

# Genetic basis of sodium exclusion and sodium tolerance in yeast. A model for plants

Rosario Haro, María A. Bañuelos, Francisco J. Quintero, Francisco Rubio and Alonso Rodríguez-Navarro

Haro, R., Bañuelos, M. A., Quintero, F. J., Rubio, F. & Rodríguez-Navarro, A. 1993. Genetic basis of sodium exclusion and sodium tolerance in yeast. A model for plants. – *Physiol. Plant.* 89: 868–874.

A yeast strain carrying disruptions in *TRK1* and *ENA* genes was very sensitive to Na<sup>+</sup> because uptake discriminated poorly between K<sup>+</sup> and Na<sup>+</sup>, and Na<sup>+</sup> efflux was insignificant. Transformation with *TRK1* and *ENA1* restored discrimination, Na<sup>+</sup> efflux and Na<sup>+</sup> tolerance. Increasing external Ca<sup>2+</sup> increased Na<sup>+</sup> tolerance almost in the same proportion in *TRK1 ena1* cells and in *trk1 ENA1* cells, suggesting an unspecific effect of this cation. By using a vacuolar ATPase mutant, the role of the vacuole in Na<sup>+</sup> tolerance was also demonstrated. The yeast model of Na<sup>+</sup> exclusion and Na<sup>+</sup> tolerance may be extended to plants.

**Key words** – Ion transport, *Saccharomyces cerevisiae*, sodium exclusion, sodium tolerance, yeast.

R. Haro, M. A. Bañuelos, F. J. Quintero, F. Rubio and A. Rodríguez-Navarro (corresponding author), Laboratorio de Microbiología, Escuela Técnica Superior de Ingenieros Agrónomos, E-28040 Madrid, Spain.

## Introduction

Most glycophytes have developed in soils with a low Na<sup>+</sup> content, and lack the mechanisms required to tolerate the water deficit and the ion excesses prevailing in saline soils (Greenway and Munns 1980). Moreover, many glycophytes are particularly intolerant of salt, being inhibited by NaCl concentrations too low (25–50 mM) to produce a significant water deficit (Lessani and Marschner 1978). In these cases the antagonistic effect of Na<sup>+</sup> to K<sup>+</sup> may be the cause of the NaCl toxicity, because K<sup>+</sup> and Na<sup>+</sup> have many chemical similarities but completely different biological functions. K<sup>+</sup> is a major component of plant cells, which requires extensive uptake from the culture medium, and Na<sup>+</sup> is a minor component, which does not require extensive uptake (Flowers and Läuchli 1983) except in those halophytes where Na<sup>+</sup> plays an osmotic role (Flowers et al. 1977, Greenway and Munns 1980, Flowers and Läuchli 1983). Therefore, an excess of Na<sup>+</sup> in the external medium may change the normal K<sup>+</sup> uptake (Lynch and Läuchli 1984), thus causing K<sup>+</sup> deficiency and Na<sup>+</sup> excess in the plant cells and,

consequently, inhibition of plant growth. Shoots and leaves are more sensitive to these cation disturbances than roots (Munns and Termaat 1986), but they are better protected than roots because there are mechanisms selecting K<sup>+</sup> in the transfer of cations from roots to shoots and leaves (Jeschke 1984). However, this selection is not absolute and may not be sufficient if the roots are overloaded with Na<sup>+</sup>. In preventing the Na<sup>+</sup> overloading of roots, a low Na<sup>+</sup> uptake and rapid Na<sup>+</sup> efflux are the basic mechanisms (Läuchli 1984). In fact, similar mechanisms of Na<sup>+</sup> exclusion are used by non-halophilic bacteria (Skulachev 1987) and by fungi (Ortega and Rodríguez-Navarro 1986, Haro et al. 1991) to survive in high saline environments.

The salinity of soils and waters is a threat for agriculture because most crop plants are glycophytes, and some of them are rather intolerant of salt. For this reason there is a demand for breeding of crops with increased salt tolerance (Shannon 1984), but salt tolerance cannot always be introduced in crop plants by plant breeding because salt tolerant varieties are not known in all species. In such cases, genetic engineering would be

Received 19 April, 1993; revised 16 August, 1993

a powerful tool if resistance could be improved by manipulating a small number of genes. This is not the case in high salt tolerance which is a complex physiological process involving many genes (Jefferies and Rudmik 1984), some of them interacting in an additive manner (Dvorák et al. 1988). But the resistance to moderate NaCl concentrations may depend on only one or two genes, encoding K<sup>+</sup> and Na<sup>+</sup> transport systems capable of protecting the roots from high Na<sup>+</sup> loads and which can be easily transferred among different plant species. This sounds simple, but the identification of the genes and the subsequent cloning requires information about the function of the K<sup>+</sup> and Na<sup>+</sup> transport systems in salt tolerance.

A molecular model giving the role of the K<sup>+</sup> and Na<sup>+</sup> transport systems in Na<sup>+</sup> tolerance in eukaryotic cell-walled organisms is not available, but the model can be established in yeast because genes encoding the K<sup>+</sup> and Na<sup>+</sup> transport systems have been cloned (Gaber et al. 1988, Haro et al. 1991, Ko and Gaber 1991, Garciadeblas et al. 1993). The Na<sup>+</sup> tolerance of yeast is a function of the internal conditions (Camacho et al. 1981), and the links between external and internal conditions are the transport systems. The gene encoding the actual Na<sup>+</sup> uptake system of yeast has not been cloned, but *TRK1* may be a gene for Na<sup>+</sup> tolerance because it determines the ratio between K<sup>+</sup> and Na<sup>+</sup> K<sub>m</sub> values (Ramos et al. 1985). The genes for Na<sup>+</sup> efflux have been studied, and they form a tandem array of four genes. One of these, *ENA1*, mediates a rapid Na<sup>+</sup> efflux, and is a gene for Na<sup>+</sup> tolerance (Haro et al. 1991, Garciadeblas et al. 1993). Furthermore, genes encoding subunits of the vacuolar ATPase have been cloned (Nelson and Nelson 1990), so that the role of the vacuole in Na<sup>+</sup> tolerance can be studied. If a model of the role of the transport systems in Na<sup>+</sup> tolerance is established in yeast, it could support advances in the understanding of Na<sup>+</sup> tolerance in plants. In fact, fungi and plant cells share the same mechanisms of membrane energization (Serrano 1988), and fungi and plant roots show striking similarities in the process of K<sup>+</sup> uptake (Rodríguez-Navarro and Ramos 1986, Benlloch et al. 1989). In addition, a yeast gene for salt tolerance is conserved in plants (Gaxiola et al. 1992), and yeast cells support functional expression of plant genes encoding transport systems (Sauer et al. 1990, Anderson et al. 1992, Riesmeier et al. 1992, Sentenac et al. 1992).

In the present paper we report the role of the genes *TRK1*, *ENA1*, and the vacuolar ATPase in the Na<sup>+</sup> tolerance of yeast. The application of the yeast model to plants is discussed.

## Materials and methods

### Strains, plasmids and media

The *Saccharomyces cerevisiae* strain DBY746 (*Mata ura3 leu2 his3 trp1*) was used as wild type. RH2.2 (*trk1Δ*) was obtained by substituting the *LEU2* gene for the 2.3-kb *Xba*I fragment of *TRK1* in DBY746. RH16.6 carries a

disruption in the *ENA* tandem array (*ena1Δ::LEU2::ena4Δ*) (Garciadeblas et al. 1993), and strain TE12 is a double mutant (*trk1Δ ena1Δ::LEU2::ena4Δ*) constructed by crossing RH2.2, RH16.6, and the wild type. The vacuolar ATPase mutant strain *LEU::vatC* is a disruption strain obtained from W303-1B (*Mata leu2 his3 ade2 trp1 ura3*) (Nelson and Nelson 1990). Plasmids pGB34 and pRH22 are Ycp50 derivatives harbouring the genes *ENA1* and *TRK1*, respectively. Cells were grown in the minimal medium arginine phosphate (Rodríguez-Navarro and Ramos 1984), which is prepared with a basic formula free of ammonium and alkali cations but was supplemented with KCl, NaCl, LiCl and CaCl<sub>2</sub> as required. In the experiments with the vacuolar mutant strain *LEU2::vatC*, ammonium was substituted for arginine because this mutant grew poorly on arginine. All media were supplemented with adenine, uracil and amino acids as required in each case. Strains with plasmids were grown in the absence of uracil because YCP50 contains the gene *URA3*.

### Na<sup>+</sup> and Li<sup>+</sup> tolerances

Tests of tolerances for Na<sup>+</sup> and Li<sup>+</sup> were performed in the arginine phosphate medium. Preliminary experiments showed that Na<sup>+</sup> and Li<sup>+</sup> concentrations of intermediate toxicity produced an inhibition of the growth rate that increased with time. Because of this complicated effect, all growth experiments were standardized by inoculating 1×10<sup>5</sup> cells in 5 ml of the medium containing Na<sup>+</sup> or Li<sup>+</sup>. Non-inhibited cultures reach 5×10<sup>6</sup> cells ml<sup>-1</sup> in 24–36 h, and at the inhibitory concentrations reported in Tab. 1, the cultures reached 5×10<sup>6</sup> cells ml<sup>-1</sup> in approximately 72 h. Twice these concentrations did not permit any appreciable growth of the corresponding strains. Strain *LEU2::vatC* showed a much slower growth than the other strains, and tolerance tests in this strain were adapted to its slower growth rate.

### K<sup>+</sup>/Na<sup>+</sup> discrimination

The capacity for discrimination between K<sup>+</sup> and Na<sup>+</sup> in uptake was estimated by discrimination tests between Rb<sup>+</sup> and Li<sup>+</sup>. The relative affinities of the alkali cation

Tab. 1. Effect of the genes *TRK1* and *ENA1* in tolerance of Na<sup>+</sup> and Li<sup>+</sup>. Arginine phosphate medium with 5 mM KCl, and either 0.2 mM or 3 mM CaCl<sub>2</sub>. Assays as described in the text.

Strain	Genes	Tolerances		
		0.2 mM Ca <sup>2+</sup>		3 mM Ca <sup>2+</sup>
		NaCl (mM)	LiCl (mM)	NaCl (mM)
TE12	<i>trk1 ena1</i>	40	0.2	60
TE12(pRH22)	<i>TRK1 ena1</i>	75	0.5	100
RH16.6	<i>TRK1 ena1</i>	125	0.5	150
TE12(pGB34)	<i>trk1 ENA1</i>	300	5	450
RH16.6(pGB34)	<i>TRK1 ENA1</i>	400	10	800

carrier of yeast for these cations (Conway and Duggan 1958, Armstrong and Rothstein 1967, Rodríguez-Navarro and Ramos 1984) as well as preliminary experiments by ourselves, demonstrated the convenience of this test, which eliminates the use of the short-lived  $^{42}\text{K}^+$  (conditions of use as given in Rodríguez-Navarro and Ramos 1984), and the use of the high amounts of  $^{22}\text{Na}^+$  (conditions of use as given in Ortega and Rodríguez-Navarro 1986) required for effective labelling in uptake experiments at high  $\text{Na}^+$  concentrations. Cells were grown overnight in arginine phosphate medium with either 0.5 mM K<sup>+</sup> or 0.5 mM K<sup>+</sup> and 200 mM Na<sup>+</sup>. Samples of cultures in mid-exponential growth phase were centrifuged, washed, and suspended in 10 mM MES brought to pH 6.0 with Ca(OH)<sub>2</sub>, containing 0.1 mM MgCl<sub>2</sub>, 0.5 mM KCl, and 2% glucose.

### Cation contents and fluxes

Samples of cells were removed by filtration, washed, acid extracted, and analysed by atomic absorption spectrophotometry as described previously (Rodríguez-Navarro and Ortega 1982, Rodríguez-Navarro and Ramos 1984). The initial rates of uptakes were directly calculated from the time courses of the cation contents of samples, taken at intervals and which were linear for more than 10 min. Figures show the results of an experiment, which was repeated 3 or 4 times; the SD of the data points in the repetitions were lower than 10% of the corresponding means. Numerical results are means  $\pm$  SD of 3 or 4 experiments.

## Results

### K<sup>+</sup>/Na<sup>+</sup> discrimination

Yeast cells take up Na<sup>+</sup> by the K<sup>+</sup>-uptake system, and the ratio between K<sup>+</sup> and Na<sup>+</sup> K<sub>m</sub> values varies depending on the growth conditions. When the K<sup>+</sup>-uptake system is in the low-affinity state, the ratio between the K<sub>m</sub> values for Na<sup>+</sup> and K<sup>+</sup> is approximately 15, but this ratio rises to approximately 300 when the system is in the high-affinity state (Ramos et al. 1985). Under the stress of abundant Na<sup>+</sup> in the culture medium (Ramos et al. 1990) or in K<sup>+</sup> starvation (Rodríguez-Navarro and Ramos 1984), the K<sup>+</sup> uptake system converts into the high-affinity state. To demonstrate that the capacity for discrimination between K<sup>+</sup> and Na<sup>+</sup> of yeast cells increases if the cells grow in the presence of Na<sup>+</sup>, the uptakes of Rb<sup>+</sup> and Li<sup>+</sup> were measured in cells suspended in 10 mM Rb<sup>+</sup> and 100 mM Li<sup>+</sup> (Fig. 1). Cells grown in the absence of Na<sup>+</sup> took up Li<sup>+</sup> at a faster rate than Rb<sup>+</sup> ( $3.0 \pm 0.2$  versus  $2.5 \pm 0.1 \mu\text{mol g}^{-1} \text{min}^{-1}$ ), but cells grown in the presence of Na<sup>+</sup> took up Rb<sup>+</sup> at a faster rate than Li<sup>+</sup> ( $7.0 \pm 0.4$  versus  $2.7 \pm 0.2 \mu\text{mol g}^{-1} \text{min}^{-1}$ ).

*TRK1* is a gene required for the expression of the high-K<sup>+</sup>-affinity mode of transport (Gaber et al. 1988), and it is thus involved in the variable discrimination

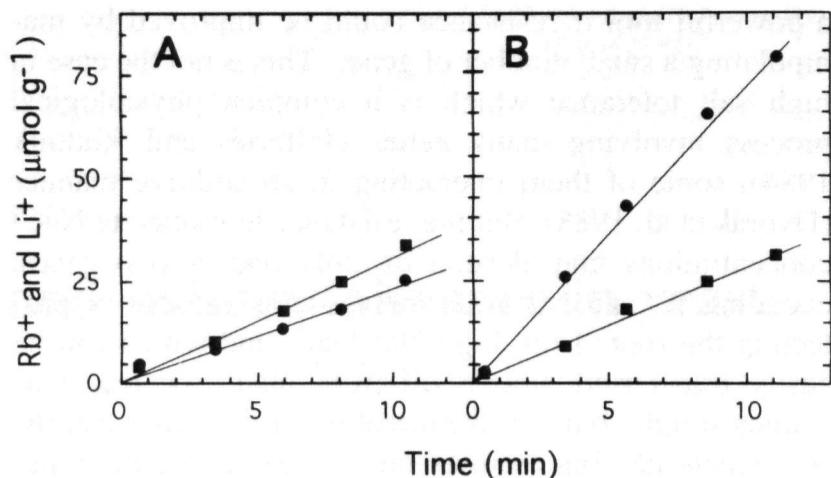


Fig. 1. Rb<sup>+</sup> and Li<sup>+</sup> uptakes in the wild type strain in the presence of 10 mM RbCl and 100 mM LiCl. Cells grown in arginine phosphate medium with (A) 0.5 mM KCl, or (B) 0.5 mM KCl and 200 mM NaCl. Uptakes measured in MES buffer with cells in the mid exponential growth phase, as described in the text. Rb<sup>+</sup> (●), Li<sup>+</sup> (■).

between Na<sup>+</sup> and K<sup>+</sup> in yeast. In *trk1* mutants the K<sup>+</sup> K<sub>m</sub> varies little, and always in the upper limit of the low-K<sup>+</sup>-affinity mode, close to the K<sub>m</sub> for Na<sup>+</sup> (Ramos et al. 1985). As predicted by these data, after either starving the cells in a K<sup>+</sup>-free medium or growing the cells in Na<sup>+</sup>, a *trk1Δ* mutant showed little change in the discrimination between Rb<sup>+</sup> and Li<sup>+</sup> during uptake. In an experiment similar to that presented for the wild type in Fig. 1, *trk1Δ* cells grown in the presence of Na<sup>+</sup> increased Rb<sup>+</sup> uptake only from  $1.0 \pm 0.1$  to  $1.5 \pm 0.1 \mu\text{mol g}^{-1} \text{min}^{-1}$ ; and they took up Li<sup>+</sup> at a faster rate than Rb<sup>+</sup> even in cells grown in the presence of Na<sup>+</sup> (Fig. 2).

### Role of *TRK1* and *ENA1* in Na<sup>+</sup> and Li<sup>+</sup> tolerances

To assess the benefits of the genes *TRK1* and *ENA1* for Na<sup>+</sup> tolerance, the growth of strains with different combinations of the alleles *TRK1/trk1Δ*, and *ENA1/ena1Δ* was tested using 5 mM K<sup>+</sup> and increasing concentrations of Na<sup>+</sup>. A strain lacking the *TRK1* gene, and the 4 genes in the *ENA* tandem array was sensitive to Na<sup>+</sup>, having its growth limit at 40 mM Na<sup>+</sup>. The presence of *TRK1*, either in a plasmid or in the chromosome, increased the tolerance in comparison with the *trk1Δ ena1Δ* strain, although in the plasmid the gene was less effective than in the

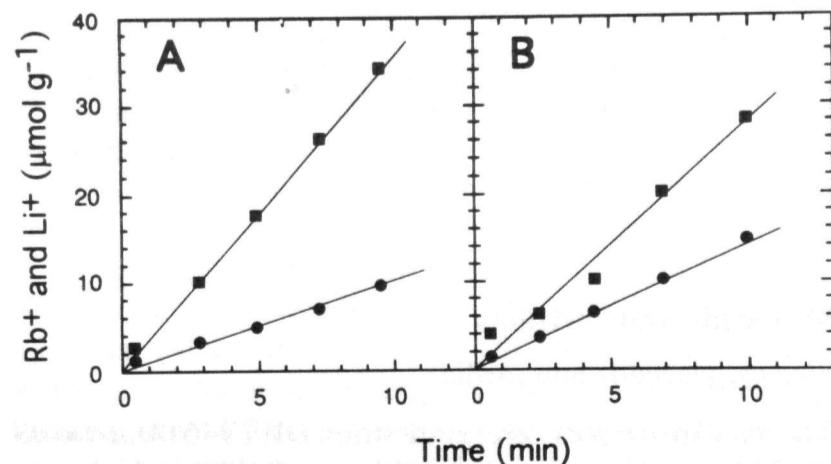


Fig. 2. Rb<sup>+</sup> and Li<sup>+</sup> uptakes in the *trk1Δ* strain (RH2.2) in the presence of 10 mM RbCl and 100 mM LiCl. Otherwise as for Fig. 1.

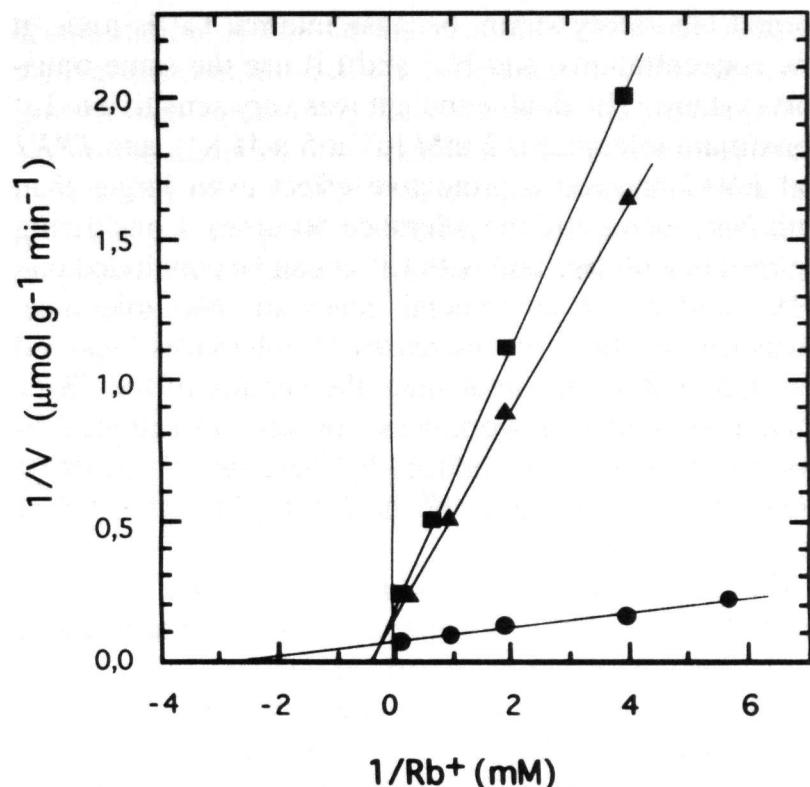


Fig. 3. Double reciprocal plot of the initial rates of  $\text{Rb}^+$  uptake in strain DBY746 grown at 0.5 mM KCl (■), 0.5 mM KCl + 200 mM NaCl (●), and 0.5 mM KCl + 20 mM LiCl (▲). Uptakes in MES buffer with cells harvested in the mid exponential growth phase. Cells grown in  $\text{Na}^+$  and in  $\text{Li}^+$  presented similar degree of inhibition.

chromosome (75 mM  $\text{Na}^+$  versus 125 mM  $\text{Na}^+$ ). The presence of *ENA1* increased the tolerance up to 300 mM  $\text{Na}^+$  (*ENA1* was always used in a plasmid since a deletion strain for *ENA2*, *ENA3* and *ENA4*, leaving intact *ENA1*, is not available), and *TRK1* and *ENA1* together increased the tolerance up to 400 mM  $\text{Na}^+$  (Tab. 1). Consistent with the fact that *TRK1* and *ENA1* encode transport systems in the plasma membrane, these genes were effective in changing the internal/external ratios of  $\text{K}^+$  and  $\text{Na}^+$  that the cells could maintain; but they did not change the toxic level of internal  $\text{Na}^+$ , and all the strains contained approximately 300  $\mu\text{mol K}^+ \text{g}^{-1}$  and 300  $\mu\text{mol Na}^+ \text{g}^{-1}$  at the limiting  $\text{Na}^+$  concentrations given in Tab. 1.

As in most laboratory strains, the wild strains used for preparing TE12 were quite tolerant to internal  $\text{Na}^+$ , this cation reaching high concentrations inside the cell without significant effects. Other strains, however, may be more sensitive to internal  $\text{Na}^+$ , and it was of interest to find out the effect of *TRK1* and *ENA1* in the protection of such strains. In the absence of a laboratory strain (genetically defined and with auxotrophic markers) sensitive to internal  $\text{Na}^+$ , we tested the  $\text{Li}^+$  tolerance of the strains given in Tab. 1. The protective effect of *TRK1* and *ENA1* on the  $\text{Li}^+$  tolerance of a laboratory strain is probably similar to the protective effect that these genes would produce on the  $\text{Na}^+$  tolerance of a strain intolerant to internal  $\text{Na}^+$ , because  $\text{Na}^+$  and  $\text{Li}^+$  are transported by the same transport systems, and low internal concentrations of  $\text{Li}^+$  are toxic in laboratory strains (J. Ramos and A. Rodríguez-Navarro, unpublished results). *TRK1* and *ENA1* were effective on protecting the cells against  $\text{Li}^+$ , but *ENA1* showed a much larger protective effect than

*TRK1* (at 5 mM  $\text{K}^+$ , *ENA1* increased the  $\text{Li}^+$  tolerance 20-fold, but *TRK1* only twofold; Tab. 1). This low effect of *TRK1* was found to occur because  $\text{Li}^+$  did not trigger the complete conversion of the  $\text{K}^+$  uptake system into the high  $\text{K}^+$ -affinity state. Figure 3 shows that yeast cells growing with an inhibitory concentration of  $\text{Li}^+$  exhibited a high  $K_m$  for  $\text{Rb}^+$ , whereas cells growing in  $\text{Na}^+$ , at a similar degree of inhibition, exhibited the characteristic low  $\text{Rb}^+$   $K_m$ . These results suggest that internal  $\text{Li}^+$  became toxic before reaching a sufficient concentration to produce the signal for the conversion of the  $\text{K}^+$  uptake system.

#### The role of $\text{Ca}^{2+}$ in $\text{Na}^+$ tolerance

$\text{Ca}^{2+}$  affects the salt tolerance of plants (Lahaye and Epstein 1971, Läuchli 1990), and divalent cations are necessary for the correct function of the  $\text{K}^+$  transport system in fungi (Rodríguez-Navarro and Sancho 1979, Sancho and Rodríguez-Navarro 1986). The arginine phosphate medium used for testing  $\text{Na}^+$  tolerance contained 0.2 mM  $\text{Ca}^{2+}$ , which is the concentration in standard minimal media for yeast (Wickerham 1951, Sherman 1991). This amount of  $\text{Ca}^{2+}$  is certainly sufficient for most conditions, but not necessarily for maximum  $\text{Na}^+$  tolerance. For this reason, we tested  $\text{Na}^+$  tolerance at 3 mM  $\text{Ca}^{2+}$ , finding that tolerance was increased (Tab. 1). The increase was statistically significant in all the strains, even in TE12, but more distinctive in the more tolerant strains.

#### Role of the vacuole in $\text{Na}^+$ tolerance

Confining  $\text{Na}^+$  in the vacuole is another mechanism that reduces the  $\text{Na}^+$  concentration in the cytoplasm, and increases the  $\text{Na}^+$  tolerance because the toxicity of  $\text{Na}^+$  in the vacuole is probably low. If the internal content of  $\text{Na}^+$  is in steady state, with an influx of  $\text{Na}^+$  of I and an efflux of  $\text{Na}^+$  of E, we can write the following equation:

$$I = E + \mu (\text{Na}^+)_c + \mu (\text{Na}^+)_v \quad (1)$$

where  $\mu$  is the specific growth rate of the cells, and  $(\text{Na}^+)_c$  and  $(\text{Na}^+)_v$  the amounts of  $\text{Na}^+$  in cytoplasm and vacuole respectively. According to this equation, a higher  $\text{Na}^+$  accumulation in the vacuole would reduce the  $\text{Na}^+$  content of the cytoplasm if I and E do not change. I depends only on the external concentrations of  $\text{K}^+$  and  $\text{Na}^+$ , but E may depend on  $(\text{Na}^+)_c$ . In the less favourable case that E exhibits first order kinetics (Rodríguez-Navarro and Ortega 1982), equation (1) can be written:

$$I = k (\text{Na}^+)_c + \mu (\text{Na}^+)_c + \mu (\text{Na}^+)_v \quad (2)$$

where k is the kinetic constant of efflux. From this equation it is evident that increasing the capacity of accumulating  $\text{Na}^+$  into the vacuole would decrease the  $\text{Na}^+$  concentration in the cytoplasm, even in the case that  $\text{Na}^+$  efflux exhibits first order kinetics.

The most elegant procedure to test the hypothesis that the capacity of the vacuole to accumulate  $\text{Na}^+$  affects the  $\text{Na}^+$  tolerance would be the use of mutants in the genes encoding the tonoplast  $\text{Na}^+$  transport systems, but such mutants have not been obtained. For this reason we used a mutant in the subunit C of the vacuolar ATPase (Nelson and Nelson 1990). In the absence of a functional ATPase, which is a dramatic defect disabling the vacuole from energization and acidification (Nelson and Nelson 1990, Klionsky et al. 1990, Serrano 1991), the function of a  $\text{Na}^+/\text{H}^+$  antiporter in the tonoplast (Blumwald and Poole 1987, Fan et al. 1989, Gabardino and DuPont 1989) must be seriously impaired. In the mutant the  $\text{Na}^+$  and  $\text{Li}^+$  tolerances were markedly decreased, such that at 0.5 mM K<sup>+</sup>, the wild type tolerated 500 mM Na<sup>+</sup> and 100 mM Li<sup>+</sup>, whereas the mutant tolerated 200 mM Na<sup>+</sup> and 15 mM Li<sup>+</sup>; thus giving reasonable support to the notion that the vacuole plays a role for the ability to tolerate Na<sup>+</sup> and Li<sup>+</sup>. Analyses of the K<sup>+</sup> and Na<sup>+</sup> contents of the wild type and the mutant, at similar degree of growth inhibition by Na<sup>+</sup> (200 mM Na<sup>+</sup> in the mutant, and 500 mM Na<sup>+</sup> in the wild type, both at 0.5 mM K<sup>+</sup>), showed a notably lower content of Na<sup>+</sup> in the mutant (50  $\mu\text{mol Na}^+ \text{ g}^{-1}$  vs 200  $\mu\text{mol Na}^+ \text{ g}^{-1}$ ), which is consistent with the lack of a Na<sup>+</sup>-rich vacuolar space.

## Discussion

Na<sup>+</sup> tolerance in yeast showed a large dependence on *ENAI*, the gene encoding a putative Na<sup>+</sup> pump (Haro et al. 1991, Garciadeblas et al. 1993), and on *TRK1*, a gene required for the expression of the high-affinity mode of K<sup>+</sup> uptake (Gaber et al. 1988). The presence of the two genes increased the tolerance over the strain lacking these genes by approximately 10-fold (Tab. 1). In the conditions of the present report *TRK1* was less effective than *ENAI*, but this does not necessarily mean that K<sup>+</sup>/Na<sup>+</sup> discrimination in the uptake is always less effective than Na<sup>+</sup> export in Na<sup>+</sup> tolerance. The higher effectiveness of export in this investigation is probably due to the high efficiency of the *ENAI* gene (Garciadeblas et al. 1993). It might be that other versions of *TRK1*, which exist in yeast (Anderson et al. 1991), bring about higher protection than the gene used in this report. In any case, the protection by *TRK1* was smaller than expected from the large *TRK1*-dependent difference between the K<sup>+</sup> and Na<sup>+</sup> K<sub>m</sub> values in Na<sup>+</sup>-grown cells. This suggests that, in high Na<sup>+</sup> media, Na<sup>+</sup> and K<sup>+</sup> uptakes deviate from the pure competition normally observed (Conway and Duggan 1958, Armstrong and Rothstein 1967, Ramos et al. 1985).

Na<sup>+</sup> tolerance of the double mutant lacking both the genes *TRK1* and *ENAI* was not low (40 mM Na<sup>+</sup> at 5 mM K<sup>+</sup>), because laboratory strains support high Na<sup>+</sup> contents (Camacho et al. 1981, and present results); and with a high Na<sup>+</sup> content, growth dilution counterbalances large influxes (see equation 1). The effect of *TRK1* and *ENAI* in a strain that was sensitive to low internal Na<sup>+</sup> concentrations was assessed by testing the Li<sup>+</sup> tolerance of a

normal laboratory strain, because internal Li<sup>+</sup> is toxic at low concentrations, and Na<sup>+</sup> and Li<sup>+</sup> use the same transport systems. The double mutant was very sensitive to Li<sup>+</sup> (maximum tolerance 0.2 mM Li<sup>+</sup> at 5 mM K<sup>+</sup>), and *TRK1* and *ENAI* showed a protective effect even larger than with Na<sup>+</sup>, increasing the tolerance 50 times. Considering the results with Na<sup>+</sup> and with Li<sup>+</sup>, it can be concluded that *TRK1* and *ENAI* are crucial genes for Na<sup>+</sup> tolerance. Furthermore, the large increases in tolerances reported here may not be maximal since the version of the *TRK1* system used in this work was not very effective. Obviously, the tolerances induced by these genes cannot be higher than the resistance of the cell to the water deficit imposed by NaCl.

The role of *TRK1* and *ENAI* in Na<sup>+</sup> tolerance is clear in yeast. The question is whether homologous genes and a similar response can be expected for salt tolerance in higher plant species. It seems evident that although many other mechanisms apart from those considered here (Gorham 1992) can protect the plants from Na<sup>+</sup>, the exclusion of Na<sup>+</sup> from the roots, either by decreasing influx or increasing efflux, would be an effective mechanism of protection. In suspension cultures of *Brassica napus* (Lefebvre 1989) and *Nicotiana tabacum* (Watad et al. 1991), the enhanced NaCl tolerance of selected cell lines has been explained by their enhanced capacity to take up K<sup>+</sup>. The molecular basis for K<sup>+</sup> and Na<sup>+</sup> fluxes in plant roots is not known, but the plant transport systems are probably similar to the systems in fungi (Serrano 1988, Benlloch et al. 1989). Cloning the genes encoding these systems would finally clarify many uncertainties about K<sup>+</sup> and Na<sup>+</sup> transport in plants. In addition, such cloning would also provide a technical approach to reduce the salt sensitivity of some plant species. The dramatic increase in Na<sup>+</sup> and Li<sup>+</sup> tolerances induced by *TRK1* and *ENAI* in yeast suggests that the genetic engineering of these genes could be a good strategy for increasing Na<sup>+</sup> tolerance in some plants.

The effect of Ca<sup>2+</sup> on Na<sup>+</sup> tolerance in yeast is not surprising considering its effects on Na<sup>+</sup> tolerance in plants (Lahaye and Epstein 1971, Läuchli 1990, and references therein). However, the present results do not support the notion that Na<sup>+</sup> produces Ca<sup>2+</sup> deficiency, as proposed for higher plants (Mass and Grieve 1987). If the primary effect of Na<sup>+</sup> were to inhibit Ca<sup>2+</sup> uptake, Na<sup>+</sup> tolerance would not be so dramatically dependent on *TRK1* and *ENAI* genes, because these genes are not related to Ca<sup>2+</sup> transport. Furthermore, *TRK1* and *ENAI* genes had a much larger effect on Na<sup>+</sup> tolerance than the concentration of Ca<sup>2+</sup> (Tab. 1). Probably Ca<sup>2+</sup> produces an unspecific effect on the membrane, improving the function of many transport systems. A more complete discussion of the effect of Ca<sup>2+</sup>, in connexion with more complex models in plants (Läuchli 1990), is beyond the scope of the present report.

The results obtained with the vacuolar ATPase mutants support the notion that confining Na<sup>+</sup> in the vacuole is also a mechanism of Na<sup>+</sup> tolerance in yeast. However,

accumulation of  $\text{Na}^+$  in the vacuole effectively reduces the  $\text{Na}^+$  concentration of the cytoplasm only during active growth (see equation 1). Therefore, the accumulation of  $\text{Na}^+$  in the vacuoles of root cells would reduce effectively the transfer of  $\text{Na}^+$  to the shoot only when the roots are growing, and the protective effect of this accumulation on crop yields may depend on the moment in which plant roots stop growing.

**Acknowledgements** – We thank N. Nelson for the generous gift of strain *LEU2::vatC*. We also thank Ana Villa for making the crosses to obtain strain TE12. This work was supported by grant PB89-0180 from Dirección General de Investigación Científica y Técnica, Spain.

## References

- Anderson, J. A., Best, L. A. & Gaber, R. F. 1991. Structural and functional conservation between the high-affinity transporters of *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. – *Gene* 99: 39–46.
- , Huprikar, S. S., Kochian, L. V., Lucas, W. J. & Gaber, R. F. 1992. Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae*. – *Proc. Natl. Acad. Sci. USA* 89: 3736–3740.
- Armstrong, W. McD. & Rothstein, A. 1967. Discrimination between alkali metal cations by yeast. II. Cation interactions in transport. – *J. Gen. Physiol.* 50: 967–988.
- Benlloch, M., Moreno, I. & Rodríguez-Navarro, A. 1989. Two modes of rubidium uptake in sunflower plants. – *Plant Physiol.* 90: 939–942.
- Blumwald, E. & Poole, R. J. 1987. Salt tolerance in suspension cultures of sugar beet. – *Plant Physiol.* 83: 884–887.
- Camacho, M., Ramos, J. & Rodríguez-Navarro, A. 1981. Potassium requirements of *Saccharomyces cerevisiae*. – *Curr. Microbiol.* 6: 295–299.
- Conway, E. J. & Duggan, F. 1958. A cation carrier in the yeast cell wall. – *Biochem. J.* 69: 265–274.
- Dvorák, J., Edge, M. & Ross, K. 1988. On the evolution of the adaptation of *Lophopyrum elongatum* to growth in saline environments. – *Proc. Natl. Acad. Sci. USA* 85: 3805–3809.
- Fan, T. W.-M., Higashi, R. H., Norlyn, J. & Epstein, E. 1989. In vivo  $^{23}\text{Na}$  and  $^{31}\text{P}$  NMR measurements of a tonoplast  $\text{Na}^+/\text{H}^+$  exchange process and its characteristics in two barley cultivars. – *Proc. Natl. Acad. Sci. USA* 86: 9856–9860.
- Flowers, T. J. & Läuchli, A. 1983. Sodium versus potassium: substitution and compartmentation. – *In Encyclopedia of Plant Physiology*, New Series, Vol 15B (A. Läuchli and R. L. Bielesky, eds), pp. 651–681. Springer-Verlag, Berlin. ISBN 3-540-12103-X.
- , Troke, P. F. & Yeo, A. R. 1977. The mechanism of salt tolerance in halophytes. – *Annu. Rev. Plant. Physiol.* 28: 89–121.
- Gabardino, J. & DuPont, F. M. 1989. Rapid induction of  $\text{Na}^+/\text{H}^+$  exchange activity in barley root tonoplast. – *Plant Physiol.* 89: 1–4.
- Gaber, R. F., Styles, C. A. & Fink, G. R. 1988. *TRK1* encodes a plasma membrane protein required for high-affinity potassium transport in *Saccharomyces cerevisiae*. – *Mol. Cell. Biol.* 8: 2848–2859.
- Garciadeblas, B., Rubio, F., Quintero, F. J., Bañuelos, M. A., Haro, R. & Rodríguez-Navarro, A. 1993. Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in *Saccharomyces cerevisiae*. – *Mol. Gen. Genet.* 236: 363–368.
- Gaxiola, R., deLarrinoa, F. I., Villalba, J. M. & Serrano, R. 1992. A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. – *EMBO J.* 11: 3157–3164.
- Gorham, J. 1992. Stress tolerance and mechanisms behind tolerance in barley. – *In Proceedings of VI International Barley Genetics Symposium* (L. Munck, ed.), pp. 1035–1049. Munksgaard, Copenhagen. ISBN 87-16-19601-5.
- Greenway, H. & Munns, R. 1980. Mechanisms of salt tolerance in non halophytes. – *Annu. Rev. Plant Physiol.* 31: 149–190.
- Haro, R., Garciadeblas, B. & Rodríguez-Navarro, A. 1991. A novel P-type ATPase from yeast involved in sodium transport. – *FEBS Lett.* 291: 189–191.
- Jefferies, R. L. & Rudmik, T. 1984. The responses of halophytes to salinity: an ecological perspective. – *In Salinity Tolerance in Plants, Strategies for Crop Improvement* (C. Staples and G. H. Toennissen, eds), pp. 213–228. John Wiley and Sons, New York, NY. ISBN 0-471-89674-8.
- Jeschke, W. D. 1984.  $\text{K}^+/\text{Na}^+$  exchange at cellular membranes, intracellular compartmentation of cations, and salt tolerance. – *In Salinity Tolerance in Plants, Strategies for Crop Improvement* (C. Staples and G. H. Toennissen, eds), pp. 37–66. John Wiley and Sons, New York, NY. ISBN 0-471-89674-8.
- Klionsky, D. J., Herman, P. K. & Emr, S. D. 1990. The fungal vacuole: composition, function, and biogenesis. – *Microbiol. Rev.* 54: 266–292.
- Ko, C. H. & Gaber, R. F. 1991. *TRK1* and *TRK2* encode structurally related  $\text{K}^+$  transporters in *Saccharomyces cerevisiae*. – *Mol. Cell. Biol.* 11: 4266–4273.
- Lahaye, P. A. & Epstein, E. 1971. Calcium and salt toleration by bean plants. – *Physiol. Plant.* 25: 213–218.
- Läuchli, A. 1984. Salt exclusion: an adaptation of legumes for crops and pastures under saline conditions. – *In Salinity Tolerance in Plants, Strategies for Crop Improvement* (C. Staples and G. H. Toennissen, eds), pp. 171–187. John Wiley and Sons, New York, NY. ISBN 0-471-89674-8.
- 1990. Calcium, salinity and the plasma membrane. – *In Calcium in Plants Growth and Development* (R. T. Leonard and P. K. Hepler, eds), The American Society of Plant Physiologists Symposium Series, Vol. 4, pp. 26–35. ISBN 0-943088-18-6.
- Lefebvre, D. D. 1989. Increased potassium absorption confers resistance to group IA cations in rubidium-selected suspension cells of *Brassica napus*. – *Plant Physiol.* 91: 1460–1466.
- Lessani, H. & Marschner, H. 1978. Relation between salt tolerance and long-distance transport of sodium and chloride in various crop species. – *Aust. J. Plant Physiol.* 5: 27–37.
- Lynch, J. & Läuchli, A. 1984. Potassium transport in salt-stressed barley roots. – *Planta* 161: 295–301.
- Mass, E. V. & Grieve, C. M. 1987. Sodium-induced calcium deficiency in salt-stressed corn. – *Plant Cell Environ.* 10: 559–564.
- Munns, R. & Termaat, A. 1986. Whole-plant responses to salinity. – *Aust. J. Plant Physiol.* 13: 143–160.
- Nelson, H. & Nelson, N. 1990. Disruption of genes encoding subunits of yeast vacuolar  $\text{H}^+$ -ATPase causes conditional lethality. – *Proc. Natl. Acad. Sci. USA* 87: 3503–3507.
- Ortega, M. D. & Rodríguez-Navarro, A. 1986. Sodium ion transport in *Neurospora crassa*. – *Physiol. Plant.* 66: 705–711.
- Ramos, J., Contreras, P. & Rodríguez-Navarro, A. 1985. A potassium transport mutant of *Saccharomyces cerevisiae*. – *Arch. Microbiol.* 143: 88–93.
- , Haro, R. & Rodríguez-Navarro, A. 1990. Regulation of potassium fluxes in *Saccharomyces cerevisiae*. – *Biochim. Biophys. Acta* 1029: 211–217.
- Riesmeier, J. W., Willmitzer, L. & Frommer, W. B. 1992. Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. – *EMBO J.* 11: 4705–4713.
- Rodríguez-Navarro, A. & Ortega, D. 1982. The mechanism of sodium efflux in yeast. – *FEBS Lett.* 138: 205–208.
- & Ramos, J. 1984. Dual system for potassium transport in *Saccharomyces cerevisiae*. – *J. Bacteriol.* 159: 940–945.

- & Ramos, J. 1986. Two systems mediate rubidium uptake in *Neurospora crassa*: one exhibits the dual-uptake isotherm. – *Biochim. Biophys. Acta* 857: 229–237.
- & Sancho, E. D. 1979. Cation exchanges of yeast in the absence of magnesium. – *Biochim. Biophys. Acta* 552: 322–330.
- Sancho, E. D. & Rodríguez-Navarro, A. 1986. The influence of calcium and magnesium ions on the exchange of monovalent cations in *Neurospora crassa*. – *Physiol. Plant.* 66: 712–716.
- Sauer, N., Friedländer, K. & Gräml-Wicke, U. 1990. Primary structure, genomic organization and heterologous expression of a glucose transporter from *Arabidopsis thaliana*. – *EMBO J.* 9: 3045–3050.
- Sentenac, H., Bonneauaud, N., Minet, M., Lacroute, F., Salmon, J.-M., Gaymart F. & Grignon, C. 1992. Cloning and expression of a plant potassium ion transport system. – *Science* 256: 663–665.
- Serrano, R. 1988. Structure and function of proton translocating ATPase of plants and fungi. – *Biochim. Biophys. Acta* 947: 1–28.
- 1991. Transport across yeast vacuolar and plasma membranes. – In *The Molecular and Cellular Biology of the Yeast *Saccharomyces**, Vol. 1 (J. R. Broach, J. Pringle and E. Jones, eds), Genome Dynamics, Protein Synthesis, and Enzymatics, pp. 523–585. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. ISBN 0-87969-355-X.
- Shannon, M. C. 1984. Breeding, selection, and the genetics of salt tolerance. – In *Salinity Tolerance in Plants, Strategies for Crop Improvement* (C. Staples and G. H. Toenniessen, eds), pp. 231–254. John Wiley and Sons, New York, NY. ISBN 0-471-89674-8.
- Sherman, F. 1991. Getting started with yeast. – *Methods Enzymol.* 194: 3–21.
- Skulachev, V. P. 1987. Bacterial sodium transport: bioenergetic functions of sodium ion. – In *Ion Transport in Prokaryotes* (B. P. Rosen and S. Silvers eds), pp. 131–164. Academic Press, New York, NY. ISBN 0-12-596935-X.
- Watad, A.-E. A., Reuveni, M., Bressan, R. A. & Hasegawa, P. M. 1991. Enhanced net K<sup>+</sup> uptake capacity of NaCl-adapted cells. – *Plant Physiol.* 95: 1265–1269.
- Wickerham, L. J. 1951. Taxonomy of yeast. – *Tech. Bull. US Dept. Agric.* No. 1029.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.