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A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast

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We have isolated a novel yeast gene, *HAL1*, which upon overexpression improves growth under salt stress. In addition, disruption of this gene decreases salt tolerance. Therefore *HAL1* constitutes a rate-limiting determinant for halotolerance. It encodes a polar protein of 32 kDa located in the yeast cytoplasm and unrelated to sequences in data banks. The expression of this gene is increased by high concentrations of either NaCl, KCl or sorbitol. On the other hand, the growth advantage obtained by overexpression of *HAL1* is specific for NaCl stress. In cells overexpressing *HAL1*, sodium toxicity seems to be counteracted by an increased accumulation of potassium. The *HAL1* protein could interact with the transport systems which determine intracellular K^+ homeostasis. The *HAL1* gene and encoded protein are conserved in plants, being induced in these organisms by salt stress and abscisic acid. These results suggest that yeast serves as a convenient model system for the molecular biology of plant salt tolerance.

Key words: K^+ transport/salt induction/salt tolerance/yeast

Introduction

The progressive salinization of irrigated land poses a threat to the future of agriculture in arid regions (Downton, 1984; Wyn Jones and Gorham, 1986). In contrast to halophytic plants living in sea water, crop plants are sensitive to low concentrations of NaCl. Nevertheless, within salt-sensitive species there is great diversity with respect to salt tolerance (Flowers *et al.*, 1977; Greenway and Munns, 1980; Downton, 1984). This genetic variability suggests that genetic engineering has the potential to improve the salinity tolerance of crops (Rains *et al.*, 1980). The methodology to generate transgenic plants is readily available (Potrykus, 1991) but the limiting factor is the isolation of genes with the capability to improve salt tolerance (halotolerance genes).

Two different approaches are currently being followed by many laboratories to isolate halotolerance genes. One is based on the cloning of genes of unknown function induced by salt and water stress (Singh *et al.*, 1989; Claes *et al.*, 1990; Piatkowski *et al.*, 1990). The other concentrates on genes that encode enzymes involved in synthesis of metabolites such as betaine and proline known to function as osmoprotectants in bacteria and plants (McCue and Hanson, 1990). However, many salt-induced genes are probably involved in non-specific stress responses against pathogens (Czarnecka *et al.*, 1984; Singh *et al.*, 1989; Neale *et al.*, 1990; Rodrigo *et al.*, 1991) and their utility to improve salt tolerance has never been demonstrated. Furthermore, in many instances accumulation of osmoprotectants such as betaine or proline does not correlate with salt tolerance (Flowers *et al.*, 1977; Greenway and Munns, 1980).

Other phenomena associated with salt tolerance and not yet investigated at the molecular level include: (i) increased activity of plasma membrane H^+-ATPase (Braun *et al.*, 1986); (ii) increased activity of the K^+ uptake system (Rush and Epstein, 1981; Watad *et al.*, 1991); (iii) increased activity of the vacuolar Na^+/H^+ antiport (Blumwald and Poole, 1987; Garbarino and DuPont, 1988) and (iv) increased activity of the vacuolar H^+-ATPase (Reuveni *et al.*, 1990).

The complexity of the cellular responses to salt stress suggest that only some critical processes may be useful in improving salt tolerance. By analogy to the rate-limiting steps of metabolic pathways, as defined by Kacser and Burns (1981), only changes in rate-limiting steps for salt tolerance would have the capability to influence growth under salt stress. The problem of phenomenological approaches (studying phenomena concomitant with salt stress) is to distinguish these critical processes from all the peripheral ones. A functional approach to the isolation of halotolerance genes would be the search for genes which by some modification (overexpression, activation by point mutations) could improve salt tolerance. This approach would identify critical processes for salt tolerance and at the same time generate the genetic tools for the improvement of it.

Although halotolerance genes have been identified in higher plants by classical genetic analysis (Abel, 1969; Downton, 1984; Wyn Jones and Gorham, 1986; Gorham *et al.*, 1987; Dvorak *et al.*, 1988), cloning of genes by complementation of the desired phenotype is still a formidable task in these organisms (Klee *et al.*, 1987; Meyerowitz, 1989). Therefore these halotolerance genes can only be used for crosses between related plant species (Epstein *et al.*, 1980; Downton, 1984).

The yeast *Saccharomyces cerevisiae* could serve as a convenient model system to isolate halotolerance genes and to uncover rate-limiting steps for growth under salt stress. This yeast shares basic ion transport mechanisms with plants (Serrano, 1985) and genes can easily be isolated by complementation (Struhl, 1983). Furthermore, the availability of multicopy plasmid vectors allows the selection of specific genes under conditions where their overexpression improves growth (Rine *et al.*, 1983). By applying a functional approach we have isolated a novel yeast gene,

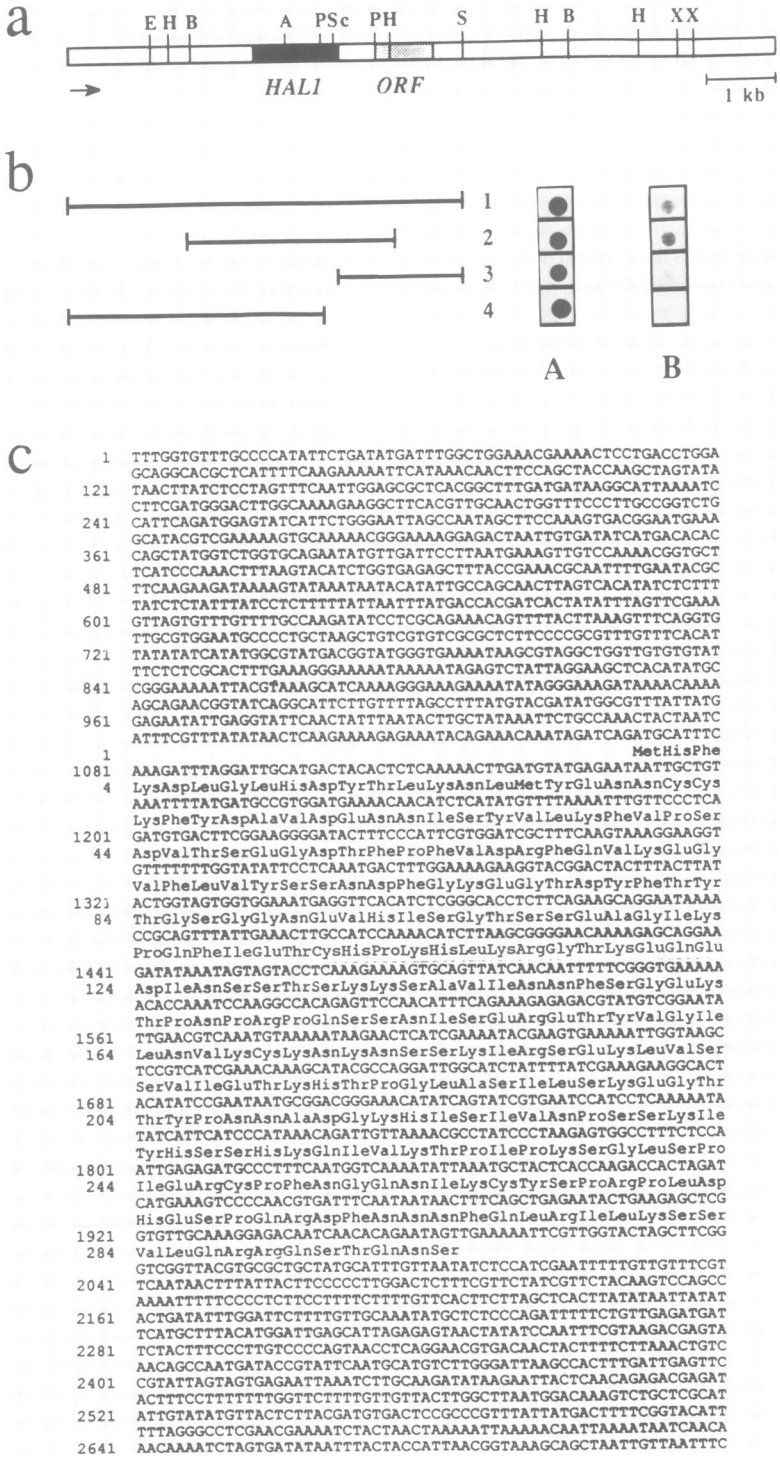

Fig. 1. Isolation and sequence of the *HAL1* locus. (**a**) Restriction map of a genomic DNA insert of 10 kb which by overexpression in multicopy plasmid YEp24 improves growth at high NaCl concentration. The coding regions of the *HAL1* gene (see below) and of a nearby open reading frame (*ORF*) are indicated. A, *Ava*I; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; Sc, *Sac*I; S, *Sal*I; X, *Xho*I. No sites for *Bam*HI, *Nco*I, *Pst*I, *Sma*I, *Sph*I and *Xba*I are present. (**b**) Localization of the halotolerance gene *HAL1* by subcloning of different restriction fragments in plasmid YEp24 and testing for growth of transformants at high NaCl. 1, fragment extending from the beginning of the insert to the internal *Sal*I site; 2, *Bgl*II–*Hin*dIII fragment; 3, *Sac*I–*Sal*I fragment ; 4, fragment extending from the beginning of the insert to the first *Pvu*II site. A, growth in normal medium; B, growth in medium supplemented with 1.5 M NaCl. (**c**) Nucleotide sequence of 2.7 kb of DNA starting at the first *Bgl*II site (part a) and containing the *HAL1* gene. The deduced amino acid sequence is indicated.

HAL1, induced by salt and corresponding to a rate-limiting step for growth under salt stress. This gene is highly conserved in higher plants, suggesting that yeast can be used as a model system for plant salt tolerance.

Results

Isolation and sequence of the HAL1 gene

Yeast cells were transformed with a genomic library made in a yeast multicopy plasmid. One transformant exhibited

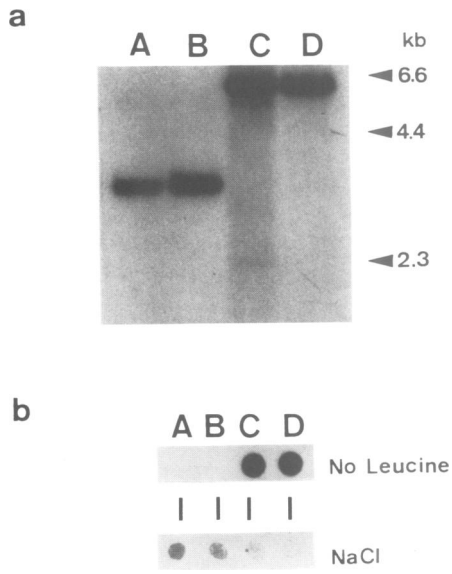

Fig. 2. Disruption of the *HAL1* gene. (**a**) Southern analysis of the four spores (A–D) of a tetrad derived from a diploid where one copy of the *HAL1* gene has been disrupted by insertion of a 2.2 kb DNA fragment containing the *LEU2* gene at the *Ava*I site (Figure 1a). Chromosomal DNA was digested with *Bgl*II and *Hin*dIII and the hybridization probe was an *Ava*I–*Sac*I fragment of 0.5 kb (see Figure 1a). The wild type and interrupted genes generate hybridizing fragments of 2.95 and 5.15 kb, respectively. (**b**) Growth of the four spores in minimal medium with either uracil as the only supplement (to test the $Leu^{+/-}$ phenotype) or with uracil, leucine and 1.2 M NaCl (to test the halotolerant phenotype). Similar results were obtained with five different tetrads.

improved growth at 1.5 M NaCl and was selected. The corresponding plasmid contained a genomic DNA fragment of 10 kb (Figure 1a). The halotolerant phenotype was conferred by a gene designated *HAL1* present in a *Bgl*II–*Hin*dIII subfragment of 2.95 kb (Figure 1b and c). It was flanked by an open reading frame not relevant for growth at high NaCl (compare phenotypes conferred by fragments 2 and 3 of Figure 1b).

HAL1 is predicted to encode a basic polar protein (isoelectric point 9.2) of 294 amino acids (32.3 kDa), with both α-helical and β-sheet secondary structure which is unrelated to any sequence in data banks (similarity search according to Lipman and Pearson, 1985). The gene is likely to be poorly expressed because only 43% of its amino acids are coded by the 25 preferred yeast codons (Bennetzen and Hall, 1982). The last 17 amino acids are essential for activity (compare phenotypes conferred by fragments 2 and 4 of Figure 1b). This terminal domain contains a potential phosphorylation site for cAMP-dependent protein kinase (Ser290, sequence RRQS). Another potential phosphorylation site was present at Thr119 (sequence KRGT).

Southern analysis indicated that *HAL1* was present in single copy in haploid cells and that no homologous genes were detected by hybridization at high stringency (Figure 2a).

Disruption of the HAL1 gene

We have replaced one of the two chromosomal copies of the *HAL1* gene in a diploid yeast by an interrupted version containing the *LEU2* gene inserted at the *Ava*I site (Figure 1a). After sporulation and tetrad dissection the gene

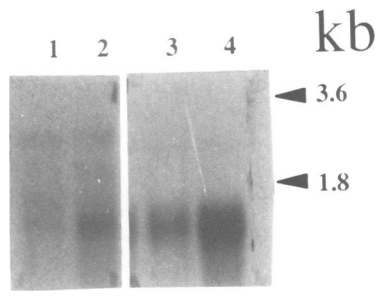

Fig. 3. Induction of the *HAL1* mRNA by NaCl. Northern analysis of the level of *HAL1* mRNA in cells containing either the single chromosomal copy of the gene (1 and 2) or, in addition, the gene cloned in multicopy plasmid YEp24 (3 and 4). Yeast cells were grown in medium with (2 and 4) or without (1 and 3) 1 M NaCl and harvested during exponential phase (absorbance at 660 nm, 0.3–0.4). The positions of ribosomal RNAs are indicated in kb.

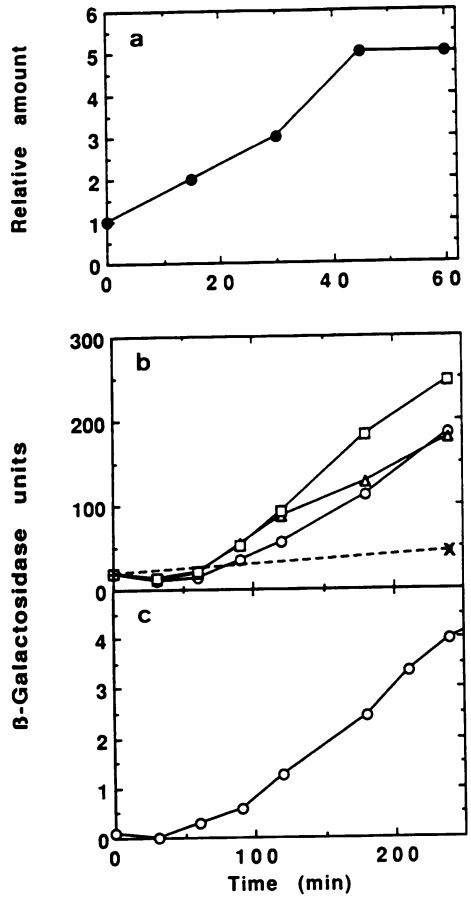

Fig. 4. Time course and specificity of induction of *HAL1*. (**a**) Northern analysis of *HAL1* mRNA levels in cells containing the cloned *HAL1* gene in multicopy plasmid YEp24. Cells were grown in medium without NaCl and when the absorbance at 660 nm reached 0.3–0.4, 1 M NaCl was added and RNA extracted at the indicated times. The hybridization signal of *HAL1* mRNA (see Figure 3) was quantified by densitometry utilizing a Quick Scan (Desaga, Heidelberg). (**b**) and (**c**) β-galactosidase activity in cells containing a fusion of the *HAL1* and *LacZ* genes in either multicopy plasmid (b) or integrated in single copy at the *HAL1* locus (c). Yeast cells were grown in medium without NaCl and when the absorbance at 660 nm reached 0.3–0.4, either 1 M NaCl (circles), 1 M KCl (squares) or 1.5 M sorbitol (triangles) were added (time zero) and the β-galactosidase activity determined at the indicated times as described in Materials and methods. The cross and the discontinuous line reflect unchanged activity in the absence of additions.

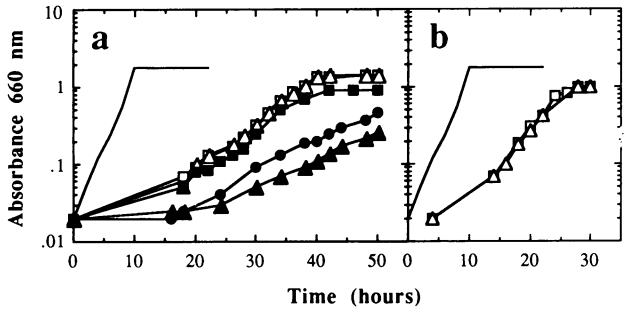

Fig. 5. Growth inhibition by NaCl, sorbitol and KCl. The continuous line reflects growth in normal medium, without additions, which is identical in all strains. Open symbols: medium supplemented with 1.5 M sorbitol (**a**) or 1 M KCl (**b**); closed symbols: medium supplemented with 1 M NaCl; circles: wild type strain RS-16; squares: cells containing the *HAL1* gene in multicopy plasmid YEp24; triangles: cells containing a disruption of the *HAL1* gene. Cells were grown for 2 days to reach stationary phase (in medium without uracil in case of plasmid-containing cells) and at time zero diluted 40 times in medium containing uracil, leucine and either 1.5 M sorbitol, 1 M NaCl or 1 M KCl. Similar results were obtained in three independent experiments.

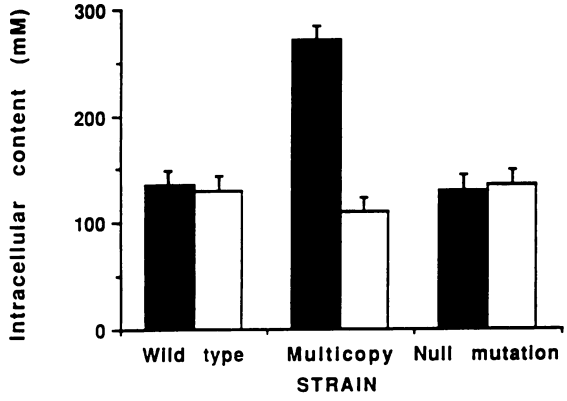

Fig. 6. Intracellular concentrations of Na^+ (open bars) and K^+ (closed bars). Exponentially growing cells in medium with 1 M NaCl were harvested with absorbance at 660 nm of 0.3–0.4. Wild type: strain RS-16; multicopy: RS-16 with the *HAL1* gene in multicopy plasmid YEp24; null mutation: gene disruption of *HAL1*. Values are the mean of three or four determinations and bars represent the standard deviations.

disruption was demonstrated by Southern analysis (Figure 2a). It was observed that the *HAL1* gene is not essential for growth under normal conditions because haploid spores containing the disrupted gene are viable (Figure 2b, 'No leucine' growth). However, growth in the presence of 1.2 M NaCl was decreased by the disruption (Figure 2b, 'NaCl' growth).

Regulation of the HAL1 gene

The expression of the *HAL1* gene is induced by osmotic stress, as demonstrated by both Northern analysis (Figures 3 and 4a) and by the activity of a reporter β-galactosidase gene fused to the *HAL1* promoter and reading frame (Figure 4b and c). The 1.3 kb hybridizing mRNA corresponds to the size predicted from the *HAL1* sequence. The induction effect was maximal at 1 M NaCl (data not shown), and amounted to ~5-fold in Northern blots (maximal induction at 45 min, Figure 4a) and >15-fold in β-galactosidase assays (activity still increasing after 250 min).

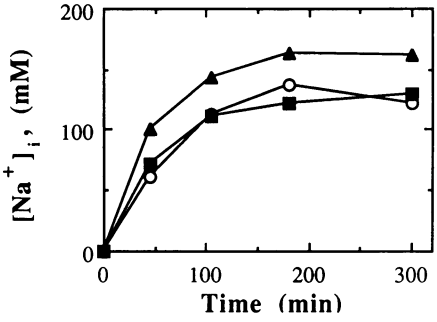

Fig. 7. Sodium uptake in strains with different *HAL1* genotypes. Yeast cells were grown in medium without NaCl and when the absorbance at 660 nm reached 0.3–0.4, 1 M NaCl was added and the intracellular Na^+ concentration determined at the indicated times. Circles: strain RS-16; squares: RS-16 with the *HAL1* gene in multicopy plasmid YEp24; triangles: gene disruption of *HAL1*. Results are the mean of two experiments with values differing by <5%.

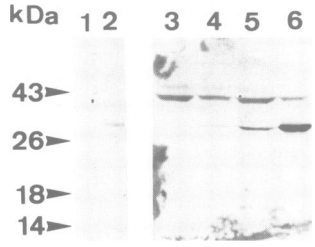

Fig. 8. Western blot of yeast homogenates with antibody against the *HAL1* protein. Each lane contained 30 μg protein. Cells overexpressing *HAL1* in multicopy plasmid were grown in normal medium (lanes 1 and 5) or in medium with 1 M NaCl (lanes 2 and 6). Lanes 3 and 4 correspond to wild type RS-16 yeast grown without (lane 3) and with (lane 4) 1 M NaCl. In lanes 1 and 2 the alkaline phosphatase reaction was developed for 3 min for normal detection sensitivity. In lanes 3–6 the reaction lasted for 4 h for maximum sensitivity but non-specific bands of ~41 kDa appeared. The positions of standards of the indicated size in kDa are shown at the left.

Similar regulation of β-galactosidase activity was observed when the gene was present in single copy on the chromosome (Figure 4c) or in multiple plasmid-borne copies (Figure 4b). The expression of *HAL1* could be induced by high concentrations of either NaCl, KCl or sorbitol (Figure 4b), suggesting that it is caused by a non-specific osmotic effect. No induction was observed by heat-shock (data not shown).

Salt tolerance and monovalent cation levels in HAL1 mutants

The growth phenotype conferred by *HAL1* alterations (overexpression and gene disruption) is specific for high concentrations of NaCl because no differences with wild type are observed in medium with high concentrations of sorbitol or KCl, which are less toxic than equivalent concentrations of NaCl (Figure 5). Therefore the physiological role of this gene must be related to sodium toxicity. Analysis of the growth curves of Figure 5 indicates that overexpression of the *HAL1* gene affects both the growth rate and the lag or adaptation phase of cultures in high NaCl medium. On the other hand the disruption of *HAL1* affects mostly the lag phase in high NaCl medium.

We have measured the intracellular Na^+ and K^+ levels of control cells and of cells with alterations in *HAL1* . As indicated in Figure 6, control cells grown in a medium with

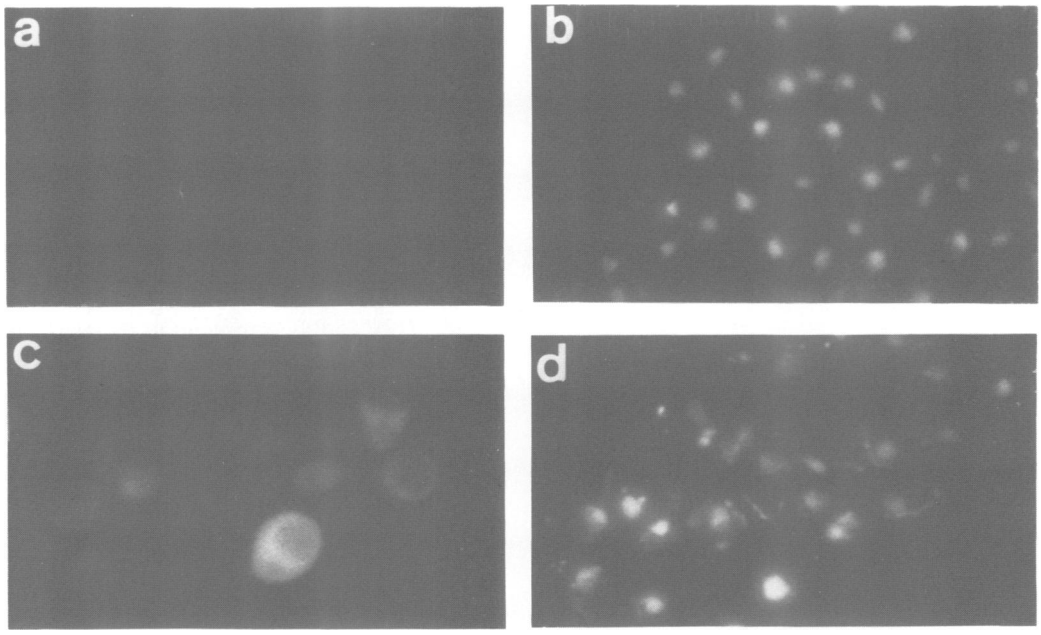

Fig. 9. Immunofluorescence of yeast cells with antibody against the *HAL1* protein. (**a**) and (**c**) Decoration of *HAL1* protein by the fluorescence of second antibody coupled to FITC (fluorescein isothiocyanate). (**b**) and (**d**) Decoration of nuclei by DAPI (4′,6′-diamidino-2-phenylindole) fluorescence. (a) and (b) Cells from strain RS-16 grown without NaCl. (c) and (d) Cells from RS-16 with the *HAL1* gene in multicopy plasmid YEp24 and grown with 1 M NaCl.

1 M NaCl contained 135 mM K^+ and 130 mM Na^+. On the other hand, cells overexpressing *HAL1* contained 270 mM K^+ and 110 mM Na^+ when grown under the same conditions. In a medium without NaCl, overexpression of *HAL1* still causes a higher intracellular K^+ (260 versus 230 mM, data not shown), although the effect was less apparent as would be expected from the much lower expression of the gene under these conditions.

The disruption of *HAL1* does not affect the levels of intracellular K^+ and Na^+ during exponential growth (Figure 6). However, in short term experiments, where the uptake of Na^+ was followed after addition of NaCl, this null mutation determines a slightly higher uptake of Na^+ (Figure 7). This could explain the extended lag phase in NaCl medium caused by this mutation.

Immunological detection of the HAL1 protein

We have raised antibodies against the protein encoded by the *HAL1* gene by expressing, in *Escherichia coli* a fusion protein between β-galactosidase and the complete *HAL1* reading frame. After rabbit immunization and affinity purification, the antibodies recognize a soluble protein of ~32 kDa induced by NaCl and overexpressed when the *HAL1* gene was present in multicopy plasmid (Figure 8). A small fraction (10−15% of total) of the *HAL1* protein was found associated with cellular membranes (data not shown) but the specificity of this association was not investigated. Immunofluorescence studies (Figure 9) indicate that this protein is mostly present in the cytosolic compartment, being excluded from nuclei and vacuoles.

The HAL1 gene and encoded protein are conserved in higher plants

Southern analysis of genomic DNA from corn and *Arabidopsis* indicates that genes homologous to the yeast *HAL1* are present in higher plants (Figure 10a). We have

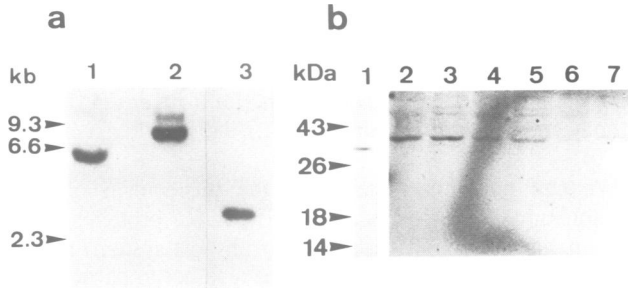

Fig. 10. Detection of *HAL1* gene and protein in higher plants. (**a**) Southern analysis of genomic DNA from yeast (1 μg, lane 3), *Arabidopsis thaliana* (6 μg, lane 2) and *Zea mays* (20 μg, lane 1) digested with *Hin*dIII, blotted to nylon and hybridized with a probe from the yeast *HAL1* gene as described in Materials and methods. The positions of standards of the indicated size kb are shown at the left. (**b**) Western analysis of homogenates from yeast (strain with the *HAL1* gene in multicopy plasmid YEp24 and grown with 1 M NaCl, lane 1), corn leaves (lanes 2, 4 and 6) and corn roots (lanes 3, 5 and 7). Corn seedlings were incubated in normal medium without supplements (lanes 6 and 7), in medium with 10 μM abscisic acid (lanes 2 and 3) and in medium with 50 mM NaCl (lanes 4 and 5). 30 μg protein were applied per lane.

recently cloned the plant homologs of the yeast *HAL1* gene from genomic libraries of *Arabidopsis thaliana* and from cDNA libraries of tomato and corn. Sequencing of a partial cDNA clone from corn showed a region of 47 nucleotides with 66% identity to yeast nucleotides 1453−1499, within the reading frame (Figure 1). This homology explains the observed cross hybridization. Outside these regions overall homology between the corn and yeast genes was low. We speculate that this conserved region must be important for the function of *HAL1*. Further analysis is needed to establish this point and to test if the plant homolog is functionally

equivalent to the yeast *HAL1* gene in terms of conferring salt tolerance. The sequence and characterization of the plant genes will be reported elsewhere (R.Gaxiola and R.Serrano, in preparation).

Western analysis of homogenates from plant tissues decorated with antibodies against the yeast *HAL1* protein shows the presence in leaves and roots of a soluble cross-reacting protein of ~35 kDa induced by both abscisic acid and salt stress (Figure 10b). Less than 5% of the cross-reacting protein was found associated with plant membranes (data not shown).

Discussion

The *HAL1* gene is an important determinant of salt tolerance in yeast because its overexpression improves growth in media with high NaCl concentrations and a null mutation decreases it. *HAL1* is the first eukaryotic gene yet described that improves salt tolerance. It was obtained by a direct functional approach: yeast were transformed by a genomic library in a multicopy plasmid and transformants were screened for growth at high NaCl concentrations. This approach can routinely be applied to microorganisms such as yeast but it would be technically difficult with higher plants. However, it has turned out that *HAL1* is highly conserved between yeast and plants, suggesting that yeast can serve as a model system for the molecular biology of salt tolerance in plants. One important observation is that the *HAL1* homolog of higher plants is induced by salt stress and by the plant hormone abscisic acid, which has been described as mediating the adaptation of plants to osmotic stress (La Rosa *et al.*, 1985; Mundy and Chua, 1988; Singh *et al.*, 1989; Guiltinan *et al.*, 1990).

We can only speculate as to the mechanism of the halotolerance conferred by *HAL1*. The *HAL1* protein seems to be neither a membrane protein (transport system) nor a nuclear transcription factor. It seems also not to participate in osmotic adjustment, the mechanism of halotolerance described for the bacterial *proB74* gene (Csonka, 1989). This is a mutation in the gene encoding γ-glutamyl kinase, the rate-limiting step in proline synthesis, resulting in an enzyme insensitive to feedback inhibition. Bacterial cells expressing this mutant enzyme overproduce proline, one of the major osmoprotectant compounds accumulated by bacteria. As expected from a non-specific osmotic protection, the growth advantage conferred by *proB74* in bacteria is shown in all kinds of osmotica (NaCl, sorbitol, sugars etc.). On the other hand, the growth advantage conferred by overexpression of *HAL1* in yeast is specific for NaCl. Overexpression of *HAL1* does not change the intracellular level of glycerol (R.Gaxiola, unpublished results), the major osmoprotectant produced by yeast (Brown *et al.*, 1986; André *et al.*, 1991).

It seems that *HAL1* is specifically involved in Na^+ tolerance. The most important feature detected in yeast cells overexpressing *HAL1* is the increased accumulation of K^+ in high NaCl medium. Under these growth conditions the intracellular Na^+/K^+ ratio is ~1 in normal cells and ~0.4 in cells overexpressing *HAL1*. This change may explain the improvement of growth because enzyme systems inhibited by salinity are sensitive to the Na^+/K^+ ratio (Greenway and Munns, 1980; Wyn Jones and Pollard, 1983) and Na^+ only becomes toxic in yeast at Na^+/K^+ cellular ratios >0.5 (Camacho *et al.*, 1981). Therefore in cells overexpressing *HAL1* sodium toxicity is counteracted by an increased accumulation of potassium. Parallel evidence from higher plants reinforces the significance of these findings because increased K^+ uptake has been correlated with salt tolerance in tobacco cell cultures (Watad *et al.*, 1990) and in tomato species (Rush and Epstein, 1981).

The molecular basis for K^+ and Na^+ transport in yeast and plants is only beginning to be understood. Uptake of monovalent cations seems to occur by voltage-dependent K^+ channels (Hedrich and Schroeder, 1989; Ramirez *et al.*, 1989) which have marginal transport activity with Na^+. They could correspond to the products of the *TRK1* (Gaber *et al.*, 1988) and *TRK2* (Ko *et al.*, 1990) genes, identified by mutations resulting in defective K^+ uptake, but this point has not been demonstrated by electrophysiology. Intracellular K^+ concentrations in fungi (Boxman *et al.*, 1985; Sanders, 1988; Ramos *et al.*, 1990) and plants (Glass, 1976) are controlled by a highly cooperative feedback inhibition of K^+ uptake by intracellular K^+. Because of negligible efflux, this implies the operation of kinetic controls instead of a thermodynamic equilibrium. This feedback inhibition is very common for nutrient transport systems of fungi and plants (Serrano, 1985; Sanders, 1988) and probably reflects the evolutionary advantage of a homeostatic mechanism independent of the fluctuating energization parameters (Sanders, 1988). The molecular basis of this regulatory mechanism is not known. It seems plausible that the *HAL1* protein somehow (directly or indirectly) interacts with the K^+ transport system and modifies the 'set point' established by the feedback. The increased Na^+ uptake of the *HAL1* disruption could also be explained by this interaction by postulating that *HAL1* also affects the specificity (Na^+/K^+ discrimination) of the system. Further studies are needed to demonstrate at the genetic and biochemical levels the posible interaction between *HAL1* and the K^+ transport system.

The present results may be significant both for the understanding of the mechanisms that determine intracellular ion homeostasis in fungi and plants and for the biotechnological improvement of salinity tolerance. In addition, the identification in yeast of a gene induced by osmotic stress may allow a detailed genetic analysis of the salt tolerance response, from the initial signal transduction to gene transcription.

Materials and methods

Yeast strain and growth medium

Saccharomyces cerevisiae strain RS-16 (Eraso *et al.*, 1987; *MAT***a**, *leu2-3,112, ura3-251,328,372*) and its derivatives described in the present work wre grown in medium containing 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco), 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid] adjusted to pH 6.0 with Tris, 30 μg/ml leucine and 30 μg/ml uracil. Solid media contained 2% agarose. Uracil or leucine were omitted when indicated. Salt tolerance was tested by addition of 1−1.5 M NaCl. For drop tests cells were grown for 2 days in liquid culture without NaCl and then 3 μl of a 1/100 dilution (~10^3 cells) were plated on medium with NaCl. Growth was recorded after 5 days at 30°C.

Isolation and sequencing of the HAL1 gene

Yeast cells were transformed (Ito *et al.*, 1983) with 30 μg DNA from a genomic library in the *URA3* multicopy plasmid YEp24 (Carlson and Botstein, 1982). About 5000 transformants were selected in a medium without uracil. Transformed cells were pooled and ~10^6 cells were plated in the above medium containing 1.5 M NaCl. After 5 days, six colonies were observed above a background of poorly growing cells. One of them (No. 6-22) was selected as the most halotolerant after retesting. Plasmid was

isolated (Ward, 1990), cloned in the recA$^-$ *Escherichia coli* strain JM109 (Yanisch-Perron *et al.*, 1985) and isolated in large scale for yeast transformation and restriction mapping. It contained an insert of ~10 kb. Restriction fragments were subcloned in plasmid YEp24, transformed into yeast and tested for salt tolerance by drop assays. The 2.95 kb *Bgl*II–*Hin*dIII fragment containing the *HAL1* gene (Figure 1a) was subcloned into pBluescript plasmid (Short *et al.*, 1988) and the two strands were sequenced with phage T7 DNA polymerase (Tabor and Richardson, 1987) using walking primers.

Disruption of the HAL1 gene

The 1.95 kb *Bgl*II–*Sac*I fragment of the *HAL1* locus (Figure 1a) was subcloned into plasmid pUC18 (Yanisch-Perron *et al.*, 1985) cut with *Bam*HI and *Sac*I. The resulting plasmid was cut with *Ava*I, blunt-ended with the Klenow fragment of DNA I polymerase and ligated to *Xho*I linkers. A 2.2 kb *Xho*I–*Sal*I fragment containing the *LEU2* gene was obtained from plasmid YEp13 (Broach *et al.*, 1979) and inserted into the artificial *Xho*I site. The resulting plasmid was cut with *Xba*I and *Sac*I before transformation of an autodiploid of strain RS-16. This was constructed by transformation with the *HO* gene in the *URA3* plasmid YCp50 (Herskowitz and Jensen, 1991). After diploidization and growth in rich medium, Ura$^-$ colonies were selected. The transformed diploid was sporulated and tetrads were dissected with a micromanipulator (Sherman and Hicks, 1991).

Southern and Northern analysis

Chromosomal DNA was prepared as described by Winston *et al.* (1983), digested with *Bgl*II and *Hin*dIII, separated by electrophoresis, blotted to nylon and hybridized with the *Ava*I–*Sac*I fragment of the *HAL1* gene (Figure 1a) labeled by the random-priming method (Feinberg and Vogelstein, 1983). High stringency conditions were employed for hybridization of yeast DNA (50% formamide, 0.75 M NaCl, 42°C, overnight) and washes (30 mM NaCl, 60°C, 1 h). For Southern analysis of plant DNA with the yeast probe, formamide was reduced to 25% and NaCl increased to 0.3 M during the washes.

RNA was extracted (Carlson and Botstein, 1982), separated by electrophoresis in formaldehyde gels, blotted to nylon and hybridized to the labeled *Ava*I–*Sac*I fragment as described above for Southern analysis of yeast DNA.

HAL1–LacZ fusions and measurement of β-galactosidase activity

The *Bgl*II–*Pvu*II fragment of the *HAL1* locus, including the promoter of the gene and most of its reading frame (Figure 1a), was fused to the *LacZ* gene in plasmids YEp358R (multicopy) and YIp358R (integrative) cut by *Bam*HI and *Sma*I (Myers *et al.*, 1986). In the case of the integrative plasmid, one transformant that contained a single copy integrated at the *HAL1* locus was selected by Southern analysis. Transformed cells were grown in normal medium up to an absorbance at 660 nm of 0.3–0.4 and then supplemented at time zero with 1 M NaCl. At the desired times, samples of 1.5–3 ml were centrifuged, resuspended in 100 μl buffer (20% glycerol, 1 mM EDTA, 1 mM DTT and 10 mM Tris–HCl pH 7.6) and permeabilized by 3 min vortexing with 6 μl of toluene/ethanol/10% Triton X-100 (1:4:1). The β-galactosidase assay and units were as described by Miller (1972).

Measurement of intracellular Na^+ and K^+ concentrations

Cells were grown in medium supplemented with 1 M NaCl up to an absorbance at 660 nm of 0.3–0.4, harvested by centrifugation, washed three times with 1.5 M sorbitol and 20 mM $MgCl_2$ and extracted with water for 30 min at 95°C. K^+ and Na^+ were determined in aliquots of the centrifuged extract with K^+- and Na^+-selective electrodes (Radiometer F2312K and G502Na, respectively) and calomel reference electrodes. A Radiometer PHM84 in the mV mode was employed. Electrodes were immersed in 10 ml 50 mM MES adjusted to pH 6.0 with Tris, 10 mM $MgCl_2$ and, to stabilize electrode response, either 0.5 mM KCl or NaCl for determination of K^+ and Na^+, respectively. Intracellular water was determined by measuring the equilibrium space of the non-metabolized sugar D-xylose (Vallejo and Serrano, 1989). One unit of absorbance of yeast suspension (determined at 660 nm with a Spectronic 20, Bausch & Lomb) corresponded to an intracellular water of 1.4 (cells grown on normal medium) or 1.7 (cells grown with 1 M NaCl) μl per ml suspension.

Antibody preparation, Western and immunofluorescence analysis

The complete reading frame of the *HAL1* gene was amplified by standard PCR methodology, utilizing as 5′ primer: 5′-CTACCATGGATTTCAAAGATTTAGGATTGCATG-3′, which introduces an *Nco*I site (underlined) at the starting ATG, and as 3′ primer: 5′-TTTCTGCAGTTTTTCAACTATTCTGTGTTGATTG-3′, which introduces a *Pst*I site (underlined) after the stop codon. The 0.9 kb amplified fragment was purified (Tautz and Renz, 1983), digested with *Nco*I and *Pst*I and subcloned into plasmid pRS-951 digested with *Nco*I and *Pst*I. This was a derivative of expression plasmid pUEX3 (Bressan and Stanley, 1987) which contained an *Nco*I linker (GCCATGGC) inserted at the *Sma*I site. Inclusion bodies containing a fusion protein of β-galactosidase and *HAL1* were injected into two rabbits for antibody production. Only one of the two rabbits reacted against the *HAL1* protein.

Specific antibodies against the *HAL1* protein were affinity purified from antisera by ammonium sulfate precipitation and binding to fusion protein immobilized on nitrocellulose (Lillie and Brown, 1987). Before utilization they were preadsorbed to the β-galactosidase produced by plasmid pRS-951 without insert.

Protein extracts from yeast cells were prepared as decribed (Serrano, 1988) and centrifuged for 1 h at 40 000 r.p.m. (Beckman rotor 65) to remove membranes. SDS–PAGE in 12% acrylamide gels (Laemmli, 1970) and Western blotting with the alkaline phosphatase system (Blake *et al.*, 1984) were performed by standard procedures.

Immunoflurescence analysis of yeast cells with affinity purified antibody against *HAL1* protein was as described by Pringle *et al.* (1991).

Preparation of DNA and protein extracts from plants

Corn seedlings (*Zea mays* L., hybrid corn variety 'Lixis', Nungesser GmbH, Darmstadt, Germany) were grown on vermiculite for 2 weeks. *Arabidopsis thaliana* plants (race Columbia) were grown on soil for 3 weeks. DNA was extracted from corn and *Arabidopsis* leaves as described by Doyle and Doyle (1990). Protein extracts were prepared from corn roots and leaves (Serrano, 1988) and centrifuged for 1 h at 40 000 r.p.m. to remove membrane.

Induction of the *HAL1* homolog in corn was tested by incubating the seedlings for 8 h in liquid medium (Murashige and Skoog, 1962) containing 0.5% sucrose and, when indicated, either 50 mM NaCl or 10 μM abscisic acid.

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