 (null *aqy1 aqy2*; background Σ 1278b; genotype: Mat α ; leu2::hisG; trp1::hisG, his3::hisG; ura352 *aqy1D*::KanMX *aqy2D*::KanMX). Provided by Peter Dahl of the S. Hohmann lab.

skn7: ATCC® Number: 4002900™ (null *skn7*; background BY4741 genotype: Mat a his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 Δ SKN7). Obtained from ATCC (www.atcc.org).

ynvw1 (dur3): (null *dur3*; background Σ 23346c; genotype: Mat a, Δ ura3, Δ dur3). Provided by Patrick Bienert of the Nicolaus von Wirén lab.

Cloning of AtPIP genes into yeast expression vectors

AtPIP coding sequences were commercially synthesised (Genscript) as gateway-enabled entry vectors (i.e. included flanking attL sites). A second set of *AtPIP* genes without the stop codon were also made, for use in GFP C-terminal fusion constructs. The *AtPIP* CoDing Sequences (CDS) were cloned into plasmids from the Advanced Gateway® adapted pRS series of yeast expression plasmids (Alberti et al., 2007) using Gateway LR Clonase II enzyme mix (Invitrogen™). The library was obtained through Addgene (<https://www.addgene.org/>)(kit #1000000011). pAG423GPD-ccdB (MG0515; HIS) or pAG426GPD-ccdB (MG0517; URA) were used for full-length CDS clones for permeability assays. pAG426GPD-ccdB-EGFP (MG0528; URA) was used for GFP fusions (i.e. *AtPIP* CDS without stop codon). All E.coli cloning steps used One Shot™ OmniMAX™ 2 T1R Chemically Competent E. coli cells (Invitrogen). All final plasmids were sanger sequenced to confirm accuracy of the clones using; Wizard® Plus SV Minipreps DNA Purification Systems (Promega), BigDye® sequencing chemistry (ThermoFisher Scientific), and ZR DNA Sequencing Clean-Up Kit (Zymo Research). Final expression vectors were transformed in yeast using the Frozen-EZ yeast Transformation Kit II (Zymo Research, Los Angeles, USA). MG0515 constructs were transformed into Δ *aqy1aqy2* and Δ *skn7* yeast strains and used for freeze-thaw, H₂O₂ and boric acid assays. MG0517 constructs were transformed into Δ *dur3* (Δ *ynvw1*) cells and used for the urea assay.

N-terminal deletion AtPIP1s

Coding sequences that removed the N-terminal cytosolic domain (amino acids 2 to 47/48) of *AtPIP1;2*, *AtPIP1;4* and *AtPIP1;5* (see Supplemental Fig. S10) were commercially synthesized and cloned as for full length CDS versions above, to produce *AtPIP1;2* Δ ₂₋₄₇, *AtPIP1;4* Δ ₂₋₄₇ and *AtPIP1;5* Δ ₂₋₄₈.

Cloning of control AQPs for different substrate assays

A yeast codon optimized version of barley *PIP1;4* (*HvPIP1;4*) was chosen as a positive control, as it has been reported to transport boric acid in yeast from agar spot and cellular boron content assessments (Fitzpatrick and Reid, 2009). The codon usage of *HvPIP1;4* was adapted for expression in the yeast genome (see CAI adaptation below), which is especially important for expressing monocot derived genes in yeast (Bienert et al., 2014). The native coding sequence of *AtTIP2;3* (AT5G47450) was cloned and used as a positive control for urea uptake assay (Dynowski et al., 2008a). Both *HvPIP1;4* and *AtTIP2;3* were cloned like the *AtPIPs*, including modification to Kozak sequences (see below). We generated and used *AtROP6* (AT4G35020) as an additional plasma membrane-localized protein control in the Y2H mbsUS. *AtKAT1* (AT5G46240) was provided as part of the Y2H mbsUS kit gifted to us by Christopher Grefen (University of Tuebingen).

Design considerations of yeast expression constructs

High AQP protein production in the yeast is essential for robust functional evaluations and to minimize potential false negative assessments of permeability (Bienert et al., 2014). To assure ample protein production, we considered several regulatory components in designing our primary *AtPIP* yeast transgenes.

Gene copy number

Gene copy number is an important component for obtaining high gene and subsequently protein expression in yeast (Chen et al., 2012). We used the Advanced Gateway® adapted pRS series of episomal yeast expression vectors (Alberti et al., 2007), to enable high-throughput cloning and to allow for multiple copies of the AQP harboring plasmids per yeast cell. The plasmid library provided a choice of origin of replication, promoter, and auxotrophic selection marker. We chose the ‘high-copy’ 2 μ origin of replication to elevate plasmid copy number, which should be bolstered by our use of the GPD promoter, yielding in the range of 20-30 plasmid copies per cell (Karim et al., 2013). Only plasmids with either the histidine (HIS) [MG0515; pAG423GPD-ccdB] or uracil (URA) [MG0517; pAG426GPD-ccdB; URA selection] selection markers were used, as these both appear to have equally minimal plasmid load burdens on yeast growth in selection medium (Karim et al., 2013).

Promoter

The choice of the right promoter is a crucial point for efficient gene expression in yeast. The AQP transgenes were all driven by the highly active promoter of the yeast *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH) ISOZYME 3* gene (*GPD*); also referred to as *TRIOSE-PHOSPHATE DEHYDROGENASE (TDH3)*. Of the most commonly used yeast promoters, *GPD (TDH3)* is among the strongest drivers of transgene expression in the glucose-rich medium of our micro-cultivation conditions (Peng et al., 2015, Lee et al., 2013, Partow et al., 2010, Da Silva and Srikrishnan, 2012). The benefits of using a stronger promoter was evident in our initial trials where enhancements in *AtPIP2;3* driven water permeability were substantially greater when using the strong *GPD* promoter over the less active *TRIOSE PHOSPHATE ISOMERASE (TPI1)* promoter (Supplemental Fig. S2).

Terminator

There was only a single choice of terminator in the Advanced Gateway® yeast vector kit, with all constructs utilizing the well-known *CYC1* yeast terminator. Although alternative terminators may help yield higher protein levels with different promoters, these appear to provide little additional benefit when using the highly active *GPD* promoter (Curran et al., 2013).

Codon compatibility

Beyond transcriptional determinants, high AQP heterologous protein production in the yeast will rely on the efficiency of translating the mRNA. This is partially dependent on the congruency of codon usage between the foreign AQP sequence and the yeast genome, and can be measured by the Codon Adaptation Index (Sharp and Li, 1987). The CAI values for all 13 *AtPIPs* against yeast codon preferences range between 0.68 - 0.74 with a GC content of 49% - 52% (Supplemental Table S1). These values are similar to their native CAI values within *Arabidopsis* (0.73 - 0.81) and also within the range of the native yeast AQP genes (*AQY1*: 0.71 CAI and 51% G+C; *AQY2*: 0.79 CAI and 46% G+C). Given this level of harmonization, we chose to clone the native *AtPIP* sequences into the yeast expression vectors.

Kozak sequence

Translation efficiency is also influenced by the Kozak sequence surrounding the AUG of the mRNA that triggers the ribosome to commence translation (Kozak, 2005). The 5'UTR sequences of all *AtPIP* transgenes were designed identical and included aspects of a Kozak sequence favoring higher protein expression in yeast (Supplemental Table S1) (Dvir et al., 2013, Li et al., 2017). Downstream of the AUG was dictated by the native *AtPIP* sequences and were generally acceptable sequence configurations (Supplemental Table S1). Most *AtPIPs* have a guanine at position +4 (i.e. AUGG), considered

quintessential for higher protein production in eukaryotes, widely used by yeast genes, and correlated with greater human AQP protein production in yeast (Kozak, 2005, Hernández et al., 2019, Öberg et al., 2009) (Supplemental Table S1). *AtPIP2;5* to *AtPIP2;8*, have an adenine or thymine at position +4 (Supplemental Table S1), generally related to more moderate protein production in yeast, but do have the favorable cytosine at +5 (Robbins-Pianka et al., 2010).

Codon Adaptation Index value calculations

CAI values were calculated using the CAI calculation tool through the CALcal Server: <http://genomes.urv.es/CALcal/> (Puigbò et al., 2008). Codon usage tables were obtained through the Kazusa database: <http://www.kazusa.or.jp/codon/> (Nakamura et al., 2000). CAI values for each of the AtPIPs against the Arabidopsis and *S. cerevisiae* genomes are presented in (Supplemental Table S1).

Protein quantification and subcellular localizations of heterologous AtPIP proteins in yeast

C-terminal GFP fusions are an effective means of evaluating heterologous protein production in cultures of viable growing intact yeast, correlating well with traditional Westerns, and for monitoring subcellular localizations of membrane bound proteins (Ghaemmamghami et al., 2003, Albano et al., 1998, Scharff-Poulsen and Pedersen, 2013, Drew et al., 2008, Drew et al., 2006). We generated a collection of *AtPIP-GFP* transgenes using the pAG426GPD-ccdB-EGFP (MG0528) vector from the Advanced Gateway® adapted pRS series of episomal yeast expression vectors (Alberti et al., 2007) that, besides the C-terminal GFP fusion, are identical in design to our primary transgenes used for the yeast permeability assays. Additionally, as the AtPIPs are of similar size to each other (278-291 aa; Supplemental Table S1) and larger than eGFP (239 aa), the production of the AtPIP-GFP chimeric protein should primarily reflect the translation efficiencies of the different AtPIPs. As such, the *AtPIP-GFP* constructs are a suitable proxy for evaluating heterologous AtPIP production and subcellular localization of our primary *AtPIP* constructs.

Quantification of AtPIP protein abundance in intact yeast

A single ~5 mm diameter colony of each *AtPIP-GFP* expressing yeast line being tested was placed in 3ml yeast nitrogen base (YNB) with 2% Glucose (v/v) and supplemented with appropriate amino drop-out (DO) (i.e. -URA), and cultured at 30°C at 230rpm for 24hrs. 50µl of overnight culture was added to 450µl of YNB medium and measured at OD₆₆₀ (OD reading should fall within the predetermined linear portion of your spectrophotometer). YNB SD DO medium was added to overnight cultures to adjust OD to a $^{Corr}OD_{660} = 5$. 2.5ml of adjusted culture was transferred to a fresh tube and centrifuge for 5mins at 3000g. The supernatant was poured off and the pellet resuspended in 220µl of appropriate YNB SD DO medium. The concentrated culture was now in the range of $^{Corr}OD_{660} = 60$. For each construct, 3 wells of a black 96-well plate were used (Nunc™ F96 MicroWell Black nontreated. Thermoscientific Cat#237105). 200µl of the yeast concentrate was placed in well 1, and 100µl of YNB SD DO medium was added to wells 2 and 3. Doubling dilutions were performed using 100µl from well 1 to well 2 and then to well 3, giving concentrations of 100%, 50% and 25%. Just prior to measurement, cells were resuspended using a multichannel pipette. GFP fluorescence was measured using an Infinite M1000 Pro plate reader (TECAN) with Gain 115, Z-position 19300, excitation wavelength 488nm, emission detection 512nm, excitation and emission bandwidth 5nm, 50 Flashes, at Flash frequency of 400 Hz, Integration time 20µs, and Settle time 50ms. GFP fluorescence readings over the 3 concentrations (i.e. 100%, 50%, 25% cell density) needed to be consistent and in the linear detection range ($R^2 \geq 0.99$). Cell concentration of the 100% well was confirmed by repeated measurements of 5µl of 100% cell concentrate to 495µl fresh YNB SD medium dilutions at OD₆₆₀. Yeast not expressing GFP were also measured to determine (minimal) background fluorescence levels. Protein abundance was reported as AtPIP-GFP fluorescence values minus background fluorescence, standardized to cell OD₆₆₀ = 1. Six independent replicate experiments were performed for each *AtPIP-GFP* expression yeast line.

AtPIP-GFP subcellular localization in yeast

Single ~5 mm diameter colonies of *AtPIP-GFP* expressing $\Delta aqy1aqy2$ yeast were used to inoculate 1ml starter culture (YNB DO with 2% Glucose v/v) and grown to stationary phase (24 hrs, 30 °C, 230 rpm). The over-night cultures were diluted 4-fold and incubated for a further 4hrs prior to GFP visualisation. 2µl of settled yeast cells were spotted on a polylysine coated slide (Thermofisher scientific) and mounted with 22x22mm cover slip and sealed with nail varnish. Sub-cellular GFP signal was visualised on a Zeiss LSM780 confocal laser-scanning microscope (Carl Zeiss) operated by Zen Black software and a DIC x40 oil immersion lens. eGFP was excited at 488nm and emission was recorded at 495-570 nm with 1.35 AU pinhole and master and digital gains identically set for all images and analysis. The SEC63-RFP ER marker was obtained through Addgene (pSM1959; Addgene plasmid #41837; donated by Susan Michaelis; (Metzger et al., 2008)). RFP was excited at 561 nm and emission was captured at 565-735 nm with 1.10 AU pinhole with a quick scan time to avoid bleaching of RFP. Over 30 cells for each of the *AtPIP-GFP* lines were scored to access membrane localisation tendencies and performed across two replicate experiments, with localisation patterns between the genotypes consistent across sessions. We also performed subcellular localisation analysis in the $\Delta skn7$ background, which concluded similar findings.

Micro-volume yeast growth system

Adjusting for non-linearity of OD measurements at high cell density

Obtaining accurate OD values of batch cultures at high cell densities ideally requires aliquots to be diluted to within the linear detection range (i.e. where OD \approx cell concentration). This is simply not feasible in a high-throughput set-up. Instead, the non-linear OD relationship can be compensated for by comparing 'recorded' ODs against 'true' ODs calculated from dilution factors (Supplemental Figure 1). Fitting a regression function will allow for the relationship between 'recorded' and 'true' ODs to be readily calculated (i.e. 'recorded' OD₆₅₀ at 200µl into 'true' OD₆₅₀ at 1cm pathlength). Such a calibration function needs to be established independently for a given machine (or at least different brand machines), as the pathlength, detector, light beam angle, aperture etc, will vary. Calibration can also be performed between two different spectrophotometers to correct/collate data collected between different machines. We used the Spectrostar Nano microplate reader (BMG Labtech, Germany) for this study, which is an affordable basic model plate reader capable of shaking, incubations, and multiple point reading, run by a versatile software program and complete with its own dedicated database (MARS – BMG Labtech).

Shaking modes

Linear, orbital and double orbital shaken modes were trialed for their capacity to agitate cultures and keep yeast in suspension. Regardless of the mode and shaking speed (up to 1000rpm), yeast eventually sedimented. We found that the dispersal of sedimentation on the bottom of the well, was markedly important for the reproducibility of growth curves. Linear (left-right) and orbital (circular) shaking were not suitable, as they produced cell clumping as ridges (linear) or a ring shape (orbital) with distinct regions absent of cells. Double orbital (figure eight) shaking produced and maintained a homogenous lawn of yeast cell sediment across the base of the well throughout the experiment, and is the only shaking mode we recommend. Shaking mode and frequency is also imperative for the continued mixing of the medium and exposure of cells to the supplemented agents being tested. A shaking velocity of 400rpm was chosen for its ability to quickly and thoroughly disperse small droplets of dye gently loaded into wells containing 200µl of yeast growth medium. A shaking duration of 5 mins prior to the OD reading (occurring usually every 10 mins) was deemed a suitable balance between sufficient mixing and considerations of wear and tear on equipment.

OD reading mode

'Orbital averaging' mode on the Spectrostar Nano microplate reader (BMG, Germany) was invoked for OD readings. In this mode, the plate reader collected data from a 4mm diameter orbit (22 read points) and reported the average value for a more stable representative OD reading with reduced deviation between replicate wells. A 650nm wavelength was chosen for OD readings as our medium and yeast generally do not absorb strongly at this longer wavelength at any time throughout the growth cycle. This means that OD readings will be dominated mainly by the single factor of scattering that will generally provide a more consistent representation of cell concentration.

Incubation

Growth assay plates were incubated at the optimal growth temperature of 30°C for yeast. The Spectrostar Nano microplate reader (BMG, Germany) heats the lid of the 96-well plate slightly warmer to avoid condensation during runs. Although the entire stage is heated, we invoked the software option to shift the plate back to the full heating plate during the initial idle incubation period of each cycle, to avoid the potential cool spot directly above the optical sensor.

Our standard incubation periods were for ~42hrs, except for urea, where growth was monitored for ~50hrs. This is because although yeast can use urea as a sole source of nitrogen, growth rates are slower than when supplied with a preferred nitrogen sources such as ammonium (Godard et al., 2007).

Default kinetic conditions for yeast growth assay

The default cycling conditions used for our yeast growth assays were: 250 cycles at 10mins per cycle (total time ~42 hrs)*; incubated at 30°C with a slightly warmer lid to avoid condensation; shaking frequency of 400 rpm; double orbital shaking mode; 5 mins shaking per cycle prior to the OD reading, with the remaining time the plate sitting idle on the incubation plate and away from the colder optical reading region; OD readings invoke orbital averaging at scan diameter of 4mm and 22 flashes per well, recording at 650nm. * 12 min cycles for urea supplementation assay (total time ~50 hrs).

Micro-culture volume and starting cell concentration

We settled on 200µl culture volumes. 100µl cultures appeared to mix insufficiently, with yeast occasionally clumping and producing erratic growth curves. 300µl was excessive, with shaking exceeding the retention capacity of the well. Cultures with higher initial cell concentrations that produced a lawn of cells provided the most reproducible growth curves. Too low of an initial cell density, produced independent scattered colonies and inconsistent growth curves often with erratic curve anomalies (e.g. sharp peaks or sudden drops). Our cultures began with an initial OD₆₅₀ of ~0.4 as measured in the 200µl volume within the 96-well plate. Ideally, the initial cell density provides a discernable lag phase for untreated cultures, but still allows sufficient growth of treated cultures within a reasonable time period.

Processing of growth curves: generating $\ln(\text{Corr.OD}_t / \text{Corr.OD}_i)$ values

Reliable yeast population growth characteristics (e.g. λ , μ , and κ) were obtained from growth curves built from frequent OD data acquisition and critical post-processing steps. Changes in growth characteristics revealed how treatments affected yeast growth and allowed quantitative comparison between successive yeast cultures. The implementation of a dynamic measuring point ϕ , allowed for standardized evaluation between different AQP expressing yeast lines, regardless of any reasonable impact that the AQP may exert on inherent growth of untreated yeast. Differential growth responses due to increased substrate diffusion into the yeast were captured by the single parameter, AUC.

Yeast growth data was processed using a custom-built fit-for-purpose pipeline in Microsoft Excel that draws on existing algorithms to process and calculate characteristics of yeast population growth curves (Warringer and Blomberg, 2003, Toussaint et al., 2006, Fernandez-Ricaud et al., 2016, Jung et al., 2015, Hall et al., 2014, Olsen et al., 2010). Most importantly, our pipeline allows for visualization

of each processing step and comparisons between treatments, for full scrutiny of obtained curves and AUC measurements. The pipeline consists of; **Removing of background:** In each experimental run, several wells are dedicated 'blanks' consisting of non-inoculate growth medium complete with appropriate chemical treatment additives. 'Blank' well values were removed from the corresponding yeast growth OD values at each time-point during the growth cycle. **OD calibration:** The OD calibration function was then applied (see: Adjusting for non-linearity of OD measurements at high cell density), providing $^{Corr.}OD_{650}$ values (Figure 1A). **Adjusting for settling time:** During the first 60 - 180 mins, there is a sharp increase in OD values (Supplementary Figure S14A). This mostly occurs due to an increase in cell size as the yeast exit from the starved G_0 state and reestablish a size permissive for replication. The OD values then settle to a 'norm', representing the actual OD of the lag-phase period. The OD values during the settling period are adjusted commensurate to the acclimated lag-phase OD (Supplementary Figure S14B). **Curve smoothing:** Several filters were applied to reduce noise across the collective OD readings of a growth curve. (i) Median filter - Considers 5 consecutive data points and replaces the middle value with the median of all 5. Median filtering minimises the impact of aberrant spike readings impacting later smoothing process and growth characteristic extractions (Supplementary Figure S14C). The first and last two data points of the entire series are cloned (i.e. extending the data beyond the recorded timepoints) to avoid edge cropping when applying this filter at the ends of the data collected. (ii) Mean filter - A sliding window of 3 consecutive data points, where the middle value in each window is replaced by the mean of the series. It is a light smoothing operation that does not distort the true data trend (Supplementary Figure S14D). (iii) Removing negative slopes filter - Sweeps each data series in a step-wise manner replacing any OD value lower than its predecessor with the predecessor itself (i.e. removing negative slopes between consecutive points) (Supplementary Figure S14E). This filter is used to compensate for occasional minor stochastic changes in OD readings brought about by, for example, bubble formation that drifts in and out of the light beam. (iv) Cubic scanning filter - As a final smoothing step, a cubic smoothing spline is applied, which is highly flexible and has useful smoothing properties that are not bound by assumed population curve behaviours. A sliding window consisting of 1 central focal data point and 30 flanking points either sides, are fitted with a cubic equation (Ax^3+Bx^2+Cx+D) and the central focal value adjusted to fit the regression line (Supplementary Figure S14F). Prior to the application of this filter, the first and last data points are cloned 30 times to prevent edge cropping when the calculation window nears the start or end of the data series. The application of these four filters provides a smoothed growth curve from which subsequent growth characteristic parameters can be more accurately derived (Supplemental Figure S14G). **Log transformation:** The smoothed growth data is then log transformed (LN) and plotted as $\ln(^{Corr.}OD_t / ^{Corr.}OD_i)$, where $^{Corr.}OD_i$ is the initial OD (Supplementary Figure S14H). $^{Corr.}OD_t / ^{Corr.}OD_i$ provides a relative assessment, easier computation of AUC values, helps correct minor variation between reps, and can be applied when OD_i are generally equivalent (which should be the case if pre-assay dilutions of samples was properly conducted). Some treatments alter the OD_i , for example the lysis of cells occurring as a result of freeze-thaw treatments (Supplementary Figure S14J). The reduced OD_i artificially inflates the OD_t/OD_i ratio compared to control and can distort comparisons of growth curves. Therefore, when reporting in $\ln(^{Corr.}OD_t / ^{Corr.}OD_i)$ mode, OD_i is derived from the untreated control (or highest essential nutrient treatment) and applied to all cultures of the given series (Supplemental Figure S14J). Our pipeline enabled toggling between $\ln(^{Corr.}OD)$ and $\ln(^{Corr.}OD_t / ^{Corr.}OD_i)$ to explore treatment effects and decide on the most appropriate output format.

Extraction of key growth characteristics

The exponential growth phase was determined using linear slopes consisting of a seven time point sliding window (Focal time-point +/- 3 flanking time points) (red data values in Supplemental Figure S15). The point of maximum slope was identified and a flanking 10% deviation was used to define the extremes of a maximum exponential range (blue data values in Supplemental Figure S15). A linear regression line was applied to this region and used to derive maximum growth rate (μ). Lag phase (λ)

was defined as the intersect point of the maximum exponential slope and the X-axis (orange in Supplemental Figure S15). Stationary phase, or carrying capacity (κ), was determined as the point the population growth rate hits virtually zero with the measuring point (ϕ) occurring just prior at the point population growth rate falls to 5% of the maximum slope (green in Supplemental Figure S15). AUC was determined using a segmented trapezoid approach. For each time interval up to the measuring point, segments of AUC was calculated using $\text{Area} = (\text{LN}(\text{OD}/\text{ODi})_n + \text{LN}(\text{OD}/\text{ODi})_{n+1})/2 \times (t_2 - t_1)$, which were then totalled to provide AUC.

Treatments of AQP expressing yeast lines

Freeze-thaw assay

Yeast are sensitive to very rapid freezing events, with the formation of intracellular ice crystals causing cell damage and death. Yeast freeze-thaw survivorship involves various factors, but is mainly associated with how rapidly water can efflux from the cell across the PM (Cabrera et al., 2020), which in turn is correlated with the abundance of water permeable AQPs (Tanghe et al., 2002, Tanghe et al., 2004, Soveral et al., 2006, Will et al., 2010). Therefore, water permeability of AQP variants can be rapidly determined in yeast by screening for improved freeze-thaw survivorship (To et al., 2015).

The common practice was for several independent yeast transformants, once identified on selection plates, to be immediately cultured, cells harvested, and stored at on glycerol at -80°C. Each independent yeast transformant was considered a biological replicate. 1ml cultures (in 14ml round bottom tubes), were grown at 30°C, shaking at 250rpm, until stationary phase for each AQP expressing line being tested. 7.5µl of each culture was spotted onto appropriate agar-based DO selection medium and incubated for 2-3 days. One-third of a spot was used to inoculate an assay starter culture consisting of 1.25ml appropriate YNB SD DO medium, at 30°C, shaking at 250rpm for 24 hours (~OD₆₅₀ 1). An aliquot of the starter culture was diluted in 1ml at 6x10⁶ cells/mL in YNB SD DO medium in a 2ml microfuge tube and incubated at 30°C for 60 mins. 250µL of each culture was aliquoted to an appropriate number of 1.5ml microfuge tubes; one tube for each round of freeze-thaw (FT) treatment being applied. The zero FT tubes were placed on ice (untreated control), while the others were progressed through (generally two) freeze-thaw cycles, and placed on ice once they reached the desired treatments. Each FT cycle consisted of the yeast culture being rapidly frozen in liquid nitrogen for 30 seconds, and thawed in a 30°C water bath for 20 mins. For each construct, 200µl of 'Untreated' and 'Treated' yeast were transferred from the 1.5ml microfuge tubes into the 96-well plate for micro-cultivation and growth detection.

H₂O₂ assay

The reactive oxygen species (ROS) hydrogen peroxide (H₂O₂), can impair yeast growth when it exceeds the protective mechanisms of the cell (Jamieson, 1998). A range of H₂O₂ concentrations added to the external growth medium were tested to determine ideal treatment doses. *AtPIP2;1*, *2;4* and *2;5* served as controls having been shown to transport H₂O₂ in yeast using both agar spot growth and low-throughput fluorescent-dye-based cellular accumulation assays (Mao and Sun, 2015, Wang et al., 2019, Dynowski et al., 2008b). We also used the *skn7* null mutant yeast strain, which is compromised in its antioxidant buffering capacity and is therefore more sensitive to H₂O₂ treatment (Morgan et al., 1997, Krems et al., 1996).

aqy1 aqy2 or *skn7* yeast expressing AtPIPs or carrying the Empty vector, were grown and diluted to 6x10⁶ cells/mL as per the Freeze-thaw assay (above). 200µL microcultures of each AtPIP/Empty vector were distributed in 96-well plate comprised of 190µL of yeast and 10µL H₂O₂ to achieved desired final treatment concentrations (with water for the 0mM H₂O₂ treatment).

Boric acid assay

Boron is essential for yeast growth, but at high concentrations is toxic. At moderate concentrations (< 80mM) it acts as a fungistatic agent, slowing down proliferation but not killing the cell (Schmidt et al., 2010, Bennett et al., 1999). A range of boric acid (BA; H₃BO₃) concentrations were tested against *aqy1 aqy2* empty vector yeast. Barley *PIP1;4* (*HvPIP1;4*) was used as a positive control, having been reported to transport BA in yeast from agar spot and cellular boron content assessments (Fitzpatrick and Reid, 2009). We codon optimized *HvPIP1;4* to favor high expression in yeast given that monocot AQP sequences express poorly in yeast (Bienert et al., 2014). The *HvPIP1;4* construct was designed with the same construct principles as the AtPIPs.

aqy1 aqy2 yeast expressing AtPIPs or carrying the Empty vector were grown and diluted to 6x10⁶ cells/mL as usual. 200μL microcultures of each AtPIP/Empty vector were distributed in 96-well plate comprised of 180μL of yeast and 20μL boric acid to achieved desired final treatment concentrations (with water for the 0mM boric acid treatment).

Urea assay

We employed the use of the urea uptake-defective strain *ynvw1* (*dur3*), which possesses a deletion of *DUR3* urea transporter, and consequently shows compromised growth in medium containing low urea concentrations as the sole nitrogen source (Liu et al., 2003). *ynvw1* yeast expressing *AtTIP2;3* (Dynowski et al., 2008a) was used as a positive control.

ynvw1 yeast spots grown on YNB plates were scraped and resuspended in 1.25mL of Yeast Basic media (YB, culture medium without nitrogen source) with 2% Glucose. Yeast cultures were diluted to 1.2x10⁷ cells/mL. 200μL microcultures for each AtPIP/Empty vector construct were distributed in 96-well plates with 190μL of yeast and 10μL urea treatment (with water being used for the 0mM urea treatment).

NaCl assay

Na⁺ uptake was quantified by spectrophotometry of lysed cell content from yeast exposed to NaCl treatment as previously described (Qiu et al., 2020) except for the following modifications: the full-length of AtPIPs were sub-cloned into MG0515 (pAG423GPD-ccdB) yeast expression destination vector driven by *GDP* (*TDH3*) promoter through LR reaction. Confirmed constructs were transformed into *Saccharomyces cerevisiae aqy1 aqy2* double mutant yeast strain using Frozen-EZ Yeast Transformation II kit (Zymo Research). The yeast culture media for growing and for performing Na⁺ uptake assays contained 2% glucose (w/v) rather than galactose.

Yeast spheroplast assays

The traditional yeast spheroplast bursting method for detection of AQP water permeability (Daniels et al., 2006), involves placing yeast spheroplasts (i.e. yeast without a cell wall) in hypotonic solution which causes water to diffuse into the cell, leading to cellular expansion and rupture, with cell lysis monitored by a loss in optical density. Spheroplast expressing water permeable AQPs rupture more readily than control cells, with the effect proportional to their capacity to transport water (Pettersson et al., 2006, Azad et al., 2009). Briefly, a 5ml culture was grown to mid to late exponential phase. 2 x 2ml aliquots were centrifuged at 1500 x g room temperature for 2 mins and supernatant removed. The pellet was resuspended in 1ml of water, centrifuge at 1500 x g and the water discarded. The pellets were combined by resuspension in 850μl of freshly made 'softening medium' (100mM Hepes-KOH, pH 9.4; 10mM DTT), incubated for 30 mins at room temperature, centrifuge at 3000 x g for 4 mins at room temperature and supernatant removed. The cells were resuspended in 850μl of 'spheroplasting medium' (1xYNB, 2% glucose, 1x amino acid, 50mM Hepes-KOH pH 7.2, 1M sorbitol), centrifuged at 1500 x g for 1 min at RT, supernatant removed, and resuspended once again in 850μl of spheroplasting medium. 400μl of cells were diluted to OD₆₅₀ 3, in spheroplasting medium, 2 μl of

zymolyase 100T lytic enzyme (Zymo Research) added, and incubated for 4 hrs at 30°C with gentle inversion. The kinetics of 20 µl of spheroplasts mixed with 480 µl spheroplast bursting medium (15% spheroplasting medium and water v/v) was measured using Cary 60 UV-VIS (Agilent) spectrophotometer with OD₆₅₀ reading at 0.1 sec intervals to determine if sufficient digestion was achieved. Actual assays were performed using 75 µl of spheroplasts for more consistent readings.

Y2H mbSUS

We used the Yeast-two-Hybrid mating-based Split-Ubiquitin System for detection of membrane bound protein-protein interactions (Y2H mbSUS) with assays preformed as described in (Grefen et al., 2007). We used the ONPG assays to detect β-Gal activity, indicative of interaction strength. We screened an AtPIP interactome library that we produced using the mbSUS vectors (Groszmann, unpublished). We generated and used *AtROP6* (AT4G35020) as an additional plasma membrane-localized protein control in the Y2H mbSUS. *AtKAT1* (AT5G46240) control was provided as part of the Y2H mbSUS kit, which was gifted to us by Christopher Grefen (University of Tuebingen).

Statistics

All statistical comparisons (e.g. ANOVA, Ttest, etc) and curve fitting applications during calibrations of the various treatments (Supplemental Figures S4, S5, S7, and S9) were performed using Origin Pro 2020 (ver. 9.7.0.188).

Bioinformatics

AtPIP protein sequences were obtained through The Arabidopsis Information Resource (TAIR) (www.arabidopsis.org). Protein sequences were imported into Geneious (ver. 11.1.5) and aligned using MUSCLE sequence aligner (Edgar, 2004). Transmembrane and loop domains were defined using TOPCONS (Tsirigos et al., 2015). The AtPIP phylogeny was generated using neighbor-joining method from MUSCLE alignments of full protein sequences, with confidence levels (%) of branch points generated through bootstrapping analysis (n = 1000) using MEGA 10 (Kumar et al., 2018). Normalized tissue-specific RNA-seq data was obtained through TRAVA (<http://travadb.org/>) (Klepikova et al., 2016) and heatmaps produced using MS Excel.

References

- ALBANO, C. R., RANDERS-EICHHORN, L., BENTLEY, W. E. & RAO, G. 1998. Green fluorescent protein as a real time quantitative reporter of heterologous protein production. *Biotechnology progress*, 14, 351-354.
- ALBERTI, S., GITLER, A. D. & LINDQUIST, S. 2007. A suite of Gateway® cloning vectors for high-throughput genetic analysis in *Saccharomyces cerevisiae*. *Yeast*, 24, 913-919.
- AZAD, A. K., SAWA, Y., ISHIKAWA, T. & SHIBATA, H. 2009. Heterologous expression of tulip petal plasma membrane aquaporins in *Pichia pastoris* for water channel analysis. *Applied and environmental microbiology*, 75, 2792-2797.
- BENNETT, A., ROWE, R., SOCH, N. & ECKHERT, C. 1999. Boron stimulates yeast (*Saccharomyces cerevisiae*) growth. *The Journal of nutrition*, 129, 2236-2238.
- BIENERT, G. P., HEINEN, R. B., BERNY, M. C. & CHAUMONT, F. 2014. Maize plasma membrane aquaporin ZmPIP2; 5, but not ZmPIP1; 2, facilitates transmembrane diffusion of hydrogen peroxide. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1838, 216-222.
- CABRERA, E., WELCH, L. C., ROBINSON, M. R., STURGEON, C. M., CROW, M. M. & SEGARRA, V. A. 2020. Cryopreservation and the Freeze–Thaw Stress Response in Yeast. *Genes*, 11, 835.

- CHEN, Y., PARTOW, S., SCALCINATI, G., SIEWERS, V. & NIELSEN, J. 2012. Enhancing the copy number of episomal plasmids in *Saccharomyces cerevisiae* for improved protein production. *FEMS yeast research*, 12, 598-607.
- CURRAN, K. A., KARIM, A. S., GUPTA, A. & ALPER, H. S. 2013. Use of expression-enhancing terminators in *Saccharomyces cerevisiae* to increase mRNA half-life and improve gene expression control for metabolic engineering applications. *Metabolic engineering*, 19, 88-97.
- DA SILVA, N. A. & SRIKRISHNAN, S. 2012. Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. *FEMS yeast research*, 12, 197-214.
- DANIELS, M. J., WOOD, M. R. & YEAGER, M. 2006. In vivo functional assay of a recombinant aquaporin in *Pichia pastoris*. *Applied and environmental microbiology*, 72, 1507-1514.
- DREW, D., LERCH, M., KUNJI, E., SLOTBOOM, D.-J. & DE GIER, J.-W. 2006. Optimization of membrane protein overexpression and purification using GFP fusions. *Nature methods*, 3, 303-313.
- DREW, D., NEWSTEAD, S., SONODA, Y., KIM, H., VON HEIJNE, G. & IWATA, S. 2008. GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in *Saccharomyces cerevisiae*. *Nature protocols*, 3, 784.
- DVIR, S., VELTEN, L., SHARON, E., ZEEVI, D., CAREY, L. B., WEINBERGER, A. & SEGAL, E. 2013. Deciphering the rules by which 5'-UTR sequences affect protein expression in yeast. *Proceedings of the National Academy of Sciences*, 110, E2792-E2801.
- DYNOWSKI, M., MAYER, M., MORAN, O. & LUDEWIG, U. 2008a. Molecular determinants of ammonia and urea conductance in plant aquaporin homologs. *FEBS letters*, 582, 2458-2462.
- DYNOWSKI, M., SCHAAF, G., LOQUE, D., MORAN, O. & LUDEWIG, U. 2008b. Plant plasma membrane water channels conduct the signalling molecule H₂O₂. *Biochemical Journal*, 414, 53-61.
- EDGAR, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research*, 32, 1792-1797.
- FERNANDEZ-RICAUD, L., KOURTCHENKO, O., ZACKRISSON, M., WARRINGER, J. & BLOMBERG, A. 2016. PRECOG: a tool for automated extraction and visualization of fitness components in microbial growth phenomics. *BMC bioinformatics*, 17, 1-15.
- FITZPATRICK, K. L. & REID, R. J. 2009. The involvement of aquaglyceroporins in transport of boron in barley roots. *Plant, cell & environment*, 32, 1357-1365.
- GHAEMMAGHAMI, S., HUH, W.-K., BOWER, K., HOWSON, R. W., BELLE, A., DEPHOURE, N., O'SHEA, E. K. & WEISSMAN, J. S. 2003. Global analysis of protein expression in yeast. *Nature*, 425, 737-741.
- GODARD, P., URRESTARAZU, A., VISSERS, S., KONTOS, K., BONTEMPI, G., VAN HELDEN, J. & ANDRÉ, B. 2007. Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. *Molecular and cellular biology*, 27, 3065-3086.
- GREFFEN, C., LALONDE, S. & OBRDLIK, P. 2007. Split-ubiquitin system for identifying protein-protein interactions in membrane and full-length proteins. *Current Protocols in Neuroscience*, 41, 5.27. 1-5.27. 41.
- HALL, B. G., ACAR, H., NANDIPATI, A. & BARLOW, M. 2014. Growth rates made easy. *Molecular biology and evolution*, 31, 232-238.
- HERNÁNDEZ, G., OSNAYA, V. G. & PÉREZ-MARTÍNEZ, X. 2019. Conservation and variability of the AUG initiation codon context in eukaryotes. *Trends in biochemical sciences*, 44, 1009-1021.
- JAMIESON, D. J. 1998. Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast*, 14, 1511-1527.
- JUNG, P. P., CHRISTIAN, N., KAY, D. P., SKUPIN, A. & LINSTER, C. L. 2015. Protocols and programs for high-throughput growth and aging phenotyping in yeast. *PloS one*, 10, e0119807.
- KARIM, A. S., CURRAN, K. A. & ALPER, H. S. 2013. Characterization of plasmid burden and copy number in *Saccharomyces cerevisiae* for optimization of metabolic engineering applications. *FEMS yeast research*, 13, 107-116.

- KLEPIKOVA, A. V., KASIANOV, A. S., GERASIMOV, E. S., LOGACHEVA, M. D. & PENIN, A. A. 2016. A high resolution map of the Arabidopsis thaliana developmental transcriptome based on RNA-seq profiling. *The Plant Journal*, 88, 1058-1070.
- KOZAK, M. 2005. Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene*, 361, 13-37.
- KREMS, B., CHARIZANIS, C. & ENTIAN, K.-D. 1996. The response regulator-like protein Pos9/Skn7 of *Saccharomyces cerevisiae* is involved in oxidative stress resistance. *Current genetics*, 29, 327-334.
- KUMAR, S., STECHER, G., LI, M., KNYAZ, C. & TAMURA, K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, 35, 1547-1549.
- LEE, M. E., ASWANI, A., HAN, A. S., TOMLIN, C. J. & DUEBER, J. E. 2013. Expression-level optimization of a multi-enzyme pathway in the absence of a high-throughput assay. *Nucleic acids research*, 41, 10668-10678.
- LI, J., LIANG, Q., SONG, W. & MARCHISIO, M. A. 2017. Nucleotides upstream of the Kozak sequence strongly influence gene expression in the yeast *S. cerevisiae*. *Journal of biological engineering*, 11, 1-14.
- LIU, L.-H., LUDEWIG, U., FROMMER, W. B. & VON WIRÉN, N. 2003. AtDUR3 encodes a new type of high-affinity urea/H⁺ symporter in Arabidopsis. *The Plant Cell*, 15, 790-800.
- MAO, Z. & SUN, W. 2015. Arabidopsis seed-specific vacuolar aquaporins are involved in maintaining seed longevity under the control of ABSCISIC ACID INSENSITIVE 3. *Journal of experimental botany*, 66, 4781-4794.
- METZGER, M. B., MAURER, M. J., DANCY, B. M. & MICHAELIS, S. 2008. Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. *Journal of Biological Chemistry*, 283, 32302-32316.
- MORGAN, B. A., BANKS, G. R., TOONE, W. M., RAITT, D., KUGE, S. & JOHNSTON, L. H. 1997. The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast *Saccharomyces cerevisiae*. *The EMBO Journal*, 16, 1035-1044.
- NAKAMURA, Y., GOJOBORI, T. & IKEMURA, T. 2000. Codon usage tabulated from international DNA sequence databases: status for the year 2000. *Nucleic acids research*, 28, 292-292.
- ÖBERG, F., EKVALL, M., NYBLÖM, M., ÖBERG, F., EKVALL, M., NYBLÖM, M., BACKMARK, A., NEUTZE, R. & HEDFALK, K. 2009. Insight into factors directing high production of eukaryotic membrane proteins; production of 13 human AQPs in *Pichia pastoris*. *Molecular membrane biology*, 26, 215-227.
- OLSEN, B., MURAKAMI, C. J. & KAEBERLEIN, M. 2010. YODA: software to facilitate high-throughput analysis of chronological life span, growth rate, and survival in budding yeast. *BMC bioinformatics*, 11, 1-8.
- PARTOW, S., SIEWERS, V., BJØRN, S., NIELSEN, J. & MAURY, J. 2010. Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*. *Yeast*, 27, 955-964.
- PENG, B., WILLIAMS, T. C., HENRY, M., NIELSEN, L. K. & VICKERS, C. E. 2015. Controlling heterologous gene expression in yeast cell factories on different carbon substrates and across the diauxic shift: a comparison of yeast promoter activities. *Microbial cell factories*, 14, 91.
- PETTERSSON, N., HAGSTRÖM, J., BILL, R. M. & HOHMANN, S. 2006. Expression of heterologous aquaporins for functional analysis in *Saccharomyces cerevisiae*. *Current genetics*, 50, 247-255.
- PUIGBÒ, P., BRAVO, I. G. & GARCIA-VALLVE, S. 2008. CAIcal: a combined set of tools to assess codon usage adaptation. *Biology direct*, 3, 1-8.
- QIU, J., MCGAUGHEY, S. A., GROSZMANN, M., TYERMAN, S. D. & BYRT, C. S. 2020. Phosphorylation influences water and ion channel function of AtPIP2; 1. *Plant, Cell & Environment*, 43, 2428-2442.

- ROBBINS-PIANKA, A., RICE, M. D. & WEIR, M. P. 2010. The mRNA landscape at yeast translation initiation sites. *Bioinformatics*, 26, 2651-2655.
- SCHARFF-POULSEN, P. & PEDERSEN, P. A. 2013. *Saccharomyces cerevisiae*-Based Platform for Rapid Production and Evaluation of Eukaryotic Nutrient Transporters and Transceptors for Biochemical Studies and Crystallography. *PLoS One*, 8, e76851.
- SCHMIDT, M., SCHAUMBERG, J. Z., STEEN, C. M. & BOYER, M. P. 2010. Boric acid disturbs cell wall synthesis in *Saccharomyces cerevisiae*. *International journal of microbiology*, 2010.
- SHARP, P. M. & LI, W.-H. 1987. The codon adaptation index-a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic acids research*, 15, 1281-1295.
- SOVERAL, G., VEIGA, A., LOUREIRO-DIAS, M. C., TANGHE, A., VAN DIJCK, P. & MOURA, T. F. 2006. Water channels are important for osmotic adjustments of yeast cells at low temperature. *Microbiology*, 152, 1515-1521.
- TANGHE, A., VAN DIJCK, P., COLAVIZZA, D. & THEVELEIN, J. M. 2004. Aquaporin-mediated improvement of freeze tolerance of *Saccharomyces cerevisiae* is restricted to rapid freezing conditions. *Applied and environmental microbiology*, 70, 3377-3382.
- TANGHE, A., VAN DIJCK, P., DUMORTIER, F., TEUNISSEN, A., HOHMANN, S. & THEVELEIN, J. M. 2002. Aquaporin expression correlates with freeze tolerance in baker's yeast, and overexpression improves freeze tolerance in industrial strains. *Applied and Environmental Microbiology*, 68, 5981-5989.
- TO, J., YEO, C. Y., SOON, C. H. & TORRES, J. 2015. A generic high-throughput assay to detect aquaporin functional mutants: potential application to discovery of aquaporin inhibitors. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1850, 1869-1876.
- TOUSSAINT, M., LEVASSEUR, G., GERVAIS-BIRD, J., WELLINGER, R. J., ABOU ELELA, S. & CONCONI, A. 2006. A high-throughput method to measure the sensitivity of yeast cells to genotoxic agents in liquid cultures. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 606, 92-105.
- TSIRIGOS, K. D., PETERS, C., SHU, N., KÄLL, L. & ELOFSSON, A. 2015. The TOPCONS web server for consensus prediction of membrane protein topology and signal peptides. *Nucleic acids research*, 43, W401-W407.
- WANG, H., ZHANG, L., TAO, Y., WANG, Z., SHEN, D. & DONG, H. 2019. Transmembrane Helices 2 and 3 Determine the Localization of Plasma Membrane Intrinsic Proteins in Eukaryotic Cells. *Frontiers in plant science*, 10, 1671.
- WARRINGER, J. & BLOMBERG, A. 2003. Automated screening in environmental arrays allows analysis of quantitative phenotypic profiles in *Saccharomyces cerevisiae*. *Yeast*, 20, 53-67.
- WILL, J. L., KIM, H. S., CLARKE, J., PAINTER, J. C., FAY, J. C. & GASCH, A. P. 2010. Incipient balancing selection through adaptive loss of aquaporins in natural *Saccharomyces cerevisiae* populations. *PLoS Genet*, 6, e1000893.