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The yeast osmosensitive mutant *fps1Δ* transformed by the cauliflower BobTIP1;1 aquaporin withstand a hypo-osmotic shock

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Abstract Osmoregulation plays an important role in cellular responses to osmotic stress in plants and in yeast. Aquaporins contribute to osmotic adjustment by facilitating transport of water or solutes across membranes. The tonoplastic water channel BobTIP1;1 (original name BobTIP26-1) genes are upregulated during dessication stress in cauliflower meristematic tissue. To investigate the physiological importance of BobTIP1;1, we expressed it in a *Saccharomyces cerevisiae* osmosensitive mutant *fps1Δ*. We showed that the defect in the yeast glycerol plasma membrane transporter is complemented by a plant cDNA encoding the aquaporin BobTIP1;1 which is localized in the vacuolar membrane of the complemented yeast cells. To our knowledge, this is the first example of a plant aquaporin for which localization in the vacuolar membrane of yeast cells is related to an osmoresistant phenotype under hypo-osmotic shock.

Keywords: Hypo-osmotic shock; Osmotic stress; Aquaporin; γ-TIP; Vacuolar membrane

1. Introduction

Water availability is of fundamental importance for all living organisms. To cope with environmental and physiological stresses, organisms must be capable of a rapid cellular adaptation for survival and growth. Facilitated transport of water or solutes across cell membranes prevents uncontrolled movements of other solutes, protons and ions which would be damageable to the cell. The Major Intrinsic Proteins (MIPs), which facilitate water and solute movement across membranes, form a large gene family in animals, plants and microbes [1,2] where they are localized in various membranes. The direction of water or solute movement is determined by osmotic differences between compartments where the proteins reside. Based on this function, members of the entire water channel family are named aquaporins (AQPs). In *Arabidopsis thaliana*, 35 different aquaporin genes have been identified which are divided into four subfamilies [3,4]. The subfamily Plasma membrane Intrinsic Proteins (PIPs) are localized in the plasma membrane while Tonoplast Intrinsic Proteins (TIPs) are localized in the vacuolar membrane. The subcellular localization of the other two subfamilies, the NIPs (NOD26-like Intrinsic Proteins) and the SIPs (Small basic Intrinsic Proteins) [5], has not yet been established. Based on expressed sequence tag frequencies and microarray expression profiles, TIP1;1 is one of the most highly expressed AQPs in maize and *A. thaliana* [6,7]. The water channel BobTIP1;1 (original name BobTIP26-1) has been localized in the vacuolar membrane (tonoplast) of cauliflower (*Brassica oleracea*, var. *botrytis* L.) meristematic tissue [8], and two genes encoding tonoplast aquaporins called BobTIP1;1-1 and BobTIP1;1-2 have been shown to be susceptible to osmotic stress [9]. They are upregulated during desiccation stress in cauliflower meristematic tissue. Furthermore, in tobacco cell suspensions the fusion protein BobTIP1;1::GFP is well targeted to the tonoplast and its overexpression in transformed tobacco cells is associated with an increased cell volume [10].

Other studies which are focused more on PIPs than TIPs have also indicated a precise regulation of the expression of the aquaporin genes under water-deficit conditions. For example, the aquaporin RWC3 probably plays a role in drought avoidance in rice by regulating water movement across the plasma membrane under conditions of water deficit [11]. Expression analyses of *A. thaliana* transcriptionally modulated genes in response to ionic stress (NaCl, K^+ and Ca^{2+}) has revealed that all aquaporins are strongly (up or down) regulated by cation stress [12]. Other treatments such as low oxygen [13], drought and cold stress [14] or iron deficiency (Stanford Microarray Database: http://afc.stanford.edu) can also affect aquaporin expression levels, indicating the importance of water channels in different physiological processes.

In comparison with other living organisms, plants have a large number of aquaporin homologues. In view of such high isoform multiplicity, the elucidation of the physiological function of the individual members by over expression or by switching off (knockout mutants antisense, RNAi) is difficult [15–17]. This can be overcome using heterologous expression systems. In most cases, the water channel activity of individual isoforms of TIPs (including BobTIP1;1) as well as PIPs or NIPs has been determined using oocytes of *Xenopus laevis* [9,18–20]. Yeasts like *Saccharomyces cerevisiae* [21–23] or *Pichia pastoris* [24] provide alternative heterologous unicellular expression systems.

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Abbreviations: TIP, tonoplast intrinsic protein; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; YP, yeast extract peptone; YPD, yeast extract peptone glucose; Gal, galactose; YNB, yeast nitrogen base; Ni–NTA, nickel–nitrilotriacetic acid; PBS, phosphate-buffered saline

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Yeast cells adjust their behaviour in response to fluctuations in environmental osmolarity by a series of molecular, physiological and morphological events commonly known as the osmotic stress response [25,26]. The most pronounced response to hyperosmotic stress appears to be the production and intracellular accumulation of osmoprotective solutes (osmolytes) like glycerol [27]. Upon a hypo-osmotic shock, glycerol is rapidly released to prevent excessive cell swelling. The adaptation to hypo-osmotic shock has been more poorly studied but it is certain that the cell wall protects yeast cells from bursting [26]. Four genes encode members of the MIP family in *S. cerevisiae* [28]: the osmoregulated glycerol facilitator *FPS1* gene, the putative channel-like *YFL054c* ORF, and two aquaporin water channel genes *AQY1* and *AQY2*. The two non-functional alleles *AQY1-2* and *AQY2-2* have been identified in the wild- type strain SP1 which is used in this work [29]. The *YFL054c*, an ORF with high homology to the *FPS1* gene, encodes a putative channel-like protein [28] which mediates glycerol passive diffusion exclusively in the presence of ethanol [30]. The Fps1p is localized in the plasma membrane where its main physiological role is to regulate glycerol export under sudden hypo-osmotic shock [31]. A *fps1* deletion mutant has been constructed by excision of a fragment from the *FPS1* gene and replacement by the *LEU2* gene [32]. Under standard conditions the SP1 *fps1*Δ mutant has no functional MIP protein and is characterized by lower survival to hypo-osmotic shocks than the wild-type strain, which is accompanied by an inability to export glycerol and an accumulation of more intracellular glycerol [31,30].

To investigate the function of BobTIP1;1 in osmoregulation during the osmotic stress response we analysed expression and localization of BobTIP1;1 in yeast cells mutated for the *FPS1* gene. Transformation of the *fps1*Δ mutant with BobTIP1;1 conferred a hypo-osmotic shock resistant phenotype to the yeast cells, showing that the defect in the yeast glycerol plasma membrane transporter can be complemented by a plant vacuolar aquaporin. Bob-TIP1;1 was localized in the vacuolar membrane of the transformed yeast mutant cells but a study of the transport kinetics of glycerol did not allow to conclude that it was involved in glycerol efflux. These results show that BobTIP1;1 corrects the effect of the *fps1*Δ mutation in yeast cells that are submitted to a hypo-osmotic stress, without replacing the glycerol facilitator Fps1p in the plasma membrane.

2. Materials and methods

2.1. Strains and growth conditions

The *S. cerevisiae* strains used in this study were wild-type SP1 (*MATa leu2 his3 ura3 trp1 ade8 can1*) and the isogenic *fps1*Δ::*LEU2* mutant (*Mata fps1::LEU2 leu2 his3 ura3 trp1 ade8 can1*) [32]. Cultures were performed using selective medium [YNB: 0.67% (w/v) yeast nitrogen base without amino acids] with 2% (w/v) glucose and supplemented as required for each particular strain at 28 °C. All cloning steps in *Escherichia coli* were performed in strain JM109 [33]. Yeast transformations were performed by the lithium acetate method [34]. Transformants were selected on selective medium lacking uracil and individual clones were grown overnight in selective medium lacking uracil.

Yeast cultures were grown at 28 °C and on a rotary shaker at 180 rpm in 1 l Erlenmeyer flasks containing 100 ml of complete liquid medium [YP: 1% (w/v) yeast extract, 2% (w/v) peptone], with 2% (w/v) glucose (YPD) or 2% (w/v) galactose (YPGal), or 1% (w/v) galactose plus 1% (w/v) glucose (YPDGal). For hyperosmotic culture conditions, the yeast cells were grown in liquid medium supplemented with 1 M sorbitol. For hypo-osmotic shock conditions, the yeast cells were transferred into the same liquid medium without sorbitol.

2.2. Cloning of BobTIP1;1

The entire reading frame of the *BobTIP1;1* cDNA [8] was amplified by PCR using 5′-GAT CTG GTA CCG ACC ATG CCG ATC-3′ and 5′-GAG ATG AGC TCG GTA GTC GGC GG-3′ as forward and reverse specific primers, respectively. The 5′ primer contains the sequence encoding a *Kpn*I restriction site and the start codon. The reverse primer is designed to mutate the stop codon and contains a *Sac*I restriction site. The amplified fragment was subcloned into pGEM-T-Easy vector (Promega) and digested with *Kpn*I and *Bam*HI restriction enzymes. The fragment was ligated into a pYES2/CT expression vector (Invitrogen) to generate constructs containing the cDNA flanked with the *GAL1* promoter and *CYC1* terminator sequences and the resulting plasmid pYES2/CT-*BobTIP1;1-V5-6xHis* was sequenced. The plasmid contains a 6xHis-tagged region and a V5 epitope downstream of the cDNA.

2.3. Yeast colony PCR

PCR amplifications were performed on individual yeast colonies using 0.2 μM of each primer (the forward and reverse specific primers were used), 800 μM of dNTPs, 50 U/ml Redtaq DNA polymerase (Sigma) and 1× Redtaq buffer (Sigma) containing 11 mM $MgCl_2$. PCR conditions were: denaturation at 94 °C for 8 min, followed by 35 cycles (94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min) and a final elongation step at 72 °C for 10 min. PCR products were analysed by electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide.

2.4. Purification of 6xHis-tagged protein

The 6xHis-tagged fusion protein was expressed in yeast cells cultured in YPDGal medium and purified on a Ni^{2+}–nitrilotriacetate column, according to the manufacturer's instructions (Qiagen). The yeast cells were initially resuspended by adding 2 ml/g fresh weight lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, pH 8). The cells were disrupted by vortexing with glass beads five times for 30 s, with intervals in ice, and the lysate was clarified by centrifugation at 15 000 *g* for 10 min, then applied to nickel–nitrilotriacetic acid (Ni–NTA) agarose beads.

2.5. Electrophoresis and Western blot immunostaining

The crude lysate and the V5-6xHis-tagged purified protein were analysed by SDS–PAGE [35] in a 12% acrylamide slab gel. Proteins were solubilized 15 min at 100 °C in a SDS loading buffer (50 mM Tris–HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 0.001% bromophenol blue, 5% glycerol). Electrophoresis was run at room temperature for 2 h at 100 V. Proteins were stained with colloidal Coomassie blue [36] or transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by electrophoresis 45 min at 15 V in a Bio-Rad (Hercules, CA) Mini-Protean III apparatus.

Western blotting was performed using mouse monoclonal anti-V5 (dilution 1:5000; Invitrogen) and horseradish peroxidase-conjugated sheep anti-mouse-IgG (dilution 1:10 000; Amersham–Pharmacia Biotech). Immunostaining was visualized using the enhanced chemiluminescence Western blotting detection reagents according to the manufacturer's instructions (Amersham–Pharmacia Biotech).

2.6. Phenotype analysis

Yeast cells were pregrown in YP medium supplemented with 2% galactose, 2% glucose or 1% galactose plus 1% glucose and 1 M sorbitol. Cells were resuspended in the same medium to an OD_{600} of 1.0. A 10-fold serial dilution of this culture was made and 5 μl of each dilution were spotted onto YP-agar plates, supplemented with 2% galactose, 2% glucose or 1% galactose plus 1% glucose, without osmoticum (hypo-osmotic shock). The cultures were incubated at 28 °C for 3 days. The data for phenotype analysis represent results of one typical experiment from at least two independent experiments.