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# The Ppz protein phosphatases regulate Trk-independent potassium influx in yeast

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**Abstract The Ppz protein phosphatases have been recently shown to negatively regulate the major potassium transport system in the yeast *Saccharomyces cerevisiae*, encoded by the *TRK1* and *TRK2* genes. We have found that, in the absence of the Trk system, Ppz mutants require abnormally high concentrations of potassium to proliferate. This can be explained by the observation that *trk1 trk2 ppz1* or *trk1 trk2 ppz1 ppz2* strains display a very poor rubidium uptake, with markedly increased $K_m$ values. These cells are very sensitive to the presence of several toxic cations in the medium, such as hygromicyn B or spermine, but not to lithium or sodium cations. At limiting potassium concentrations, addition of EGTA to the medium improves growth of these mutants. Therefore, our results indicate that, in addition to their role in regulating Trk potassium transporters, Ppz phosphatases (essentially Ppz1) positively affect the residual low affinity potassium transport mechanisms in yeast. These findings may provide a new way to elucidate the molecular nature of the low affinity potassium uptake system in yeast as well as a useful model to analyze the function of plant or mammalian potassium channels through heterologous expression in yeast.**


*Keywords:* Protein phosphatases; Potassium uptake; Trk1/Trk2 transporter; Yeast

## 1. Introduction

*Saccharomyces cerevisiae PPZ1* and *PPZ2* genes encode two structurally similar Ser/Thr protein phosphatases which are involved in a variety of cell processes, such as salt tolerance, maintenance of cell integrity and regulation of cell cycle at the $G_1$/S transition (see [1] for a review). The biological role of Ppz1 is more important than that of Ppz2, as deduced by the fact that deletion of the latter does not result in significant phenotypes, although it intensifies the phenotypes associated to the absence of *PPZ1*. For instance, cells lacking Ppz1 are tolerant to high concentrations of sodium or lithium cations, while *ppz2* cells are not. However, a double *ppz1 ppz2* mutant is even more tolerant than a single *ppz1* strain. It has been recently proposed that most of the phenotypes produced by deletion of the Ppz phosphatases could be explained by a negative role of these proteins on the Trk1/Trk2 potassium transporters [2,3].

Trk1 and Trk2 are membrane proteins responsible for the high affinity potassium uptake in *S. cerevisiae*. Trk1 appears to be the most important component in this uptake, as mutation of this gene results in a significant defect in potassium transport and the inability to grow under limiting concentrations of this cation [4–6]. Mutation of *TRK2* aggravates the requirement for potassium of a *trk1* mutant. It has been documented that defects in potassium transport result in increased sensitivity to sodium or lithium cations [7–10]. Furthermore, cells lacking the high affinity transporters show highly hyperpolarized membrane potential [11].

The viability of *trk1 trk2* cells revealed the existence of additional forms to transport potassium, although the nature of this transport remains still obscure. Two main alternatives have been postulated in the past few years. For instance, the existence of a calcium-blocked, non-specific cation channel named NSC1 has been proposed as the primary low-affinity potassium uptake route [12–14]. However, the molecular nature of this transporter has not been determined. In contrast, an ectopic, non-specific uptake that would occur through sugar and/or amino acid permeases has been postulated [11,15].

In this work, we present data supporting the notion that the Ppz phosphatases (essentially Ppz1) also influence the low affinity potassium uptake observed in the absence of the Trk transporters. In this case, however, the phosphatases would act as positive modulators of the transport.

## 2. Materials and methods

### 2.1. Yeast strains and growth conditions

Yeast cells were grown, unless otherwise stated, at 28 °C in YPD medium, complete minimal medium (CM) lacking the appropriate requirements for selection or in the ammonium-phosphate $K^+$- and $Na^+$-free minimal medium [16,17]. When indicated, media were supplemented with KCl. All yeast strains used in this work are listed in Table 1. Strains MAR71 and MAR72 were constructed by transforming strains ESV210 (*trk1::LEU2*) and ESV220 (*trk2::HIS3*), respectively, with the *ppz1* ::*URA3* and *ppz2* ::*TRP1* disruption cassettes described in [18] and [19]. Strains MAR70, MAR62, and MAR73 were obtained by transformation of strain ESV212 (*trk1::LEU2 trk2::HIS3*) with the mentioned disruption cassettes.

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Table 1
Yeast strains used in this work

| Name | Relevant genotype | Source/reference |
|---|---|---|
| DBY746 | *MATα ura3-52 leu2-3, 112 his3-* Δ1 *trp1-* Δ239 | D. Botstein |
| JA31 | DBY746 *ppz1* ::*URA3 ppz2* ::*TRP1* | [28] |
| ESV212 | DBY746 *trk1* ::*LEU2 trk2* ::*HIS3* | [22] |
| MAR70 | DBY746 *trk1* ::*LEU2 trk2* ::*HIS3 ppz1* ::*URA3* | This work |
| MAR62 | DBY746 *trk1* ::*LEU2 trk2* ::*HIS3 ppz2* ::*TRP1* | This work |
| MAR71 | DBY746 *trk1* ::*LEU2 ppz1* ::*URA3 ppz2* ::*TRP1* | This work |
| MAR72 | DBY746 *trk2* ::*HIS3 ppz1* ::*URA3 ppz2* ::*TRP1* | This work |
| MAR73 | DBY746 *trk1* ::*LEU2 trk2* ::*HIS3 ppz1* ::*URA3 ppz2* ::*TRP1* | This work |

### 2.2. Growth experiments

To test the growth capacity of the yeast cells on solid media, plates were spotted with 3–10 μl of diluted cultures and incubated for the indicated periods of time. To calculate the doubling time, cells were grown in liquid ammonium phosphate medium supplemented with 100 mM KCl, washed twice with sterile cool distilled water and inoculated ($10^5$ cells/ml) in the same medium containing different concentrations of KCl. Density of the cultures ($A_{550}$) was monitored at different times [20]. To evaluate the effect of external calcium levels on growth, cells were cultured overnight in YPD, washed and resuspended at $A_{620}$ 0.05 in ammonium phosphate medium supplemented with KCl. Aliquots were distributed in 96-well plates, incubated for 24 h and the optical density determined. Under these conditions, the $A_{620}$ of the cultures varied from 0.2 to 0.9, according to the strain and the treatment.

### 2.3. $K^+$ content of cells and $Rb^+$ transport experiments

The $K^+$ content of cells grown in ammonium phosphate supplemented with 10 mM KCl was determined by collecting them on Millipore filters, which were rapidly washed with 20 mM $MgCl_2$. The cells were then extracted with acid and analyzed by atomic emission spectrophotometry, essentially as described in [21]. The experiments were repeated 3–6 times and the standard deviations calculated.

The time course of $Rb^+$ uptake of actively growing cells was studied in ammonium phosphate medium supplemented with 20 mM KCl. When the $A_{550}$ of the culture reached values of 0.3, RbCl (50 mM) was added to the medium (zero time) and samples of cells were withdrawn at various times thereafter [21]. The kinetic constants for rubidium transport were determined in $K^+$-starved cells. To this end, the different strains were grown in ammonium phosphate medium supplemented with 100 mM KCl, resuspended in $K^+$-free medium, and grown for 5 additional hours. Then, the cells were centrifuged, washed, and resuspended in uptake buffer, consisting of 10 mM MES (brought to pH 5.8 with $Ca(OH)_2$), 0.1 mM $MgCl_2$, 2 mM $CaCl_2$, and 2% glucose (w/v). The required amount of RbCl was added to the buffer at zero time and samples of cells were withdrawn at various times. Cells were treated as described for determining the $K^+$ content. The velocity values are expressed as nmol/min/mg of dry weight of cells. All experiments were repeated at least three times.

## 3. Results

### 3.1. Ppz phosphatase mutants show increased potassium requirement in the absence of Trk transporters

To evaluate the effect of the absence of Ppz phosphatases in the ability of cells lacking the Trk potassium transporters to grow under limiting potassium concentrations, the *PPZ1* and *PPZ2* genes were disrupted in a *trk1 trk2* background. These strains were spotted on ammonium phosphate plates supplemented with different concentrations of potassium chloride. As observed in Fig. 1, upper panel, wild-type and *ppz1 ppz2* cells were able to grow even at very low concentration of added potassium (0.2 mM), while the strains lacking the *trk1 trk2* transporters did not. Trk-deficient cells or *trk1 trk2 ppz2* mutants grew when the concentration of potassium was increased to 5 mM. Interestingly, mutation of *PPZ1* in Trk-deficient cells did not allow growth at 5 mM KCl, but required a higher amount (50 mM) of added potassium. The quadruple mutant did show a similar phenotype to that of the *ppz1 trk1 trk2* strain. To quantify these effects more accurately, a similar experiment was performed in liquid medium containing different amounts of added potassium (Fig. 1, lower panel). As it can be observed, the absence of Ppz1 function severely affects growth at limiting potassium as denoted by the increase in the doubling time. Additional mutation of *PPZ2* slightly aggravates the growth defect. These phenotypes are eliminated by increasing potassium in the medium.

The growth defect of Trk Ppz mutants could be attributed to a defect in the transport of this cation. To evaluate this possibility, we grew different mutants in ammonium phosphate medium containing 20 mM potassium, 50 mM rubidium chloride was added and the time-course of rubidium uptake (widely used as a $K^+$ analog in transport experiments) was

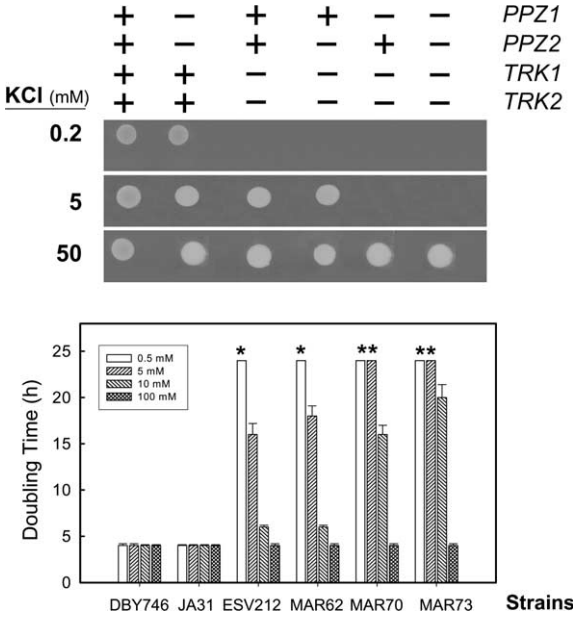


Fig. 1. *Upper panel*: DBY746 wild-type cells and the indicated isogenic derivatives were spotted in ammonium phosphate plates containing different concentrations of potassium chloride. Growth was monitored after 3 days. *Lower panel*: The indicated strains were inoculated in liquid ammonium phosphate medium containing different concentrations of potassium chloride and $A_{550}$ determined periodically for at least 48 h. Asterisks denote strains that did not significantly proliferate after 48 h of culture. As both panels are aligned, the corresponding genotype for each strain appears at the top of the upper panel.

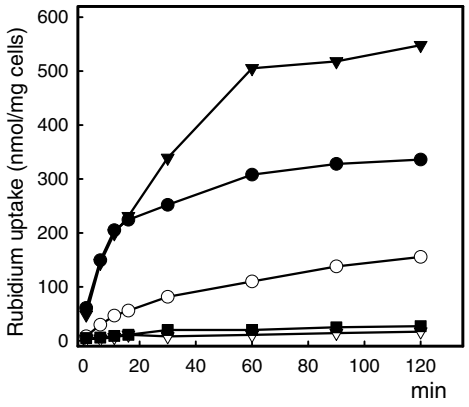


Fig. 2. Wild type strain DBY746 (-●-) as well as ESV212 (*trk1 trk2*, -○-), JA31 (*ppz1 ppz2*, -▼-), MAR70 (*ppz1 trk1 trk2*, -■-) and MAR73 (*ppz1 ppz2 trk1 trk2*, -▽-) isogenic derivatives were grown in ammonium phosphate medium containing 20 mM potassium chloride. At time zero, rubidium chloride was added to achieve a concentration of 50 mM, aliquots of the cells taken at the indicated times and the amount of internal rubidium were determined. A representative experiment is shown.

determined. As shown in Fig. 2, lack of Ppz phosphatases increased rubidium uptake while, as expected, Trk mutants showed a dramatic reduction in uptake. Remarkably, under the conditions tested the uptake of strain MAR70 (*ppz1 trk1 trk2*) and that of the quadruple mutant was virtually null. Similar results were obtained at different rubidium concentrations or when cells were grown in SD medium. Strain MAR72 (*ppz1 ppz2 trk2*) behaved similarly to the *ppz1 ppz2* mutant, while uptake of strain MAR71 (*ppz1 ppz2 trk1*) was similar to that of the quadruple mutant strain (not shown). Measurement of the intracellular potassium levels under limiting extracellular potassium (Table 2) revealed that strain, MAR70 (*ppz1 trk1 trk2*) and MAR73 (*ppz1 ppz2 trk1trk2*) accumulated less potassium than the wild-type strain or even than the *trk1 trk2* mutant.

Table 2
Effects on the potassium content and on the kinetic parameters for rubidium uptake of Trk and Ppz mutations

| Strain | Genotype | Potassium content (nmol/mg) | Rubidium uptake kinetic parameters | |
|---|---|---|---|---|
| | | | $K_m$ (mM) | $V_{max}$ (nmol/mg/min) |
| DBY746 | *PPZ1 PPZ2 TRK1 TRK2* | 405 ± 33 | 0.2 ± 0.03 | 28.5 ± 3.5 |
| JA31 | *ppz1 ppz2 TRK1 TRK2* | 577 ± 26 | 0.2 ± 0.03 | 30.0 ± 3.2 |
| ESV212 | *PPZ1 PPZ2 trk1 trk2* | 380 ± 27 | 14.0 ± 2.4 | 10.1 ± 2.3 |
| MAR62 | *PPZ1 ppz2 trk1 trk2* | 391 ± 43 | 16.2 ± 2.1 | 9.2 ± 3.7 |
| MAR70 | *ppz1 PPZ2 trk1 trk2* | 344 ± 38 | 66.9 ± 3.9 | 7.5 ± 1.4 |
| MAR73 | *ppz1 ppz2 trk1 trk2* | 329 ± 45 | 70.0 ± 5.4 | 7.1 ± 2.0 |

For potassium content measurements, cells were grown in ammonium phosphate medium supplemented with 10 mM KCl. For rubidium uptake experiments, cells were potassium-starved and $K_m$ and $V_{max}$ determined as described under Experimental. Data are means ± SD from three independent experiments.

To characterize the nature of the effect of lack of Ppz phosphatases in a Trk-deficient background, uptake of rubidium was quantified under conditions that would allow determining the kinetic parameters of the transport. As shown in Table 2, deletion of *PPZ2* did not significantly alter the $K_m$ or the $V_{max}$ of a *trk1 trk2* strain. In contrast, deletion of *PPZ1* drastically increased the $K_m$ of the transport (almost 5-fold) and somewhat reduced the $V_{max}$. These parameters were very similar in the quadruple mutant.

### 3.2. Ppz phosphatase mutants show altered sensitivity to toxic cations in the absence of Trk transporters

The results presented above reinforce the notion that Ppz phosphatases (essentially Ppz1) are negative effectors of the Trk potassium transporters. Remarkably, in the absence of Trk1 and Trk2, our data show that the absence of the phosphatases drastically worsens the transport of potassium. We considered that the very poor transport observed in the quadruple mutant should reflect in the tolerance of these cells to toxic cations. To test this hypothesis, different mutants were grown in the presence of hygromycin B, spermine or tetramethylammonium (TMA), as well as in diverse concentrations of sodium or lithium chloride. We observed (Fig. 3) that, as previously reported [2,11], *trk1 trk2* mutants are sensitive to low concentrations of hygromycin B, spermine or TMA (not shown). The absence of Ppz1 (but not that of *PPZ2*) further increased sensitivity to these compounds. In contrast, lack of Ppz1 (or deletion of both *PPZ* phosphatases genes) increased tolerance to both sodium and lithium cations of Trk-deficient cells.

Low affinity potassium transport has been documented in the absence of Trk transporters and some reports have linked this phenomenon to the presence of calcium cations in the medium. We then decided to analyze the possible relationship

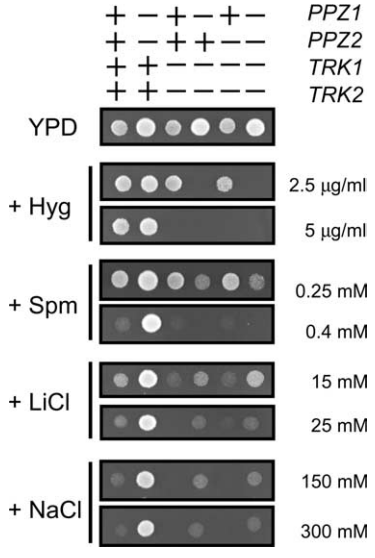


Fig. 3. Effect of mutation of Trk potassium transporters and Ppz phosphatases on tolerance to toxic cations. The wild-type strain DBY746 and its derivatives JA31 (*ppz1 ppz2*), ESV212 (*trk1 trk2*), MAR70 (*ppz1 trk1 trk2*), MAR62 (*ppz2 trk1 trk2*) and MAR73 (*ppz1 ppz2 trk1 trk2*) were spotted on YPD plates containing the indicated concentrations of different toxic cations. Hyg, hygromycin B; Spm, spermine. Growth was monitored after 3 days.

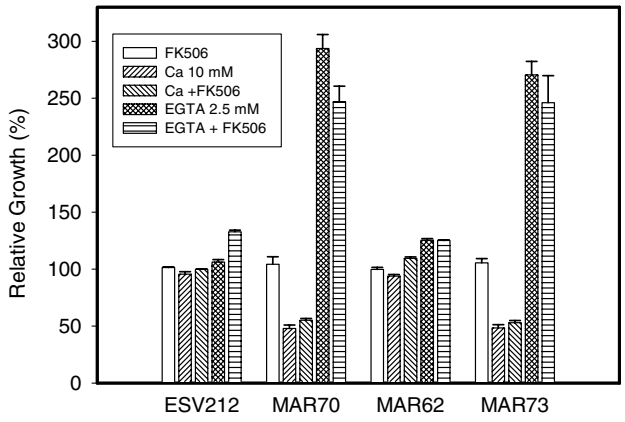


Fig. 4. Functional interactions between calcium and the Trk and Ppz mutations. Cells were inoculated ($A_{620}$ 0.05) in ammonium phosphate medium supplemented with 10 mM KCl (plus the indicated compounds) and grown for 24 h. FK506 was used at 1.5 μg/ml. Data represent the percentage of growth with respect to that of cells that received no treatment and expressed as means ± SEM from six independent determinations.

between this divalent cation and the growth defect observed in the Trk mutants lacking Ppz phosphatases. Results in Fig. 4 show that, under the conditions tested, addition of calcium barely affects growth of ESV212 (*trk1 trk2*) or MAR62 (*ppz2 trk1 trk2*) cells. However, calcium worsened the growth defect of strains MAR70 (*ppz1 trk1 trk2*) and MAR73 (*ppz1 ppz2 trk1 trk2*), and this was not prevented by incubation of the cells with the calcineurin inhibitor FK506. These effects on growth were not observed when cells were cultured in the presence of higher potassium concentrations (100 mM). In contrast, addition of EGTA to the medium significantly improved growth of strains MAR70 and MAR73, but did not affect ESV212 or MAR62 cells. At high potassium (100 mM), none of the strains were affected by EGTA (not shown).

## 4. Discussion

Several lines of evidence appeared in the last two years have provided support to the notion that the Ppz protein phosphatases act as negative effectors on potassium uptake through regulation of the high-affinity potassium transporters Trk1/Trk2. For instance, potassium content in *ppz1 ppz2* cells is higher than in wild-type cells under different growth conditions (i.e., saline stress) and this increase is abolished in *trk1 trk2* double mutants [2,3,22]. In addition, strong overexpression of Ppz1 results in decreased rubidium uptake [2]. We have confirmed the high content of potassium of a *ppz1 ppz2* strain in the absence of salt stress and our data also indicate that the single *ppz1* mutant (but not a *ppz2* strain) already shows a large accumulation of potassium, suggesting that Ppz1 has the major role for Trk regulation. Furthermore, we find that *ppz1 ppz2* cells display a substantially increased rubidium uptake (Fig. 2), a phenotype that is also evident in a single *ppz1* mutant (not shown). All these results agree with the idea that the Ppz phosphatases negatively regulate Trk potassium transporters.

On the light of the results described above, it could be expected that the absence of the Ppz phosphatases in cells deficient in the Trk-dependent potassium transport would have no effect on potassium requirements. However, we observe that these cells have increased potassium needs and this effect is almost as intense when only *PPZ1* is deleted in a *trk1 trk2* background (Fig. 1). The increased potassium requirement can be justified by the observation that *ppz1 ppz2 trk1 trk2* cells display a marginal rubidium uptake, much poorer than that of *trk1 trk2* mutants. These findings suggest that the Ppz phosphatases are positive regulators of the Trk-independent, low affinity potassium uptake. It has been described that decreased potassium transport caused by deletion of *TRK1* and *TRK2* results in hyperpolarization of the cell membrane [11] and in increased sensitivity to toxic cations, such as hygromycin B, spermine or TMA [2,11,23,24]. Accordingly, lack of Hal4 and Hal5 protein kinases, described as positive activators of Trk function, yields cells hypersensitive to this type of compounds [24], while deletion of the Ppz phosphatases results in increased tolerance to these cations [2]. On the basis of these precedents and our results, it could be expected that strain MAR73 would display higher sensitivity to hygromycin B or spermine than the double *trk1 trk2* mutant, a phenotype that is indeed observed (Fig. 3). We also show that the lack of Ppz1 or both Ppz1 and Ppz2 phosphatases in a Trk-deficient background does no result in increased sensitivity to sodium and lithium when compared with the *trk1 trk2* mutant, but instead in higher tolerance. This difference in behavior can be explained on the light of the recent finding that lack of Ppz phosphatases results in increased expression of the *ENA1* gene in a Trk-independent, calcineurin-dependent fashion and that a *ppz1 trk1 trk2* mutant, upon saline stress, contains less internal sodium than a *trk1 trk2* strain [22]. Because *ENA1* encodes a P-type $Na^+$-ATPase acting as a major sodium and lithium efflux system, transcriptional activation of this mechanism by the absence of Ppz phosphatases could explain the improvement in tolerance for these specific cations.

Our results demonstrate that Ppz phosphatases, and specifically Ppz1, influence low-affinity, Trk-independent potassium transport. Very little is known about the nature of this transport, which has not been elucidated at the molecular level. Patch-clamp analysis allowed identifying a major low-affinity potassium uptake process, termed NSC1 [12–14], which is inhibited by divalent cations, such as calcium. Interestingly, we observe that calcium cations worsen growth of strain MAR73 at low potassium concentrations (10 mM), while addition of the chelating agent EGTA drastically improves growth, and that these effects disappear at high (100 mM) potassium levels. This reinforces the notion that calcium negatively affects low affinity, Trk-independent potassium uptake. We recently reported that lack of Ppz1 phosphatase results in sensitivity to calcium ions due to activation of the calcineurin pathway [22]. However, it must be noted that the growth defect described here is observed at rather low concentrations of external calcium, which do not affect growth of Ppz mutants ([22] and data not shown), and it is not abolished by chemical inhibition of calcineurin with FK506 (Fig. 4). Therefore, the modulation of the low affinity potassium uptake by calcium seems to be an event independent of the activation of the calcineurin pathway. In addition, our data suggest that the effect of calcium cations on the low affinity potassium transport cannot be mediated by the Ppz phosphatases. It can be proposed that either both the effect of calcium and the phosphatases are independent events or, alternatively, that the phosphatases might influence calcium homeostasis and, as a consequence, affect low affinity potassium transport.

Our findings suggest a dual role for the Ppz phosphatases in potassium transport: inhibition of Ppz phosphatase activity would result in activation of the high-affinity Trk transporters and inhibition of the low affinity potassium uptake, while activation of the phosphatases would result in the opposite situation. This regulatory switch might include the inhibitory Ppz subunits Hal3 [22,25] and Vhs3 [26], which so far appear to govern all Ppz functions. Interestingly, it has been reported that deletion of *SKY1*, encoding a SR protein kinase, is able to improve growth of *trk1 trk2* cells on low potassium [27], a phenotype just opposed to the one described here for the Ppz phosphatases. This raises the possibility that the kinase and the phosphatases may have contrary functions, perhaps by controlling the phosphorylation state of a target(s) relevant for potassium uptake. In any case, the identification of the Ppz phosphatases as positive regulators of the Trk-independent, low affinity potassium uptake in yeast adds new players to this cellular function and may provide a way to elucidate the elusive molecular nature of this phenomenon. In addition, the ability to generate strains, such as MAR70 and MAR73, displaying a near-zero potassium influx offers a useful tool to analyze heterologous expression of potassium channels in yeast.

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