# The Pzh1 protein phosphatase and the Spm1 protein kinase are involved in the regulation of the plasma membrane $H^+$-ATPase in fission yeast

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**Abstract We have previously shown that the mutation of the *Schizosaccharomyces pombe* PPZ-like protein phosphatase encoded by the gene *pzh1*$^+$ results in increased tolerance to sodium and in hypersensitivity to potassium ions. A similar phenotype has also been reported for deletants in the *spm1/pmk1* gene, encoding a mitogen-activated protein (MAP) kinase. We have found that the sodium tolerance phenotype of *pzh1* deletants is stronger than that of *spm1* mutants, and both effects are additive. Therefore, most probably both gene products mediate different pathways on sodium tolerance. In our hands, mutation of the kinase does not alter the tolerance to potassium, but it yields cells more tolerant to magnesium ions. While in budding yeast the mutations are synthetically lethal, fission yeast cells lacking both the phosphatase and the kinase genes are viable. Interestingly, their ability to export $H^+$ to the medium is greatly impaired (although not that of *pzh1* or *spm1* single mutants). We have observed that, although the amount of the $H^+$-ATPase in the plasma membrane is not altered, the activity of the enzyme is lower than normal and cannot be induced by glucose. These observations suggest that the activity of the $H^+$-ATPase in fission yeast might be regulated by phospho-dephosphorylation mechanisms that might involve the *pzh1*$^+$ and *spm1*$^+$ gene products.**



*Key words:* Protein phosphorylation; Salt tolerance; Protein phosphatase; Proton efflux; $H^+$-ATPase; *Schizosaccharomyces pombe*

## 1. Introduction

The mechanisms that drive ion and nutrient uptake in fungi are powered by the active efflux of $H^+$ from the inside to the outside of the cell (see [1] for a review). This electrochemical proton gradient is built by proton transporting P-type ATPases. The plasma membrane $H^+$-ATPase has been shown to be essential in both budding [2] and fission yeast [3]. In fission yeast, two genes (*pma1*$^+$ and *pma2*$^+$) encode plasma membrane $H^+$-ATPases. Although functionally interchangeable, they are differentially expressed, the function of Pma1 being predominant [3].

Budding and fission yeast rely on different mechanisms for excluding $Na^+$ ions [4]. In the budding yeast *Saccharomyces cerevisiae*, active sodium efflux relies on the P-type ATPase encoded by the *ENA1* gene (the first member of the *ENA/PMR2* locus), which is induced upon exposure of the cells to high sodium or lithium, and to alkaline pH [5–7]. The Ser/Thr Ppz1 and Ppz2 protein phosphatases define a most interesting regulatory pathway for *ENA1* expression [8–10]. These gene products (particularly Ppz1) exert a negative regulatory effect on the expression of the ATPase gene. Therefore, cells lacking PPZ1 are hypertolerant to sodium and lithium ions, although they do not show altered tolerance to potassium ions [9]. On the contrary, in the fission yeast *Schizosaccharomyces pombe*, efflux of lithium and sodium is mediated by the $H^+/Na^+$ antiporter encoded by the *sod2* gene [11,12] and, consequently, cells lacking the antiporter are highly sensitive to these cations.

Despite these differences, we have recently identified and cloned from fission yeast the gene *pzh1*$^+$, encoding a PPZ-like protein phosphatase [13]. This gene product is also involved in cation tolerance, since cells lacking *pzh1* are hypertolerant to sodium and lithium, as well as hypersensitive to potassium. Preliminary analysis suggests that the phosphatase functions in a pathway that is unrelated to the Sod2 antiporter [13]. Interestingly, a phenotype similar to that described for *pzh1* mutants has been ascribed to cells defective in the Pmk1/Spm1 mitogen-activated protein (MAP) kinase [14], although this has not been confirmed by other authors [15]. The *pmk1/spm1* gene has also been recently involved in chloride resistance [16]. The similarity in phenotypes of *pzh1* and *spm1/pmk1* mutants (as well as the discrepancies reported in the case of the kinase) prompted us to undertake a more detailed analysis of the possible functional relationship between both gene products. In this paper we show that they define different pathways on sodium tolerance and that they seem to be involved in the regulation of the glucose-induced activation of the plasma membrane $H^+$-ATPase.

## 2. Materials and methods

### 2.1. Strains, recombinant DNA techniques and gene disruptions

*Escherichia coli* cells (strain NM522 and DH5α) were grown at 37°C in LB medium (supplemented with 50 μg/ml ampicillin for plasmid selection). *Schizosaccharomyces pombe* cells were grown at 28°C in YES (0.5% yeast extract, 3% glucose, plus 225 μg/ml of adenine, uracil and leucine) or essential minimal medium (EMM) supplemented with the necessary requirements [17]. The pH of the medium was buffered at 5.2 with 20 mM MES.

*E. coli* cells were transformed using the calcium chloride method [18] and *S. pombe* cells were transformed by a modification of the lithium acetate method [19]. Restriction reactions and other standard recombinant DNA techniques were performed essentially as described [18]. Strains described in this paper derive from the wild-type strain 117 (*h*$^-$ *ade6-M210 ura4-D18 leu1-32*). Gene disruptions were made by using the one-step gene disruption method [20]. The construction of strain LB2 (*pzh1::ura4*$^+$) has been described previously [13]. Strain LB5, which carries a deletion of the *spm1*$^+$ gene, was generated by using a previously described *spm1::LEU2* cassette [15]. Strain LB7

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(*pzh1::ura4*$^+$ *spm1::LEU2*) was constructed by transformation of strain LB2 with the *spm1::LEU2* disruption cassette. All disruptions were verified by PCR analysis.

### 2.2. Determination of salt sensitivity and ion contents of the yeast strains

Sensitivity to the different salts was monitored on agar plates and liquid cultures. Dot tests were performed on plates containing different amounts of salts as previously described [13]. The tolerance in liquid cultures was evaluated by diluting fresh cultures with media (to achieve an optical density of 0.030). One hundred μl of the diluted cultures were placed in 96-well microtiter plates and 100 μl of fresh media, containing different concentrations of salts were added. Cells were grown for 20 h with shaking and growth determined by measuring the optical density of the cultures at 630 nm. Relative growth was calculated as the ratio between growth in the presence or the absence of added salts and expressed as a percentage. The intracellular ion ($Li^+$, $Na^+$, $K^+$, $Rb^+$) content of the cells was determined as previously described [21,22]. Briefly, samples of cells were filtered, washed with 20 mM magnesium chloride, treated with acid, and the cations analyzed by atomic absorption spectrophotometry.

### 2.3. Measurement of proton flux and plasma membrane ATPase activity

For proton flux measurements, cells were grown in buffered YES medium, harvested, washed twice with ice-cold distilled water and resuspended in 10 ml of glycyl glycine buffer (glycyl glycine 10 mM, magnesium chloride 10 mM, adjusted at pH 4.5 with HCl) to give an optical density of 1. Cells were maintained in suspension by stirring at 28°C. After five minutes of pre-incubation to allow the pH of the medium to stabilize, glucose (100 mM) was added to trigger proton flux through activation of the $H^+$-ATPase [23,24]. Proton flux was determined by continuously monitoring the pH of the medium.

The activation of plasma membrane ATPase by glucose was performed as described previously for *S. cerevisiae* [23,25] with minor modifications. Briefly, cells were grown in YES medium and harvested by centrifugation at 4°C in the mid-exponential phase ($OD_{660}$ = 1.0). Pellets were then resuspended in distilled water with or without glucose (0.1 M). After a 10-min incubation, a concentrated solution of Tris-EDTA (pH 8.0) containing dithiothreitol and protease inhibitors [23] was added, and the suspensions were rapidly frozen at −80°C. After thawing, cells were disrupted with the aid of glass beads. The total membrane fraction preparation was obtained and the ATPase activity was determined at pH 6.5 as previously described [23] using two different concentrations of ATP (1 and 2 mM). Protein concentration in the total membrane fraction was measured by Bradford's procedure [26] using bovine serum albumin as the standard.

### 2.4. Immunological techniques

For immunodetection of the plasma membrane $H^+$-ATPase, cells (50 ml) were grown on YES medium up to an optical density of one, collected by centrifugation, and resuspended in 1 ml of fresh medium. The suspension consisted of 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 60 mM potassium chloride, 0.5 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. Two ml of ice-cold glass beads was added and the cells disrupted by vortex for three min at 4°C. Homogenates were diluted with 2 ml of GTED buffer (10 mM Tris-HCl, pH 7.5, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol), vortexed briefly and centrifuged 5 min at 750×*g*. Supernatants were then centrifuged at 27 000×*g* and pellets resuspended in 200 μl of GTED buffer. Aliquots (40 μl, about 30 μg of protein) were run on 7.5% SDS-polyacrylamide gels [27]. For immunological detection, proteins were transferred to Immobilon P membranes (Millipore), blocked and incubated with a 1:2000 dilution of a polyclonal antibody raised against the *S. cerevisiae* plasma membrane $H^+$-ATPase (kindly provided by Dr. F. Portillo). Immunoreactive proteins were identified with luminescence substrates (ECL, Amersham).

## 3. Results and discussion

### 3.1. Analysis of the functional relationship of the Pzh1 phosphatase and the Spm1 MAP kinase in salt tolerance

The initial report [14] describing the existence in fission yeast of the novel MAP kinase gene *pmk1*$^+$ (also known as *spm1*$^+$) suggested that this kinase might be involved in the regulation of salt tolerance, since the mutation of this gene resulted in increased tolerance to sodium and decreased tolerance to potassium. These phenotypes are strikingly similar to those found in our laboratory for mutants in the *pzh1* phosphatase [13]. However, the increased tolerance to sodium of the kinase mutant was not confirmed in a different report [15]. These facts prompted us to undertake a more detailed study to define the role of this kinase on salt tolerance and its functional relationship with Pzh1.

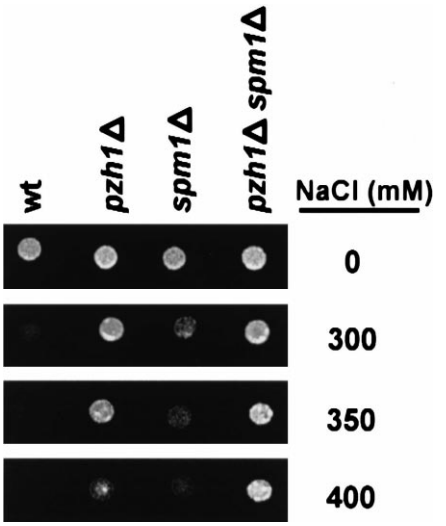


Fig. 1. Comparison of the effects of the *pzh1* and *spm1* mutations on sodium tolerance. *S. pombe* wild-type cells and strains LB2 (*pzh1::ura4*$^+$), LB5 (*spm1::LEU2*) or LB7 (*pzh1::ura4*$^+$ *spm1::LEU2*) were grown overnight on liquid YES medium. The cell suspensions were diluted to an $OD_{595}$ of 0.05 and 3 μl deposited on YES plates containing the indicated amounts of NaCl. Growth was monitored after 48 h at 30°C.

In our hands, deletion of the *spm1/pmk1* gene results in increased tolerance to sodium ions (Fig. 1), according to that described by Toda et al. [14], although the effect is relatively weak. It has been claimed that the existence of a *ura4*$^-$ mutation in the genetic background used in [15] might account for the different phenotype reported by these authors in comparison to that reported by Toda et al., who used an *ura4*$^+$ strain [14]. Although we have also verified that the presence or absence of the *ura4*$^+$ marker slightly alters sodium tolerance (not shown), in our case the *spm1* mutation has been made in an *ura4*$^-$ background. Therefore, the differences described in the previous reports are difficult to explain solely on the basis of this specific background difference. We could not observe differences between wild-type and *spm1Δ* cells in tolerance to potassium below 1 M KCl, although *spm1Δ* cells were more sensitive to higher concentrations of the salt (not shown). This probably does not reflect a difference in tolerance specific for potassium, but the failure of the *spm1Δ* mutants to grow at high osmolarity [15].

As can be observed in Fig. 1, the increase in tolerance to sodium as a result of the mutation of *spm1* is clearly smaller than that of *pzh1Δ* cells. In fact, the differences in sodium or potassium fluxes, or intracellular content of these cations when comparing wild type and *spm1* mutants (not shown) were very small, whereas changes in these parameters are readily detectable in *pzh1* cells (Balcells et al., manuscript in preparation). In addition, cells carrying a deletion in both the

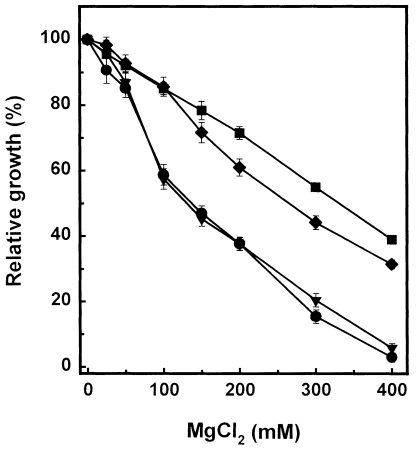


Fig. 2. Mutation of the *spm1/pmk1* kinase gene yields cells hypertolerant to magnesium ions. Strains 117 (wild type, – ● –), LB2 (*pzh1::ura4*$^+$, – ▼ –), LB5 (*spm1::LEU2*,– ■ –) and LB7 (*pzh1::ura4*$^+$ *spm1::LEU2*, – ◆ –) were grown in the presence of different concentrations of magnesium chloride and the effect of the salt on cell growth determined as described in Section 2. Results are mean ± S.E.M. of 6 independent experiments.

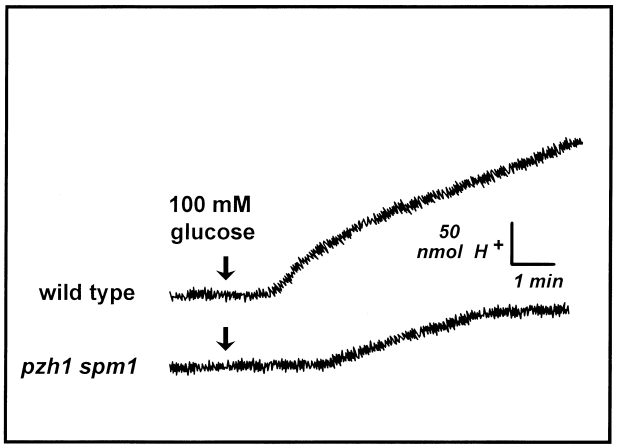


Fig. 3. Impaired proton efflux in *pzh1 spm1* mutants. Proton flux for wild type (strain 117) or *pzh1 spm1* (strain LB7) mutants was determined by continuously monitoring the pH of the medium after activation of the plasma membrane H$^+$-ATPase with 100 mM glucose.

kinase and the phosphatase genes are more tolerant than *pzh1* mutants, suggesting an additive effect. Therefore, the mechanisms that might mediate the effect of Spm1 on sodium tolerance seem to be independent from those of Pzh1.

Wild type, as well as strains carrying deletion in *pzh1, spm1* or both genes have also been tested for magnesium tolerance (Fig. 2). Deletion of the phosphatase gene does not alter tolerance to magnesium chloride. However, cells defective in Spm1 function, are clearly more tolerant to this salt. This effect is observed even at relatively low concentrations of magnesium chloride. Therefore, the difference is not provoked by the presence of chloride ions because, as we mentioned above, wild-type and *spm1*$^-$ cells grow similarly in the presence of up to 1 M KCl. The deletion of the phosphatase in as *spm1*$^-$ background results only in a small decrease in tolerance. It is remarkable that a previous study on *spm1* mutants [14], in which magnesium tolerance was tested, failed to detect this phenotypic alteration. In fission yeast, changes in fluxes of $Mg^{2+}$ have been described prior to cell division [28]. Altered $Mg^{2+}$ sensitivity has been described in *S. pombe sep1* mutants, which are defective in a transcription factor homologue of the HNF-3/forkhead DNA-binding-domain family and display aberrant cell shape and septum formation [29,30]. It is remarkable that the Spm1/Pmk1 kinase has been postulated to have a role in cytokinesis, cell wall metabolism and morphogenesis, that is, in functions that are directly related to progress into cell cycle.

### 3.2. *The pzh1 spm1 mutant shows a phenotype of decreased proton flux that can be attributed to an impaired plasma membrane H$^+$-ATPase activity*

As a part of a wide screening for phenotypic changes related to mutations in the *pzh1* or *spm1* genes, we have tested the ability of these mutants to induce proton efflux as a result of glucose-induced activation of the plasma membrane H$^+$-

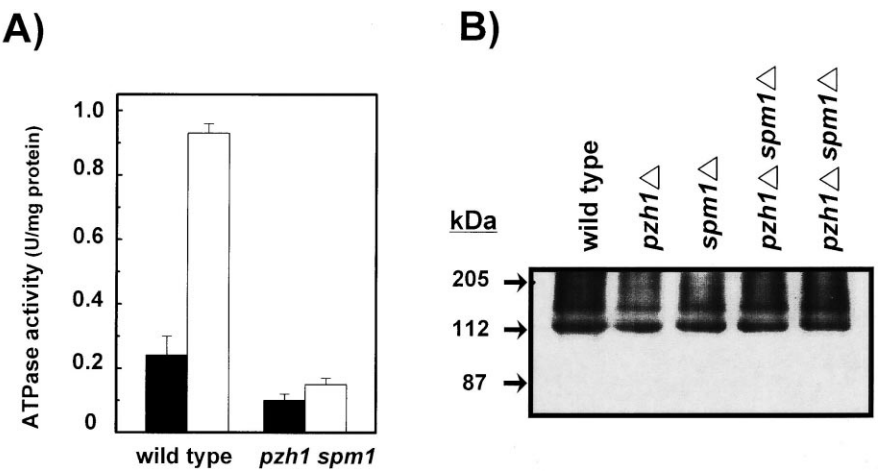


Fig. 4. Mutation of the *pzh1* and *spm1* genes affects the activity of the plasma membrane H$^+$-ATPase. A: Determination of the activity of the plasma membrane H$^+$-ATPase in wild-type or LB7 cells untreated (closed bars) or treated with 100 mM glucose (open bars). One unit of ATPase activity is equivalent to 1 μmol of phosphate released/min. Data are mean ± S.E.M. of three independent experiments. B: Immunodetection of the plasma membrane H$^+$-ATPase. Crude preparations of plasma membranes from the indicated strains were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted as described in Section 2.

ATPase. We have not observed changes in proton efflux in cells lacking the Pzh1 phosphatase or the Spm1 kinase (not shown). However, in the double *spm1 pzh1* mutant the proton efflux process induced by glucose is clearly impaired (Fig. 3). This situation is revealed by a 3- to 4-fold reduction in the total amount of protons released to the medium after 10 min of glucose addition. To test if the reduced proton efflux was due to a defect in the activity of the membrane $H^+$-ATPase, we have determined this activity in partially purified membranes from wild type and *spm1 pzh1* mutants. As shown in Fig. 4A, the ATPase in *spm1 pzh1* cells is slightly less active in the absence of glucose and, in addition, is virtually unable to show the characteristic activation in response to glucose [24]. As expected for yeast cells with low levels of ATPase activity, *spm1 pzh1* double mutants displayed a growth defect on acidic medium (below pH 4). The impaired ATPase activity is not due to a defect in the amount of catalytic protein at the plasma membrane, since immunoblot experiments showed equivalent amounts of the immunoreactive 110-kDa protein (Fig. 4B). Although a detailed kinetic analysis has not been performed, preliminary data suggest that the *spm1 pzh1* mutations might not alter the $K_m$ for ATP of the ATPase (not shown).

It has been proposed that Spm1/Pmk1 could be the fission yeast homolog of the budding yeast Slt2/Mpk1 MAP kinase, although they might differ in certain cellular functions [14,15]. In *S. cerevisiae*, cells lacking both the Slt2/Mpk1 kinase and the Ppz1 phosphatase cannot grow [31]. However, we show here that the *spm1 pzh1* mutant is viable and grows normally under standard conditions. This argues again in favor of the notion that both Spm1 and Pzh1 proteins are not functionally identical to their budding yeast counterparts. Nevertheless, the kinase and the phosphatase still retain a functional connection in fission yeast, because the absence of both genes results in cells with a plasma membrane $H^+$-ATPase insensitive to glucose-induced activation. This finding suggests that protein phosphorylation events might regulate this process in fission yeast. In fact, involvement of phosphorylation mechanisms in the regulation of the glucose-induced activation of the ATPase has been documented in *S. cerevisiae*, and it has been proposed that two independent phosphorylating pathways should exist: one that involves casein kinase-1, and another that involves an 'up-regulating' kinase, yet uncharacterized [32]. Recent evidence also suggests that, in *S. cerevisiae*, the Ser/Thr protein phosphatase calcineurin regulates Pma1 activity [33,34].

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