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The *SAL1* Gene of Arabidopsis, Encoding an Enzyme with 3′(2′),5′-Bisphosphate Nucleotidase and Inositol Polyphosphate 1-Phosphatase Activities, Increases Salt Tolerance in Yeast

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A cDNA library in a yeast expression vector was prepared from roots of Arabidopsis exposed to salt and was used to select Li^+-tolerant yeast transformants. The cDNA *SAL1* isolated from one of these transformants encodes a polypeptide of 353 amino acid residues. This protein is homologous to the HAL2 and CysQ phosphatases of yeast and *Escherichia coli*, respectively. Partial cDNA sequences in the data bases indicate that rice produces a phosphatase highly homologous to SAL1 and that a second gene homologous to *SAL1* exists in Arabidopsis. The SAL1 protein expressed in *E. coli* showed 3′(2′),5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities. In yeast, *SAL1* restored the ability of a *hal2/met22* mutant to grow on sulfate as a sole sulfur source, increased the intracellular Li^+ tolerance, and modified Na^+ and Li^+ effluxes. We propose that the product of *SAL1* participates in the sulfur assimilation pathway as well as in the phosphoinositide signaling pathway and that changes in the latter may affect Na^+ and Li^+ fluxes.

INTRODUCTION

Irrigation of land in semiarid regions frequently increases the salt content of the soil because the salts dissolved in water accumulate when the water evaporates. If increased salt content occurs, it has a detrimental economic effect because most crop plants have decreased productivity in saline soils. Although improvement of salt tolerance in crop plants has been pursued for a long time, traditional plant breeding has had limited success. The heterologous transfer of genes offers new possibilities to improve the salt tolerance of plants, but this approach requires prior basic research to identify the genes to be transferred and, in most cases, the functions that they fulfill. Therefore, the physiological basis of salt tolerance, the genes induced by salt stress, and the genes involved in the synthesis of osmoprotectants have been the subject of many recent studies (Singh et al., 1989; Claes et al., 1990; McCue and Hanson, 1990; Piatkowski et al., 1990; Delauney and Verma, 1993; Rhodes and Hanson, 1993).

One strategy to identify genes conferring salt tolerance uses yeast (Gaxiola et al., 1992; Haro et al., 1993). In yeast, the genes encoding K^+ and Na^+ transport systems are necessary for correct K^+ uptake and Na^+ and Li^+ exclusion from the cells (Haro et al., 1993); the gene encoding the regulatory subunit of calcineurin (Mendoza et al., 1994) and those encoding the PPZ1 and PPZ2 protein phosphatases (Posas et al., 1995) are necessary for the correct expression of the ENA1 ATPase mediating Na^+ efflux; *HAL1* is involved in the regulation of cation content (Gaxiola et al., 1992); and *HAL2* encodes the 3′,5′-bisphosphate nucleotidase, a salt-sensitive enzyme whose overexpression probably counteracts the decrease in activity produced by toxic levels of Na^+ or Li^+ (Gläser et al., 1993; Murguía et al., 1995).

The genes encoding the K^+ and Na^+ transport systems in the epidermal and cortical cells of roots and the genes involved in the regulation of the expression and function of these transporters could be determinants of salt tolerance in plants. In addition, the plant genes homologous to the *HAL1* or *HAL2* genes of yeast may play an important role in salt tolerance. To isolate these genes, we constructed a cDNA library from roots of Arabidopsis exposed to NaCl and used it to complement the Na^+ and Li^+ sensitivities of yeast strains defective in Na^+ and Li^+ effluxes. Using this approach, we identified the *SAL1* cDNA. The expression of this cDNA in *Escherichia coli* produced an enzyme with 3′(2′),5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities. It is likely that both activities function in the protection from Li^+ toxicity, but that only the inositol phosphatase activity functions in the protection from Na^+ toxicity.

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RESULTS

Cloning of *SAL1*

In yeast, the effluxes of Na^+ and Li^+ are mediated by the ATPases encoded by a tandem of four genes: *ENA1*, *ENA2*, *ENA3*, and *ENA4* (Haro et al., 1991; Garciadeblas et al., 1993). Strain G19 carries a disruption of the four genes (*ena1*Δ::*HIS3*::*ena4*Δ) and is very sensitive to Na^+ and Li^+. This strain was transformed with a cDNA library in plasmid pYES2 that was prepared from roots of Arabidopsis exposed for 24 hr to 100 mM NaCl. This plasmid carries *URA3* as a marker gene and the galactokinase *GAL1* promoter for controlled expression of the cDNA inserts. Cells of transformant colonies (5×10^4) selected on uracil-free SD medium (synthetic medium containing glucose) were incubated for 24 hr in uracil-free SG medium (synthetic medium containing galactose) and then plated on the same medium supplemented with 10 mM LiCl. The colonies emerging in <7 days were transferred simultaneously to plates of SD and SG media both supplemented with 10 mM LiCl. Plasmids from the colonies that tolerated Li^+ only in galactose were reintroduced into G19, and Li^+ tolerance was tested again in glucose and in galactose. One of these plasmids, pAT18 with a cDNA insert of 1.3 kb, consistently enabled the mutant to grow on 10 mM LiCl with galactose as carbon source. This cDNA in pAT18 was designated *SAL1*. High-stringency DNA gel blot hybridization indicated that *SAL1* is present in the genome of Arabidopsis, and RNA gel blot hybridization showed that *SAL1* is expressed as a transcript of ~1.4 kb (data not shown). Densitometric analysis of RNA blots of roots, leaves, and stems indicated that *SAL1* mRNA was 1.5-fold more abundant in leaves and stems, and that exposing the plants to 100 mM NaCl for 24 hr slightly increased *SAL1* mRNA (data not shown).

Sequence analysis of *SAL1* cDNA revealed an open reading frame of 1059 bp that could encode a protein of 353 amino acids and 37.5 kD. The deduced amino acid sequence of SAL1 shows significant homology with the putative products of the gene *MET22/HAL2* of yeast (Gläser et al., 1993) and *cysQ* of *E. coli* (Neuwald et al., 1992) (36.1 and 30% identity, 55.7 and 53.3% similarity, respectively; data not shown). Figure 1 shows the most notable sequence homologies, which were in the motifs conserved in 3′(2′),5′-bisphosphate nucleotidases, inositol phosphatases, and some other proteins of unknown function (Neuwald et al., 1991; Glasser et al., 1993). A data base search for protein sequences related to SAL1 revealed high homology with two sequences translated from the 5′ ends of two partial sequences of cDNAs from rice and Arabidopsis (Expressed Sequence Tag data base accession numbers 21,306 and 7103, respectively). These partial sequences correspond only to the N terminus of the putative proteins and do not reach the conserved regions of the family of phosphatases. Therefore, the high homology suggests that the corresponding genes may encode phosphatases homologous in function to SAL1.

Phosphatase Activity of SAL1

The gene *MET22/HAL2* encodes the 3′(2′),5′-bisphosphate nucleotidase enzyme of yeast, which is necessary for the assimilation of sulfate (Murguía et al., 1995). Null mutants in this gene do not grow in the absence of an organic sulfur source (Thomas et al., 1992; Gläser et al., 1993). The homology of the translated amino acid sequences of SAL1 and HAL2 suggested that these proteins may have homologous functions. Therefore, we transformed pAT18 into a *hal2*Δ mutant yeast strain (RS-1051) and tested the growth on sulfate as the sole sulfur source. Because RS-1051 does not grow on galactose, we used a synthetic medium with 2% glucose and 2% galactose. As shown in Figure 2, pAT18 complemented the defect of the *hal2*Δ mutation, even though the SAL1 protein is probably present at low levels when the cells are in this medium (Yocum et al., 1984).

To obtain the SAL1 protein, the cDNA insert in pAT18 was cloned into plasmid pT7-7 under the control of the Φ10 promoter of T7 bacteriophage, and the resulting plasmid, pT7-28,

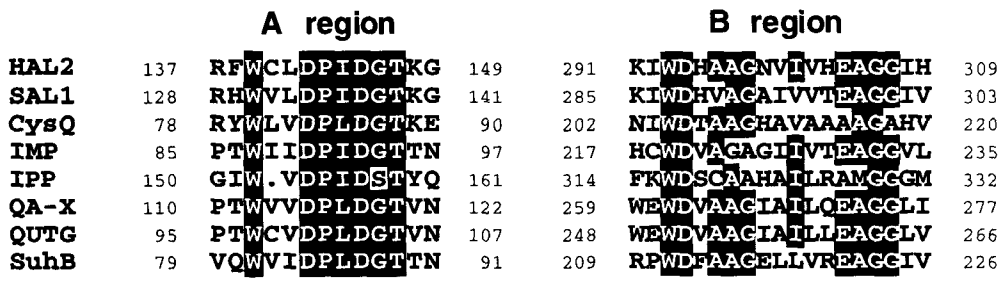

Figure 1. Alignment of the Translated Amino Acid Sequence of the *SAL1* cDNA with 3′(2′),5′-Bisphosphate Nucleotidases, Inositol Phosphatases, and Homologous Proteins.

Identical amino acids in the two motifs (regions A and B) identified by Neuwald et al. (1991) are in black boxes. HAL2, yeast 3′(2′),5′-bisphosphate nucleotidase (Gläser et al., 1993); CysQ, putative 3′(2′),5′-bisphosphate nucleotidase of *E. coli* (Neuwald et al., 1992); IMP, bovine inositol 1-phosphatase (Diehl et al., 1990); IPP, bovine inositol polyphosphate 1-phosphatase (York and Majerus, 1990); QA-X, *Neurospora crassa* QA-X (Geever et al., 1989); QUTG, *Aspergillus nidulans* QUTG (Lamb et al., 1990); SuhB, *E. coli* SuhB (Yano et al., 1990).

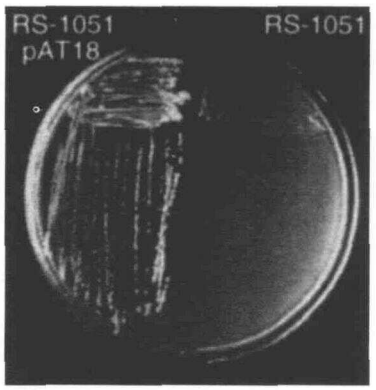

Figure 2. Growth Test on Sulfate as a Sole Source of Sulfur.

Growth was measured in SD medium containing 2% glucose and 2% galactose. Results with strains RS-1051 (*hal2/met22* mutant) and RS-1051 transformed with pAT18 are shown.

was transformed into strain BL21(DE3) of *E. coli*. Figure 3 shows the SDS-PAGE of cell-free extracts from isopropyl β-D-thiogalactopyranoside–induced cells, revealing the presence of a protein of ~38 kD, the size expected from the translation of the *SAL1* open reading frame. Associated with the presence of this protein, the *E. coli* extract exhibited phosphatase activity on 3′-phosphoadenosine 5′-phosphate (PAP). Therefore, we purified the enzyme by affinity chromatography in PAP–agarose, increasing the activity of the extract 10-fold. Consistent with the activity of HAL2 phosphatase (Murguía et al., 1995), the SAL1 protein hydrolyzed 2′-PAP and 3′-phosphoadenosine 5′-phosphosulfate (PAPS). In addition, the SAL1 protein hydrolyzed inositol 1,4-bisphosphate and inositol 1,3,4-trisphosphate but not inositol 1-phosphate, thus exhibiting the typical activity of an inositol polyphosphate 1-phosphatase, which is an important enzyme in the phosphoinositide signaling pathway (Majerus, 1992).

However, as summarized in Table 1, the SAL1 protein did not show broad phosphatase activity, and phosphate esters, such as ATP, fructose 1,6-bisphosphate, or inositol hexaphosphoric acid, were not hydrolyzed. Extracts from transformants with pT7-7, the plasmid without the *SAL1* cDNA insert, treated in the same manner, lacked significant phosphatase activity and did not show the 38-kD protein when subjected to SDS-PAGE. Homogenates of Arabidopsis leaves purified by several steps of precipitation and affinity chromatography with PAP–agarose contained a protein that comigrated with SAL1 when subjected to SDS-PAGE (Figure 3) and showed 3′,5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities.

Figure 4 shows the activity of the SAL1 phosphatase expressed in *E. coli* as a function of pH and Mg^{2+} concentration. Under conditions of maximal activity, pH 7.5, and 1 mM Mg^{2+}, the K_m value for PAP hydrolysis was too low to be calculated with precision, but we estimated that the half-maximal rate was reached at 2 to 10 μM PAP. With 1,4-inositol bisphosphate, the SAL1 phosphatase exhibited hyperbolic kinetics, with a K_m of 90 μM. With both substrates, Li^+ was a strong noncompetitive inhibitor and Na^+ was a weak noncompetitive inhibitor. The data shown in Figure 5 indicate that inhibitory concentrations of Li^+ were in the submillimolar range (0.20 mM for 50% inhibition), whereas inhibitory concentrations of Na^+ were in the submolar range (0.2 M for 50% inhibition). The inhibition by Li^+ decreased ~10-fold in the presence of 100 mM K^+ (from 50 to 5% inhibition at 0.20 mM Li^+), whereas the inhibition by Na^+ was imperceptibly affected by 100 mM K^+ (data not shown).

pAT18 Increases the Intracellular Tolerance to Li^+

Transformation with pAT18 and growth on galactose increased the Li^+ tolerance both of the mutant strain (G19) lacking Li^+ efflux (from 0.5 to 2.5 mM) and of the wild-type strain W303.1B (from 10 to 50 mM) approximately fivefold. Li^+ or Na^+ tolerance increases if either the intracellular content or the intracellular toxicity of these cations decreases (Haro et al., 1993). The latter cause has been proposed to explain the increase in salt tolerance produced by the overexpression of HAL2 phosphatase in yeast (Murguía et al., 1995). To address the question of why the SAL1 protein increased Li^+ tolerance, we compared the Li^+ contents of the cells transformed with *SAL1* (strain G19 transformed with pAT18) with control cells (G19 transformed with pYES2) when both types of cells were exposed to concentrations of Li^+ at the limits of tolerance (2.5 and 0.5 mM Li^+, respectively). Although both strains grew at

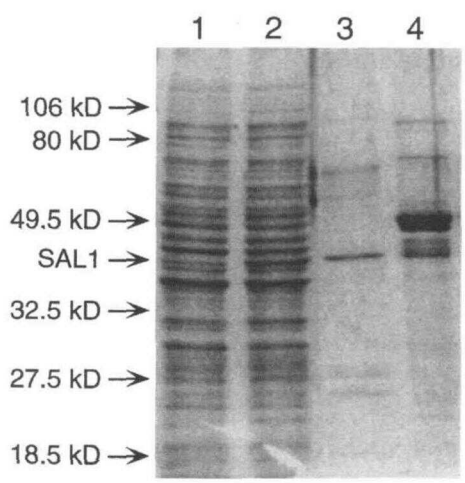

Figure 3. SDS-PAGE Analysis of the *SAL1* Gene Product.

Lane 1 contains protein extracts from *E. coli* transformed with plasmid pT7-7 (control); lane 2, protein extracts from *E. coli* transformed with plasmid pT7-28 (containing the coding region of the *SAL1* cDNA); lane 3, same extracts as given for lane 2 after purification with PAP–agarose; lane 4, leaf extracts of Arabidopsis purified with PAP–agarose. Numbers on the left indicate the molecular masses of markers in kilodaltons.

Table 1. Substrate Specificity of the Phosphatase Activity of the SAL1 Protein Expressed in *E. coli* and Purified as Described in the Text

Substrate[a]	Activity[b]
PAP	100
2′-PAP	105
PAPS[c]	180
Inositol 1,4-bisphosphate	34
Inositol 1,3,4-trisphosphate	37
Inositol 1-monophosphate	0
Inositol hexaphosphate	0
ATP	0
Fructose 1,6-bisphosphate	0

[a] All compounds were used at 0.5 mM.

[b] Percentage of the activity on PAP. Assays at 25 mM Tris-HCl, pH 7.5, and 1 mM Mg^{2+}. The specific activity on PAP was 4 μmol min^{-1} mg^{-1} of protein.

[c] Commercial form as Li^+ salt (4.0 mol of Li^+ per mol). We compared the activity with this compound and the activity with PAP plus 2.0 mM Li^+.

approximately the same rate under these conditions, the Li^+ content in the cells transformed with pAT18 was much higher than in control cells (27 versus 8 nmol mg^{-1}). Treatment of the cells with cytochrome *c* to determine the Li^+ content in the cytoplasm and vacuole (Okorokov et al., 1980) showed that the excess of Li^+ in the cells transformed with pAT18 was not in the vacuole. Taken together, these results indicate that intracellular Li^+ was less toxic in transformants with pAT18.

pAT18 Increases Li^+ and Na^+ Effluxes

Transformation of pAT18 into G19 did not increase the tolerance to Na^+, whereas transformants of W303.1B showed a modest but clear increase (from 300 to 500 mM NaCl in arginine phosphate [AP] medium). Because the lack of Na^+ efflux is the only characteristic that differentiates G19 from W303.1B (both strains are entirely isogenic, except for the *ena1*Δ::*HIS3*::*ena4*Δ mutation in G19), the increase in Na^+ efflux and not a change in the intracellular toxicity of Na^+ seemed to cause the increase in the Na^+ tolerance in pAT18 transformants of W303.1B. Because Na^+ and Li^+ use the same transport systems, the increase in Li^+ efflux should also participate in the increase in Li^+ tolerance produced by pAT18 in strain W303.1B. However, a role for the increase in Li^+ efflux in the increase in Li^+ tolerance in W303.1B transformed with pAT18 could not be shown because the ability of pAT18s to decrease intracellular toxicity masked the effect of the increase of Li^+ efflux.

To confirm that pAT18 increased Li^+ efflux in W303.1B, we performed Li^+ uptake experiments. The data presented in Figure 6 show that pAT18 decreased the net uptake of Li^+ in W303.1B. Because the initial rate of uptake was not affected, we concluded that pAT18 increased Li^+ efflux without affecting Li^+ influx (a simple mathematical treatment of the data allows calculation of influx and efflux in uptake experiments; see Rodríguez-Navarro and Asensio, 1977). To test whether pAT18 affected the expression of the *ENA1* gene, as *ENA1* expression is affected by calcineurin (Mendoza et al., 1994) as well as by the PPZ1 and PPZ2 phosphatases (Posas et al., 1995), we transformed pAT18 into a yeast strain carrying a chromosome integrant of an *ENA1–lacZ* gene and tested the β-galactosidase activity under different conditions. We found that pAT18 did not affect the expression of the *ENA1*–lacZ gene, either under basal conditions or in the presence of Li^+ or Na^+. The lack of effect of pAT18 on the expression of the *ENA1–lacZ* gene suggested that the SAL1 protein affected the activity of the ENA ATPases, possibly by affecting a signaling pathway regulating the enzyme.

Of the two enzyme activities of the SAL1 protein, the 3′(2′),5′-bisphosphate nucleotidase, which is necessary for sulfate assimilation, is not known to be involved in regulatory processes. On the contrary, inositol polyphosphate 1-phosphatase plays an important role in the phosphoinositide signaling pathway. It is of interest that this pathway is involved in the regulation of K^+ uptake in yeast (Alijo and Ramos, 1993) and in sugar beet storage tissue slices (Srivastava et al., 1989). In addition, in Arabidopsis another enzyme in this pathway, phosphatidylinositol-specific phospholipase C, is induced by salt (Hirayama et al., 1995). To address the possibility that phosphoinositides regulate Li^+ efflux in yeast, we measured Li^+ efflux with the

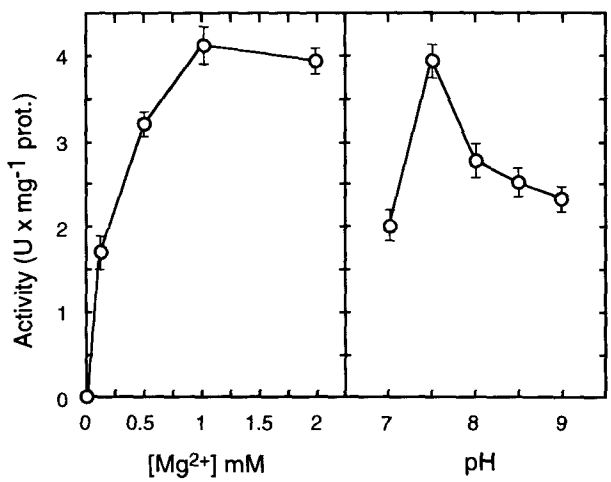

Figure 4. Effect of Mg^{2+} Concentration and pH on the 3′,5′-Bisphosphate Nucleotidase Activity of the *SAL1* Gene Product.

The SAL1 protein was expressed in *E. coli* and purified by affinity chromatography with PAP–agarose. Assays were performed with 0.5 mM PAP. Data represent means ± SE.

(Left) 25 mM Tris-HCl, pH 7.5.

(Right) 1 mM $MgCl_2$ and 25 mM Tris-HCl up to pH 8.5; 25 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid–Tris, pH 9.0. One unit (U) hydrolyzes 1 μmol of PAP per min per mg of protein (prot.).

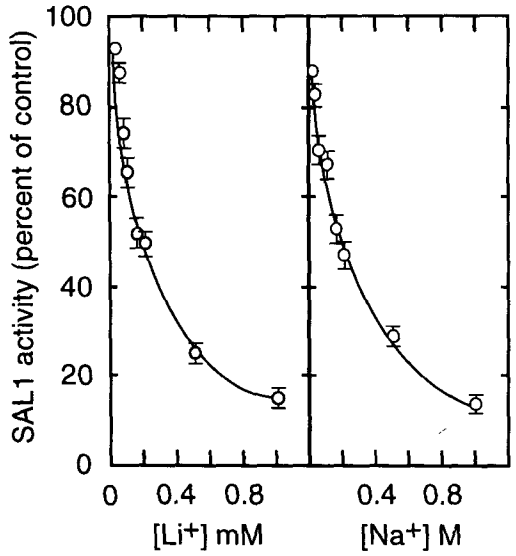

Figure 5. Inhibition of the 3′,5′-Bisphosphate Nucleotidase Activity of the *SAL1* Gene Product by Li^+ and Na^+.

The SAL1 protein was expressed in *E. coli* and purified by affinity chromatography with PAP–agarose. Assays were performed with 0.5 mM PAP, 1 mM Mg^{2+}, 25 mM Tris-HCl, pH 7.5, as in Figure 4.

wild-type strain W303.1B and with the mutant strain G19 in the presence and absence of drugs affecting the phosphoinositide signaling pathway. These experiments were designed to distinguish specific effects on the efflux affecting only W303.1B from other kinds of effects that should affect both strains. Figure 7 shows that compound 48/80, an inhibitor of phosphatidylinositol-specific phospholipase C (Bronner et al., 1987), increased Li^+ efflux only in strain W303.1B, mimicking the effect of pAT18. These results support the notion that phosphoinositides regulate the activity of the ENA ATPases.

DISCUSSION

The *SAL1* gene of Arabidopsis encodes a novel enzyme with two different activities, 3′(2′),5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase (Table 1). In yeast, sulfate assimilation is initiated by the activation of sulfate through adenosine 5′-phosphosulfate and PAPS. The removal of sulfate from PAPS produces PAP, and this compound has to be recycled to AMP by the 3′(2′),5′-bisphosphate nucleotidase encoded by the *HAL2/MET22* gene (Murguía et al., 1995). In a yeast expression vector, the *SAL1* cDNA complemented the *hal2/met22* mutation, indicating that the SAL1 3′(2′),5′-bisphosphate nucleotidase is fully functional in yeast. Assimilation of sulfate in plants presumably follows the same pathway as in yeast (Schmidt and Jäger, 1992), and the characterization of the *SAL1* gene of Arabidopsis gives direct experimental support to this notion. Furthermore, the existence of a homologous gene in rice suggests that this conclusion may apply to other higher plants.

The inositol polyphosphate 1-phosphatase activity of SAL1 suggests that it participates in the phosphoinositide signaling system. In animals, the inositol polyphosphate 1-phosphatase enzyme is specific to the phosphoinositide signaling system. It hydrolyzes inositol 1,4-bisphosphate and inositol 1,3,4-trisphosphate to inositol 4-phosphate and inositol 3,4-bisphosphate, respectively, in the pathway that converts the signaling compound inositol 1,4,5-trisphosphate into inositol (for review, see Majerus, 1992). In plants, the characteristics of this signaling system are under study (see reviews by Drøbak, 1992; Coté and Crain, 1993; Welters et al., 1994), and currently it is difficult to predict the number and the characteristics of the enzymes involved in the turnover of the inositol phosphates. Therefore, to date there is no test to determine whether the SAL1 phosphatase participates in the plant phosphoinositide signaling system. However, because the only function known for inositol polyphosphate 1-phosphatases in animals is in the phospho-

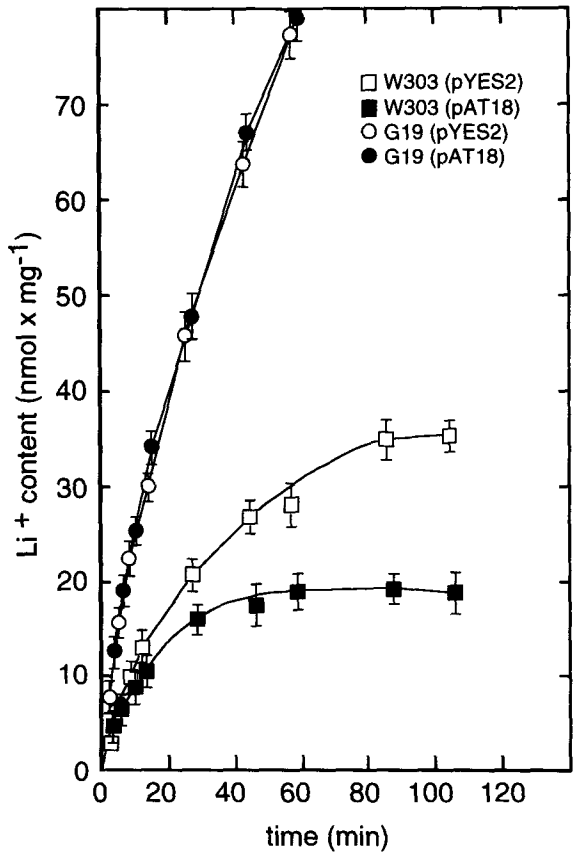

Figure 6. Effect of the *SAL1* cDNA on the Net Uptake of Li^+.

Shown are time courses of the Li^+ content of strains W303.1B and G19 transformed with pAT18 and pYES2 exposed to 50 mM Li^+ in AP medium with 1 mM K^+. Data represent means ± SE.

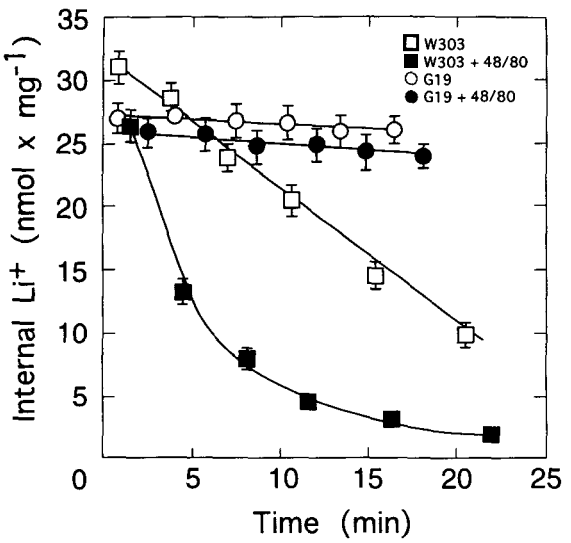

Figure 7. Effect of Compound 48/80 on Li^+ Efflux in Yeast.

Cells of the wild-type strain W303.1B and of the efflux mutant G19 were grown in AP medium with 3.0 mM K^+ and transferred to AP medium containing 2% glucose, 1 mM K^+, and 25 mM Li^+ (for G19) or 50 mM Li^+ (for W303.1B). After 50 min, the Li^+-loaded cells were transferred to AP medium with 2% glucose and 10 mM K^+, and the loss of Li^+ was followed in the absence and in the presence of 5 μg mL^{-1} of compound 48/80. Data represent means ± SE.

inositide signaling system, we propose an analogous function for the plant enzyme.

Certainly, the K_m value of SAL1 for inositol 1,4-bisphosphate is higher than the K_m value for the animal inositol polyphosphate 1-phosphatases, 90 μM versus 5 to 9 μM inositol 1,4-bisphosphate (Inhorn and Majerus, 1987; York and Majerus, 1990). However, this discrepancy with the K_m value of animal enzymes should be viewed with caution, not only because plant and animal phosphoinositide signaling systems may be different, but also because an enzyme with two functions may be under complex processes of regulation and the in vitro K_m value may be quite different from the K_m value in vivo. Furthermore, the existence of another gene in Arabidopsis encoding a protein homologous to SAL1 suggests that it might encode another inositol polyphosphate 1-phosphatase and that the understanding of the function of the SAL1 phosphatase is impossible without considering the function of the homologous enzyme.

The dual activity of SAL1 leads to another intriguing question about the connection between these two activities in the plant cell. Inositol phosphatases and 3′(2′),5′-bisphosphate nucleotidases are related phosphatases, according to sequence homologies (Gläser et al., 1993). In this sequence homology group, inositol polyphosphate 1-phosphatases, inositol 1-phosphatases, and 3′(2′),5′-bisphosphate nucleotidases might be more related than the other members because they share a striking and specific sensitivity to Li^+ (Hallcher and Sherman, 1980; Inhorn and Majerus, 1987; Murguía et al., 1995). It is possible that in some plants, only one enzyme has evolved to act both as 3′(2′),5′-bisphosphate nucleotidase and as inositol polyphosphate 1-phosphatase.

The capacity of the *SAL1* cDNA to increase the Li^+ tolerance of yeast may be due to two different mechanisms: a decrease in the intracellular toxicity of Li^+ and an increase in the efflux of the cation. The cause for the decrease in intracellular toxicity has not been investigated; however, the sensitivity of the SAL1 3′(2′),5′-bisphosphate nucleotidase activity to Li^+ is almost identical to that found for the HAL2 3′(2′),5′-bisphosphate nucleotidase, and the *SAL1* cDNA complemented the *hal2* mutation, as discussed above. Therefore, the decrease in the intracellular Li^+ toxicity can be explained, as in the case of the HAL2 phosphatase (Murguía et al., 1995), by the intracellular increase of the 3′(2′),5′-bisphosphate nucleotidase activity. The increase in the efflux of Li^+ was dependent on a functional ENA system (Figure 6), which is the system mediating Na^+ and Li^+ efflux in yeast (Haro et al., 1991; Garciadeblas et al., 1993). Our finding that compound 48/80, an inhibitor in phosphatidylinositol phospholipase C (Bronner et al., 1987), increased Li^+ efflux in a wild-type strain but not in an isogenic strain carrying an *ena1*Δ::*HIS3*::*ena4*Δ mutation (Figure 7) indicates that the phosphoinositide signaling system regulates the ENA system. Taken together, the two sets of results suggest that the expression of the SAL1 inositol polyphosphate 1-phosphatase in yeast alters the state of the endogenous phosphoinositide signaling system, and this alteration results in the enhancement of the ENA efflux system.

The involvement of the phosphoinositide signaling system in the regulation of the yeast ENA system is a novel finding; the next step would be to determine whether this signaling system is also involved in the regulation of Na^+ efflux in higher plants. Current information on Na^+ efflux in plants is not sufficient to allow a serious discussion of this point. However, it is worth considering that in Arabidopsis, a cDNA encoding a putative phosphatidylinositol-specific phospholipase C is induced by salinity (Hirayama et al., 1995). In sugar beet storage tissue slices, phosphoinositides enhance K^+ uptake, mimicking a hypertonic mannitol shock (Srivastava et al., 1989). Moreover, in *Dunaliella salina* (Einspahr et al., 1988) and carrot cells (Cho et al., 1993), osmotic shocks produce changes in the metabolism of phosphatidylinositol.

The SAL1 phosphatase of Arabidopsis and the HAL2 phosphatase of yeast are both inhibited by submillimolar concentrations of Li^+. However, SAL1 was 10-fold less sensitive to Na^+ than HAL2 was (50% inhibition at 200 and 20 mM Na^+; compare Figure 5 in this report with Figure 3 in Murguía et al., 1995). Therefore, it is unlikely that, as proposed for yeast (Murguía et al., 1995), the 3′(2′),5′-bisphosphate nucleotidase activity and sulfate assimilation are the targets of Na^+ toxicity in Arabidopsis. However, as in the case of *HAL2* in yeast, the overexpression of *SAL1* might increase Na^+ tolerance in plants if it activates Na^+ efflux.

METHODS

Strains, Methods, and Media

The yeast (*Saccharomyces cerevisiae*) strains W303.1B (*Matα ura3 his3 leu2 ade2 trp1*), its derivative G19 (*Matα ura3 his3 trp1 ade2 ena1Δ::HIS3::ena4Δ*) obtained by gene disruption, and the *hal2Δ* strain RS-1051 (*MATα ura3 leu2 hal2::URA3*) were used in this study. The minimal media SD and SG, and the arginine phosphate medium (AP) have been described by Rodríguez-Navarro and Ramos (1984) and Sherman (1991). AP medium with either glucose or galactose as a carbon source but free of K^+ and Na^+ in their basal forms were supplemented with KCl, NaCl, and LiCl, as required. Growth tests were normally performed by inoculation of a 50-μL drop of a cell suspension containing 10^5 to 10^6 cells onto the surface of the indicated agar medium and recording the growth after 7 days. *Escherichia coli* was grown on standard Luria-Bertani medium (Sambrook et al., 1989).

DNA Manipulation and Sequence Analysis

Manipulation of nucleic acids was done by standard protocols (Sambrook et al., 1989) or, when appropriate, by following the manufacturer's instructions. DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (1977), as modified for use with Sequenase (U.S. Biochemical Corp.). DNA sequence data for comparative analysis were obtained from GenBank (release 83 [6/94]). Peptide sequences were obtained from GenBank or SwissProt (release 83 [3/94]). Protein comparisons were performed, using the BESTFIT, GAP, and FastA (updated FastP) algorithms from the University of Wisconsin Computer Group (Madison, WI; Devereux et al., 1984, and updates). For blot hybridizations, genomic DNA was first digested with SacI, HindIII, and BamHI. Then, after electrophoresis and blotting to a nylon membrane, it was hybridized at high stringency (50% formamide and 0.75 M NaCl at 42°C) with the 1.1-kb ClaI-XbaI fragment of pAT18 labeled with phosphorous-32 by the random-priming method (Feinberg and Vogelstein, 1983). For RNA blot hybridizations of the *SAL1* transcript, total RNA was isolated and electrophoresed by use of formaldehyde–agarose gels, blotted to a nylon membrane, and hybridized as described above. To ensure that equivalent quantities of RNA of equal quality were loaded onto gels and that transfer was complete, the ethidium bromide staining intensity of the ribosomal bands was evaluated before and after transfer.

The GenBank accession number for the nucleotide sequence of *SAL1* cDNA is U40433.

Library Construction and Selection of Transformants

Plants grown for 30 days on Murashige and Skoog medium (Murashige and Skoog, 1962) were exposed for 24 hr to modified Murashige and Skoog medium containing Na^+ instead of K^+ salts and supplemented with 100 mM NaCl. The roots of these plants were frozen in liquid nitrogen, the RNA was extracted as described by Ecker and Davis (1987), and poly(A)$^+$ mRNA was purified in oligo(dT)-cellulose columns (Clontech Laboratories, Palo Alto, CA). cDNA was synthesized with use of a commercial cloning system kit (Stratagene) and inserted into the yeast expression vector pYES2 (Invitrogen). The resulting plasmids were then transformed into strain MC1061 of *E. coli*. Transformants of the yeast strain G19 with plasmids purified from the library were selected on SD medium without uracil. A sample of the emerging colonies was incubated in SG medium without uracil for 24 hr and plated on SG medium containing 10 mM Li^+. Colonies able to grow in this medium but unable to grow in SD-Li^+ were isolated. The plasmids isolated from these clones were reintroduced into mutant G19 to demonstrate that the Li^+ tolerance depended on the plant cDNA.

Cation Contents

Cells were collected in Millipore membrane filters (Millipore Corp., Bedford, MA), rapidly washed with 20 mM $MgCl_2$ solution, acid extracted, and analyzed by atomic emission spectrophotometry. Uptake experiments were performed in AP medium.

Purification and Assay of SAL1 Phosphatase

Expression of *SAL1* in *E. coli* was performed as described by Tabor (1990). The 1143-bp ClaI-XmnI fragment from pAT18 was treated to produce blunt ends and inserted into vector pT7-7 digested with NdeI and treated to produce blunt ends. The plasmid containing the insert in the correct orientation was transformed into *E. coli* strain BL21(DE3). Cultures of the transformant strain in Luria-Bertani medium (A_{550}, 1.0) were induced with 1 mM isopropyl β-D-thiogalactopyranoside for 4 hr, washed, and suspended in lysis buffer (30% sucrose, 0.25 M Tris, pH 8.0, 0.5M KCl, 25 mM EDTA, 5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 25 μg mL^{-1} chymostatin). The extracts were sonicated, treated with RNase H and DNase I, and centrifuged for 20 min at 12,000 rpm in a microcentrifuge. The SAL1 phosphatase was purified from the supernatant by PAP–agarose (Sigma) affinity chromatography, as described by Ramaswamy and Jakoby (1987).

Purification of 3′,5′-bisphosphate nucleotidase from Arabidopsis leaves was performed as described for tomato leaves by Murguía et al. (1995). Assays of phosphatase activity were performed as described in the same reference.

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