

# The Wheat cDNA *LCT1* Generates Hypersensitivity to Sodium in a Salt-Sensitive Yeast Strain<sup>1</sup>

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Salinity affects large areas of agricultural land, and all major crop species are intolerant to high levels of sodium ions. The principal route for Na<sup>+</sup> uptake into plant cells remains to be identified. Non-selective ion channels and high-affinity potassium transporters have emerged as potential pathways for Na<sup>+</sup> entry. A third candidate for Na<sup>+</sup> transport into plant cells is a low-affinity cation transporter represented by the wheat protein *LCT1*, which is known to be permeable for a wide range of cations when expressed in yeast (*Saccharomyces cerevisiae*). To investigate the role of *LCT1* in salt tolerance we have used the yeast strain G19, which is disrupted in the genes encoding Na<sup>+</sup> export pumps and as a result displays salt sensitivity comparable with wheat. After transformation with *LCT1*, G19 cells became hypersensitive to NaCl. We show that *LCT1* expression results in a strong decrease of intracellular K<sup>+</sup>/Na<sup>+</sup> ratio in G19 cells due to the combined effect of enhanced Na<sup>+</sup> accumulation and loss of intracellular K<sup>+</sup>. Na<sup>+</sup> uptake through *LCT1* was inhibited by K<sup>+</sup> and Ca<sup>2+</sup> at high concentrations and the addition of these ions rescued growth of *LCT1*-transformed G19 on saline medium. *LCT1* was also shown to mediate the uptake of Li<sup>+</sup> and Cs<sup>+</sup>. Expression of two mutant *LCT1* cDNAs with N-terminal truncations resulted in decreased Ca<sup>2+</sup> uptake and increased Na<sup>+</sup> tolerance compared with expression of the full-length *LCT1*. Our findings strongly suggest that *LCT1* represents a molecular link between Ca<sup>2+</sup> and Na<sup>+</sup> uptake into plant cells.

Salinity affects large areas of agricultural land as a result of intensive irrigation. Although a diverse range of plant species thrives on saline soils, all major crop species are intolerant to high levels of salt. Sodium ions in particular are toxic to living cells if accumulated in the cytoplasm. Considerable progress in understanding plant salt tolerance at a cellular level has been made in the areas of signal transduction (Liu and Zhu, 1998; Halfter et al., 2000; Liu et al., 2000), H<sup>+</sup>-coupled Na<sup>+</sup> export (Apse et al., 1999; Shi et al., 2000), and compatible solutes (Rentsch et al., 1996; Nuccio et al., 1998). Although there remains uncertainty regarding the principle pathway by which Na<sup>+</sup> enters root cells, electrophysiological and molecular studies have started to reveal possible routes for Na<sup>+</sup> uptake in plants.

Electrophysiological investigations have uncovered the presence of non-selective cation channels in the plasma membrane of various cell types and plant species (Amtmann and Sanders, 1999; Tyerman and Skerrett, 1999). These channels are freely Na<sup>+</sup> permeable; although, to date, their molecular identities remain unknown. High-affinity transporters might also play a role in Na<sup>+</sup> uptake. At a molecular level, *HKT1* was originally identified, in a yeast (*Saccharomyces*

*cerevisiae*) functional complementation screen of a wheat cDNA library, as a cDNA encoding a high-affinity K<sup>+</sup> transporter (Schachtman and Schroeder, 1994). It was subsequently shown that *HKT1* is a K<sup>+</sup>-Na<sup>+</sup> symporter that can mediate low-affinity Na<sup>+</sup> transport at high external Na<sup>+</sup> (Rubio et al., 1995; Gassmann et al., 1996), and a *HKT1* homolog from *Arabidopsis* has recently been reported as functioning solely as a Na<sup>+</sup> transporter (Uozumi et al., 2000). Nevertheless, the membrane location and function of *HKT1* in planta have yet to be established.

A further possible pathway for Na<sup>+</sup> uptake might be encoded by the *LCT1* gene. *LCT1* was cloned from a wheat cDNA library by functional complementation of a yeast strain defective in high-affinity K<sup>+</sup> uptake (Anderson et al., 1992; Schachtman et al., 1997). In contrast to *HKT1*, *LCT1* conferred K<sup>+</sup> uptake capacity only at K<sup>+</sup> concentrations of 1 mM K<sup>+</sup> and above, indicating relatively low affinity for K<sup>+</sup>. *LCT1* has no homology with any protein described to date, although the predicted secondary structure, with eight to 10 membrane-spanning domains, clearly indicates a membrane location. Southern analysis shows that the *LCT4* gene is present in the wheat genome and *LCT1*-mRNA was detected by reverse transcriptase-PCR in wheat leaf and root tissue (Schachtman et al., 1997). In accordance with the notion that *LCT1* encodes a plasma membrane cation transporter, *LCT1*-transformed yeast cells exhibit increased unidirectional influx of a wide range of cations including not only K<sup>+</sup>, Na<sup>+</sup>, and Ca<sup>2+</sup>, but also

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various heavy metals (Schachtman et al., 1997; Clemens et al., 1998).

The observation that LCT1 mediates Na<sup>+</sup> influx led to the suggestion that LCT1 plays a role in salt sensitivity of wheat (Schachtman et al., 1997). However, to support a role of LCT1 in salinity stress, four fundamental questions remain to be answered. Is LCT1-mediated Na<sup>+</sup> uptake at high external Na<sup>+</sup> concentrations (>10 mM) large enough to cause salt toxicity? Does LCT1-mediated transport of other cations, i.e. K<sup>+</sup>, counter-balance or aggravate physiological consequences of Na<sup>+</sup> uptake? What is the effect of external Ca<sup>2+</sup> on LCT1-mediated Na<sup>+</sup> uptake? In which membrane and cell types is LCT1 located in planta?

Attempts to identify the subcellular localization of LCT1 using antibodies and GFP-fusion constructs unfortunately have so far been unsuccessful (D.P. Schachtman, unpublished data). In a similar manner, the first three questions cannot be studied in planta since transformation and gene inactivation are still problematic in wheat. We, therefore, continued to use yeast as a heterologous expression system for LCT1. Yeast has proven to be a useful model system with respect to salt toxicity targets and signaling pathways involved in salt tolerance (Serrano, 1996; Serrano et al., 1999). Since wild-type yeast is very salt tolerant due to effective Na<sup>+</sup> export by the plasma membrane-located Na<sup>+</sup> pumps ENA1 through 4 (Haro et al., 1991, 1993), our experiments were carried out with the yeast strain G19, which is disrupted in the *ENA1-4* genes (Quintero et al., 1996). G19 cells are similar to plant cells in that they possess the Na<sup>+</sup>/H<sup>+</sup> antiport systems, NHA1 and NHX1, residing in the plasma membrane and the tonoplast, respectively (Nass et al., 1997; Bañuelos et al., 1998), as well as efficient K<sup>+</sup> uptake systems, TRK1 and 2 (Gaber et al., 1988; Ko and Gaber 1991). As a result, the salt sensitivity of G19 is comparable with that of wheat. We show here that expression of LCT1 in G19 cells leads to salt hypersensitivity due to increased accumulation of intracellular Na<sup>+</sup> and net loss of intracellular K<sup>+</sup>. Low concentrations of external Ca<sup>2+</sup> did not alleviate LCT1-induced salt stress, but Na<sup>+</sup> uptake was inhibited by high concentrations of Ca<sup>2+</sup> as well as K<sup>+</sup> and Cs<sup>+</sup>.

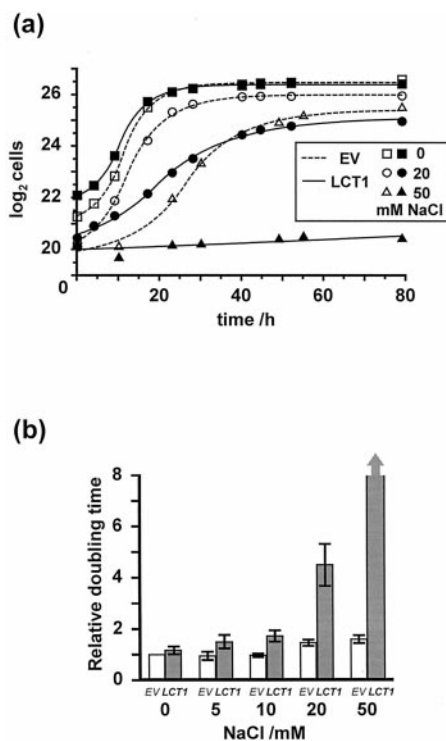
The lack of general homology between LCT1 and known proteins precludes predictions regarding domains involved in transport function or regulation. The N terminus of LCT1 hosts the only domain that has homology to known motifs: a sequence rich in Pro, Glu, Ser, and Thr (PEST) sequence was recognized with high score between amino acids 123 and 140 (Schachtman et al., 1997). PEST sequences have been correlated with rapid degradation of proteins and it has been suggested that they represent recognition sites for specific proteases (Rogers et al., 1986; Rechsteiner and Rogers, 1996). It is tempting to hypothesize that N-terminal protein cleavage at the

PEST sequence is involved in regulation of LCT1 (Schachtman et al., 1997). To test this hypothesis we have constructed N-terminal deletion mutants of LCT1 that mimic protein cleavage at the PEST site. We found that yeast strains transformed with N-terminal-deleted cDNAs exhibited decreased uptake capacity for Na<sup>+</sup> and Ca<sup>2+</sup>. The results are discussed with respect to future engineering of salt tolerance in wheat.

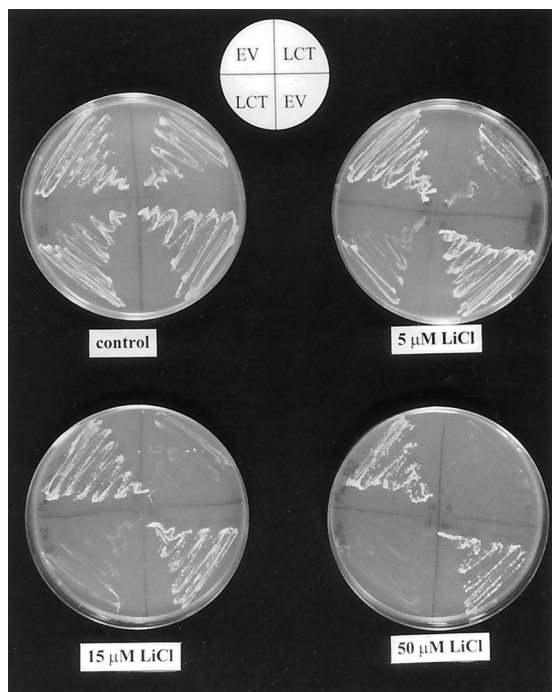
## RESULTS

### LCT1-Transformed Yeast Cells Are Hypersensitive to Na<sup>+</sup> and Li<sup>+</sup>

The genes encoding plasma membrane Na<sup>+</sup> export pumps have been deleted in the yeast strain G19 and, therefore, it is more sensitive to external Na<sup>+</sup> concentration than wild-type yeast (Bañuelos et al., 1998). Any gene product facilitating Na<sup>+</sup> uptake into G19 cells should further increase salt sensitivity. LCT1-transformed cells were significantly more sensitive to



**Figure 1.** Salt sensitivity of G19 yeast transformed with LCT1. a, Typical growth of LCT1-transformed G19 cultures (black symbols and lines) and G19 cultures transformed with the empty pYES2 plasmid (EV, white symbols and dashed lines) in liquid AP medium containing 1 mM KCl, 0.2 mM CaCl<sub>2</sub>, and various concentrations of NaCl. b, Mean doubling times of G19 cultures transformed with EV (white bars) and LCT1 (black bars) in various concentrations of NaCl. Data are standardized with respect to the doubling times of EV cultures in Na<sup>+</sup>-free medium in the same experiment. Values for LCT1-transformed cultures in 50 mM NaCl were >100. Means of nine, three, four, five, and three experiments (for 0, 5, 10, 20, and 50 mM NaCl, respectively) ± SE.



**Figure 2.**  $\text{Li}^+$  hypersensitivity of *LCT1*-transformed cells. Growth of G19 cells transformed with *EV* and *LCT1* on plates containing AP medium with different concentrations of  $\text{LiCl}$ . Note that growth of *LCT1*-transformed cells is reduced at  $\text{Li}^+$  concentrations as low as  $5 \mu\text{M}$ .

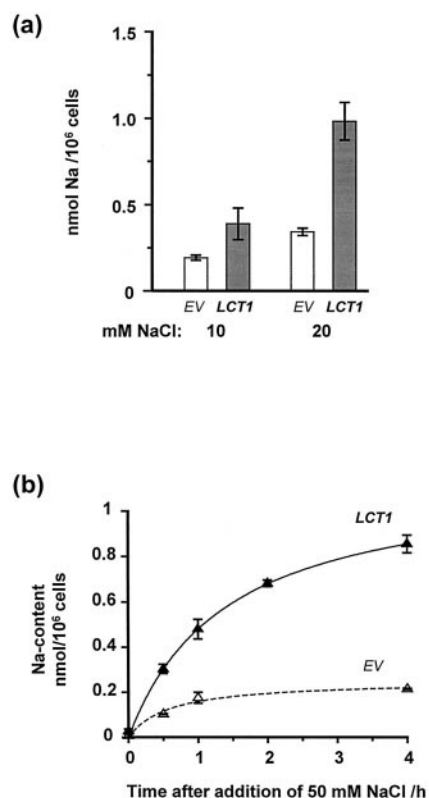
elevated external concentrations of  $\text{Na}^+$  than cells transformed with the empty plasmid (*EV*). Figure 1 shows typical growth curves and mean doubling times during exponential growth for *EV*- and *LCT1*-transformed cells in liquid Arg phosphate (AP) medium containing 1 mM  $\text{KCl}$ , 0.2 mM  $\text{CaCl}_2$ , and various concentrations of  $\text{NaCl}$ . Growth of *LCT1*-transformed cultures was similar to growth of control cultures when no  $\text{NaCl}$  was present, but strongly reduced in 20 mM  $\text{NaCl}$ . No growth of *LCT1*-transformed cultures occurred in 50 mM  $\text{NaCl}$ , whereas control cultures still grew well in these conditions.

Lithium is toxic to all living cells at very low cytoplasmic concentrations binding to  $\text{Na}^+$ -sensitive enzymes with high affinity (Serrano, 1996). In wild-type yeast,  $\text{Li}^+$  is removed from the cytoplasm by the ENA pumps, and G19 cells are more sensitive to  $\text{Li}^+$  than wild-type yeast (Haro et al., 1991). Figure 2 shows that transformation with *LCT1* further increased  $\text{Li}^+$  sensitivity of the yeast mutant strain. External  $\text{LiCl}$  inhibited growth of *LCT1*-transformed yeast at concentrations as low as  $5 \mu\text{M}$ , whereas *EV*-transformed yeast grew well with  $50 \mu\text{M}$   $\text{LiCl}$  in the growth medium.

#### ***LCT1*-Transformed Yeast Accumulates Significantly More Sodium Than *EV*-Transformed Cells**

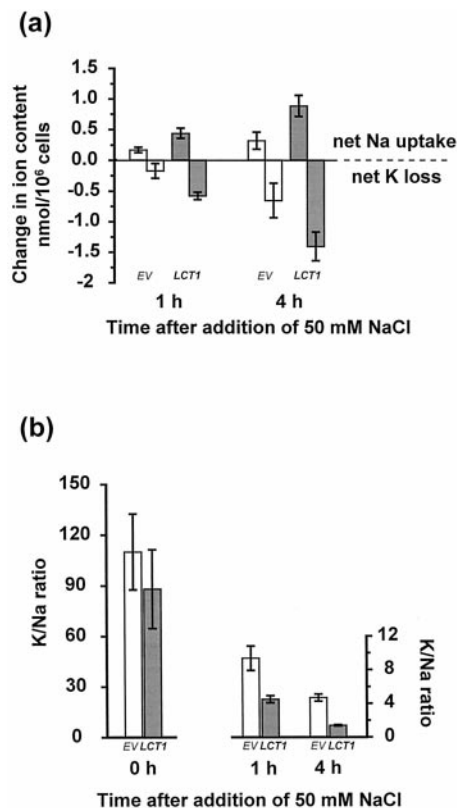
Reduced growth of *LCT1*-transformed cells in saline medium correlated with increased  $\text{Na}^+$  contents

of the cells. Figure 3a demonstrates that after growth of the cultures in liquid AP medium with 10 mM or 20 mM  $\text{NaCl}$ , *LCT1*-transformed cells contained two or three times more  $\text{Na}^+$  than the controls. Figure 3b shows the time course of  $\text{Na}^+$  accumulation in *LCT1*-transformed cells and controls over the first 4 h after the addition of 50 mM  $\text{NaCl}$ . For this experiment cultures were grown to the early stationary phase in  $\text{Na}^+$ -free AP medium before 50 mM  $\text{NaCl}$  was added. Net  $\text{Na}^+$  uptake rates during the first 30 min and  $\text{Na}^+$  accumulation after 4 h were increased by factors of three and four in *LCT1*-transformed cells compared with control cells. Enhanced  $\text{Na}^+$  uptake by *LCT1*-transformed cells was not due to cell death, as viability of yeast during salt exposure was confirmed by methylene-blue staining.



**Figure 3.**  $\text{Na}^+$  content of *LCT1*-transformed cells after growth in  $\text{NaCl}$ -containing media. a, Intracellular  $\text{Na}^+$  content of G19 cultures transformed with *EV* (white bars) or *LCT1* (black bars) after growth in liquid AP medium containing 10 or 20 mM  $\text{NaCl}$ . For this experiment, precultures of G19 cells grown in  $\text{Na}^+$ -free medium were diluted to an  $\text{OD}_{600}$  of 0.05 and were exposed to different  $\text{NaCl}$  concentrations. Two days later cells were harvested in the stationary phase. Means of three experiments  $\pm$  SEM are shown. b, Time course of  $\text{Na}^+$  accumulation in *EV*- (dashed curve) and *LCT1*- (black curve) transformed cells after addition of 50 mM  $\text{NaCl}$ . Prior to the experiment, the cultures had grown to the early stationary phase in  $\text{Na}^+$ -free liquid AP medium. Means of three experiments  $\pm$  SE are shown.

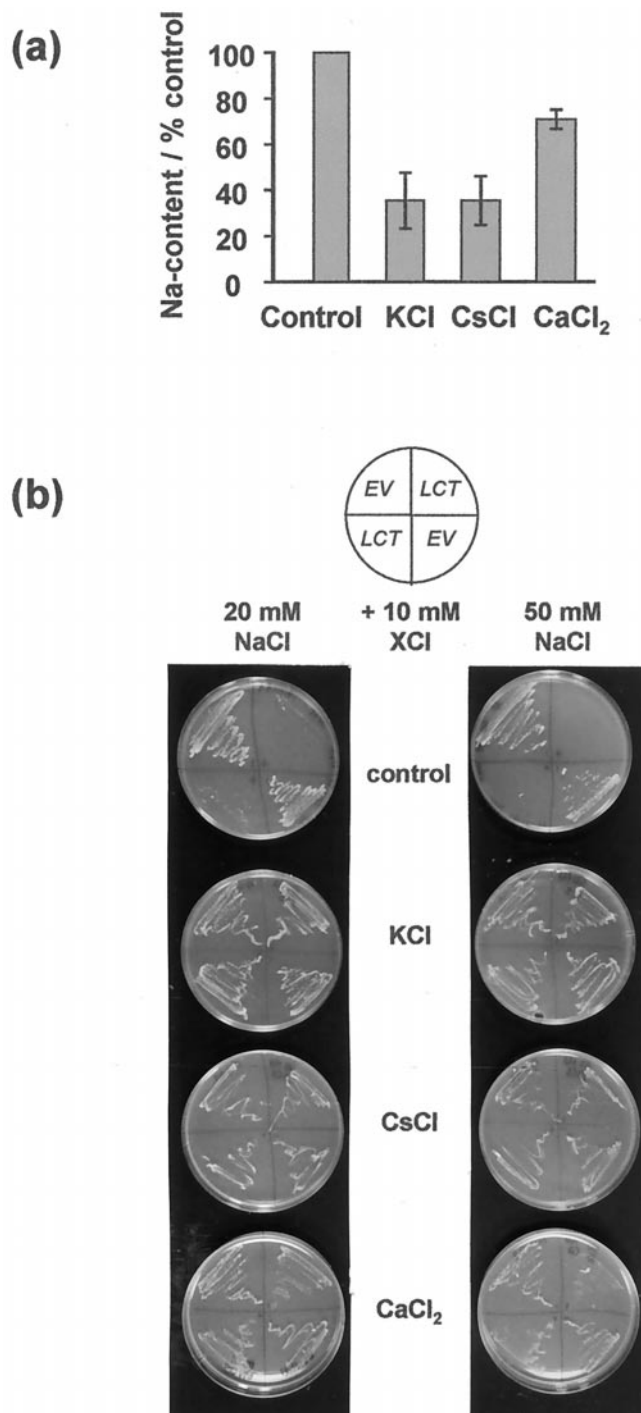




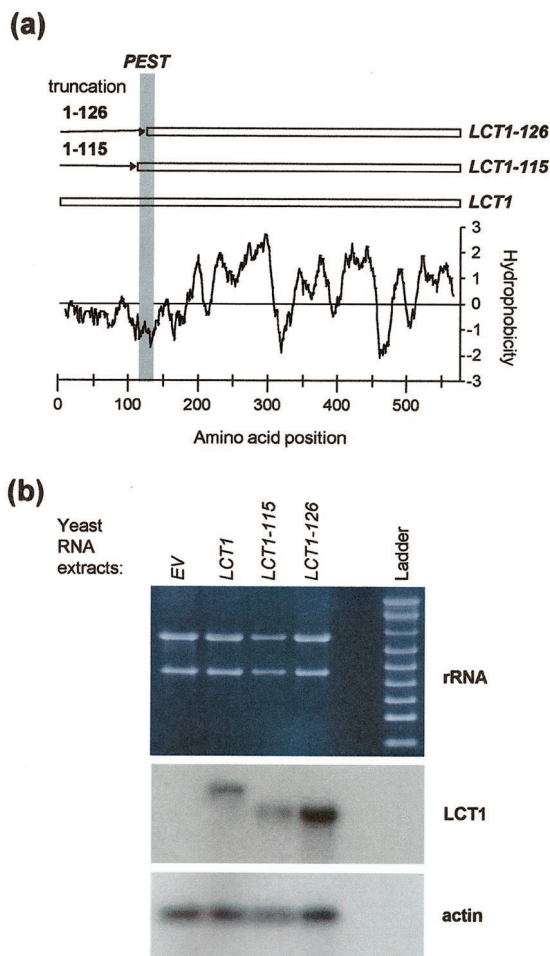
**Figure 4.** Changes in Na<sup>+</sup> and K<sup>+</sup> content of *LCT1*-transformed cells after addition of 50 mM NaCl. a, Net Na<sup>+</sup> uptake and K<sup>+</sup> loss of G19 cells transformed with *EV* (white bars) or *LCT1* (black bars) at 1 and 4 h after addition of 50 mM NaCl. Values were calculated by subtracting Na<sup>+</sup> and K<sup>+</sup> contents at time 0 from Na<sup>+</sup> and K<sup>+</sup> contents at the given time. Means of five experiments ± SEM are shown. b, Intracellular K<sup>+</sup>/Na<sup>+</sup> ratios in G19 cells transformed with *EV* (white bars) and *LCT1* (black bars) before and after addition of 50 mM NaCl. Means of five experiments ± SE are shown.

***LCT1*-Transformed Yeast Cells Lose More Potassium upon Salt Treatment Than *EV*-Transformed Cells**

It is the intracellular K<sup>+</sup>/Na<sup>+</sup> ratio rather than simply the absolute intracellular concentration of Na<sup>+</sup> that determines salt tolerance in yeast and plant cells. To investigate the possible role of K<sup>+</sup> in enhanced salt sensitivity of *LCT1*-transformed cells we measured cellular K<sup>+</sup> content in normal growth conditions (1 mM K<sup>+</sup>) and during salinity stress (1 mM K<sup>+</sup> + 50 mM Na<sup>+</sup>). When grown without NaCl in the medium, there was no significant difference in the K<sup>+</sup> contents of *EV*- and *LCT1*-transformed cells. Addition of 50 mM Na<sup>+</sup> to the external medium caused not only a rise of intracellular Na<sup>+</sup>, but also a net loss of intracellular K<sup>+</sup> (Fig. 4a). This decrease in intracellular K<sup>+</sup> was significantly larger in *LCT1*-transformed cells than in *EV*-transformed cells. Net uptake of Na<sup>+</sup> and net loss of K<sup>+</sup> upon salt treatment resulted in dramatically smaller intracellular K<sup>+</sup>/Na<sup>+</sup> ratios in *LCT1*-transformed cells than in control cells (Fig. 4b). Addition of 50 mM KCl instead of NaCl did not change intracellular K<sup>+</sup> contents (data not



**Figure 5.** Effect of K<sup>+</sup>, Cs<sup>+</sup>, and Ca<sup>2+</sup> on Na<sup>+</sup> contents and Na<sup>+</sup> sensitivity of *LCT1*-transformed cells. a, Na<sup>+</sup> contents of *LCT1*-transformed G19 cells 7 h after addition of 50 mM NaCl alone (control) or together with 50 mM KCl, CsCl or CaCl<sub>2</sub>. Values are standardized with respect to Na<sup>+</sup> contents in 50 mM NaCl with no other cations added. Means of three experiments ± SE are shown. b, Addition of 10 mM KCl, CsCl, or CaCl<sub>2</sub> restores growth of *LCT1*-transformed cells on agar plates containing AP medium with 20 or 50 mM NaCl added.



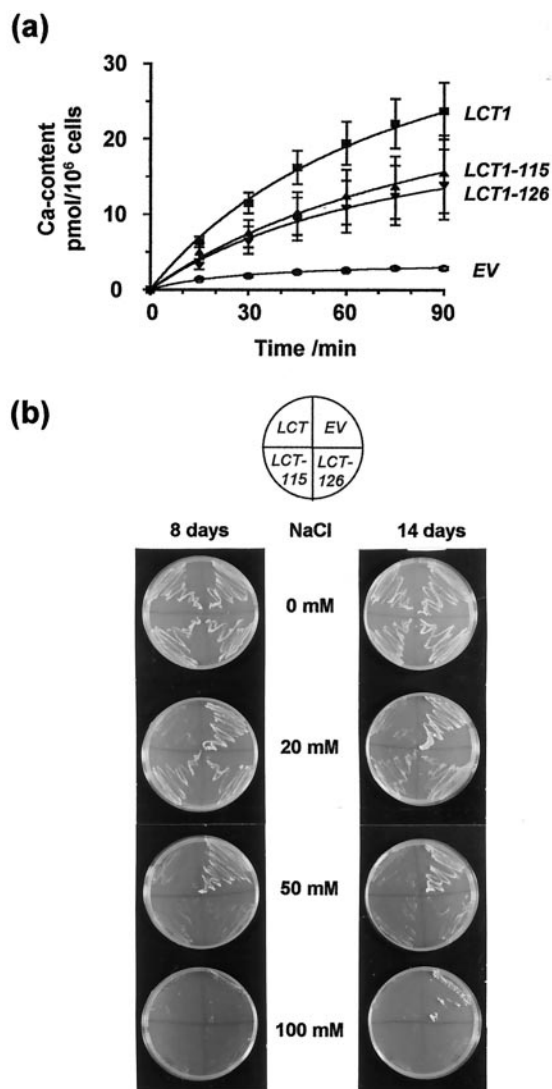
**Figure 6.** Construction of N terminally truncated *LCT1* genes and expression in yeast. **a**, Positions of PEST sequence and N-terminal truncations on the hydropathy plot of *LCT1*. **b**, Agarose-formamide gel and northern blots of total RNA extracted from G19 cells transformed with *EV* (first lane), full length *LCT1* (second lane), and N-terminal truncations *LCT1-115* (third lane), and *LCT1-126* (fourth lane). Top, Bands for ribosomal RNA on agarose-formaldehyde gel. Middle, Hybridization of blot with *LCT1* probe. Bottom, Hybridization with actin probe. Note that differences in band intensities for *LCT1* constructs are mainly due to different loading.

shown). Addition of 50 mM KCl together with 50 mM NaCl decreased  $\text{Na}^+$  uptake (see below) and  $\text{K}^+$  loss, but  $\text{K}^+/\text{Na}^+$  ratios were still lower in *LCT1*-transformants than in *EV*-transformed cells (data not shown).

#### Effect of Other Cations on *LCT1*-Induced $\text{Na}^+$ Uptake and Salt Sensitivity

To further investigate the interaction between  $\text{Na}^+$  and other cations in *LCT1*-transformed cells, intracellular  $\text{Na}^+$  contents in yeast in liquid culture were measured after incubation in 50 mM NaCl, added alone or with 50 mM KCl, CsCl, or  $\text{CaCl}_2$ . Figure 5a shows that *LCT1*-induced  $\text{Na}^+$  accumulation was strongly inhibited by KCl and CsCl, which reduced

$\text{Na}^+$  contents by more than 60%, whereas  $\text{Ca}^{2+}$  decreased  $\text{Na}^+$  accumulation only by about 25%. The same tendency was found for  $\text{Na}^+$  accumulation through endogenous transporters in *EV*-transformed cells, but *ses* were large due to the small absolute  $\text{Na}^+$  contents in these cells (data not shown). In accordance with the measured decrease of *LCT1*-induced  $\text{Na}^+$  accumulation by liquid cultures, addition of KCl, CsCl, or  $\text{CaCl}_2$  restored growth of *LCT1*-transformed yeast on agar plates with elevated NaCl



**Figure 7.**  $\text{Ca}^{2+}$  uptake and  $\text{Na}^+$  sensitivity of yeast cells transformed with wild-type *LCT1* or N-terminally truncated *LCT1* genes. **a**,  $^{45}\text{Ca}^{2+}$  uptake of JKmc cells transformed with *EV*, wild-type *LCT1*, or N-terminally truncated *LCT1* genes (*LCT1-115*, *LCT1-126*, compare with Fig. 6) in liquid uracil-free synthetic medium (SD) medium containing 100  $\mu\text{M}$   $\text{CaCl}_2$ . Means of nine experiments  $\pm$  SE are shown. **b**, Growth of G19 cells transformed with *EV*, wild-type *LCT1*, or N-terminally truncated *LCT1* (*LCT1-115* and *LCT1-126*) on plates containing AP medium with various concentrations of NaCl. Note that the truncated *LCT1* constructs resemble *EV* on 20 mM NaCl and *LCT1* on higher NaCl. Growth after 8 and 14 d is shown to demonstrate sustainability of phenotypes.

concentrations (Fig. 5b). With 10 mM CsCl or CaCl<sub>2</sub>, *LCT1*-transformed yeast could grow at NaCl concentrations up to 50 mM. 10 mM KCl allowed growth of *LCT1*-transformed cells even at 100 mM NaCl (data not shown). Addition of KCl and CaCl<sub>2</sub> also increased the Na<sup>+</sup> tolerance of *EV*-transformed cells.

Growth of *LCT1* transformants on plates containing 50 μM LiCl was restored by addition of KCl and CaCl<sub>2</sub>, but not by addition of NaCl (all 10 mM, data not shown). Ca<sup>2+</sup> concentrations lower than 10 mM did not rescue growth of *LCT1*-transformed cultures in media containing 50 μM Li<sup>+</sup> or 50 mM Na<sup>+</sup> (data not shown).

#### Deletion of the N Terminus Alleviates *LCT1*-Induced Phenotypes

The predicted *LCT1* protein sequence has no overall homology to any other known protein and, therefore, predictions about functionally important regions are difficult. Only the hydrophilic N terminus hosts a known motif, a so-called PEST sequence, which may represent a recognition site for proteases (Rogers et al., 1986; Schachtman et al., 1997). To mimic N-terminal protein cleavage at this site we constructed mutant clones of *LCT1* in which the N terminus was cleaved at two positions in the PEST sequence (Fig. 6a). Transcription of the constructs was verified by northern analysis of transformed G19 cells (Fig. 6b). Taking into account the amounts of RNA blotted onto nitrocellulose membrane (indicated by total rRNA and actin mRNA), transcription of the two *LCT1* truncations appeared to be similar to the transcription of the full-length *LCT1*, although expression of *LCT1*-126 may be slightly higher. For analysis of the mutants we first used a yeast strain, here called JKmc, which is deleted in *MID1* and *CCH1*, two yeast genes involved in Ca<sup>2+</sup> influx and Ca<sup>2+</sup>-dependent mating processes (Iida et al., 1994; Fischer et al., 1997). Ca<sup>2+</sup> uptake, which is usually very small in JKmc cells, is significantly enhanced by *LCT1*, as determined in flux experiments with <sup>45</sup>Ca<sup>2+</sup> (Fig. 7a, compare with Clemens et al., 1998). Transformation of JKmc with N-terminally truncated *LCT1* showed decreased rates of <sup>45</sup>Ca<sup>2+</sup> uptake compared with the full-length *LCT1* transformants, but <sup>45</sup>Ca<sup>2+</sup> uptake was still larger than in control cells (Fig. 7a). We were interested to find out whether this phenotype of N-terminally truncated *LCT1* clones was maintained in G19 cells. G19 cells transformed with *LCT1*-115 and *LCT1*-126 were significantly less sensitive to salt than cells transformed with full-length *LCT1*. At high external NaCl concentrations (50 mM), neither of yeast-expressing the truncated *LCT1* constructs grew, indicating that the mutant cDNAs are translated into functional proteins. However, at intermediate NaCl concentrations (20 mM), cells transformed with *LCT1*-115 and *LCT1*-126 grew consistently better than yeast containing

full-length *LCT1* (Fig. 7b). Our results indicate that Ca<sup>2+</sup> and Na<sup>+</sup> uptake are reduced in the mutant *LCT1* transformants compared with full-length *LCT1* transformants.

## DISCUSSION

### Uptake of Na<sup>+</sup>, Li<sup>+</sup>, and Cs<sup>+</sup> by *LCT1*

We have shown here that heterologous expression of *LCT1* induces salt hypersensitivity in a salt-sensitive yeast mutant strain. Poor growth of the *LCT1* transformants at NaCl concentrations above 20 mM was clearly correlated with increased levels of intracellular Na<sup>+</sup> and, therefore, in yeast, *LCT1* is localized in the plasma membrane where it directly mediates or facilitates Na<sup>+</sup> uptake into the cell. Stabilization of intracellular Na<sup>+</sup> levels can be explained with Na<sup>+</sup> export through Na<sup>+</sup>/H<sup>+</sup> antiporters in the plasma membrane and tonoplast (Nass et al., 1997; Bañuelos et al., 1998). Medium acidification during culture growth probably further increases Na<sup>+</sup> extrusion by Nha1.

*LCT1* also facilitates the uptake of Li<sup>+</sup> and Cs<sup>+</sup> across the plasma membrane of transformed yeast cells. Li<sup>+</sup> is known to be very toxic when present in the cytoplasm (Serrano, 1996). The toxic effect of external Li<sup>+</sup> on *LCT1*-transformed cells was still visible at medium concentrations as low as 5 μM even though Ca<sup>2+</sup> and K<sup>+</sup> were present at 40 and 200 times higher concentrations than Li<sup>+</sup>, respectively (see Fig. 2). The fact that Na<sup>+</sup> did not rescue growth of *LCT1*-transformed yeast on Li<sup>+</sup> confirms that Li<sup>+</sup> acts as a "hypersodium" ion, exacerbating its toxic effect at the same sites as Na<sup>+</sup>. Thus, even if Na<sup>+</sup> competes for Li<sup>+</sup> in uptake, the resulting additive toxic effect of both ions remains the same.

Inhibition of Na<sup>+</sup> uptake by equimolar concentrations of Cs<sup>+</sup> indicates permeability of *LCT1* for Cs<sup>+</sup>. Contamination of soils over large areas of Europe with caesium radioisotopes after the accident at the nuclear power plant at Chernobyl in 1986 has been a major concern. Radioactive Cs<sup>+</sup> enters the food chain via accumulation in plants. A recent review on Cs<sup>+</sup> uptake by plants postulates that voltage-independent non-selective ion channels, as well as high-affinity K<sup>+</sup> transporters, are the main pathways for Cs<sup>+</sup> entry into plant roots (White and Broadley, 2000). It appears that in addition to the aforementioned transporters, *LCT1* has to be considered with respect to low-affinity uptake of Cs<sup>+</sup> in wheat. Cs<sup>+</sup> has been reported to inhibit plant growth (White and Broadley, 2000). It is interesting that in our experiments, high concentrations of Cs<sup>+</sup> were not toxic for yeast (see Fig. 5b). A possible explanation is that Cs<sup>+</sup> toxicity in plants is primarily indirect via blockage of K<sup>+</sup> uptake through Cs<sup>+</sup>-sensitive, K<sup>+</sup> inward-rectifying channels (Ichida and Schroeder, 1996). This channel type is not present in yeast (Rodríguez-Navarro, 2000).



### Role of K<sup>+</sup> in LCT1-Induced Salt Stress

Beneficial effects of K<sup>+</sup> uptake through LCT1 on salt-stressed yeast only occurred when the K<sup>+</sup>/Na<sup>+</sup> ratio in the external medium was greater than 0.1. Under these conditions, LCT1-mediated K<sup>+</sup> uptake had a dual effect, inhibition of Na<sup>+</sup> transport through LCT1 and maintenance of high intracellular K<sup>+</sup> levels. As a result, K<sup>+</sup> was more effective in rescuing salt-hypersensitive cells than other cations.

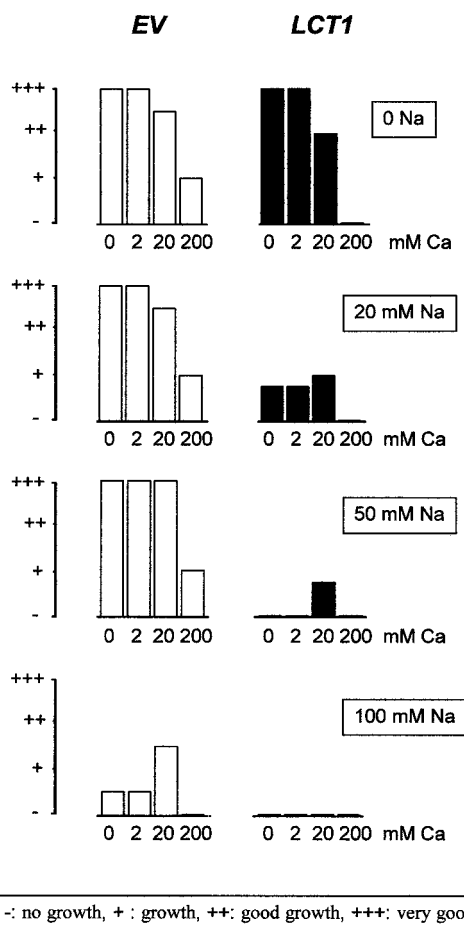
At low external K<sup>+</sup>/Na<sup>+</sup> ratios, the physiological effect of LCT1-induced Na<sup>+</sup> accumulation was aggravated by decreased levels of intracellular K<sup>+</sup>, indicating exchange of intracellular K<sup>+</sup> by Na<sup>+</sup>. K<sup>+</sup> efflux may occur through LCT1 itself or via K<sup>+</sup>/H<sup>+</sup> exchange by Nha1 (Bañuelos et al., 1998) or, if the membrane is depolarized, through the K<sup>+</sup> outward-rectifying channel Tok1 (Ketchum et al., 1995; Bertl et al., 1998). The fact that K<sup>+</sup> loss was always slightly larger than Na<sup>+</sup> accumulation points to a sustained membrane depolarization upon NaCl exposure in accordance with a very positive electrochemical equilibrium potential for Na<sup>+</sup>.

An intracellular K<sup>+</sup>/Na<sup>+</sup> ratio of >0.5 has been shown to be required for growth of yeast (Camacho et al., 1981). A similar, if not higher, cytoplasmic K<sup>+</sup>/Na<sup>+</sup> ratio is probably necessary for the functioning of metabolically active plant cells. In fact, K<sup>+</sup> deficiency and low cytoplasmic K<sup>+</sup>/Na<sup>+</sup> ratio are considered to be major components of plant salt stress (Maathuis and Amtmann, 1999; Schachtman and Liu, 1999). LCT1-induced K<sup>+</sup> loss and its contribution to a low intracellular K<sup>+</sup>/Na<sup>+</sup> ratio in yeast exemplifies the inverse effects that one non-selective low-affinity transporter can exert on Na<sup>+</sup> and K<sup>+</sup> contents of a cell when external K<sup>+</sup>/Na<sup>+</sup> ratios are low. This finding is highly relevant for plant cells, which possess a similar suite of K<sup>+</sup> efflux pathways as yeast (Rodríguez-Navarro, 2000).

### Na<sup>+</sup> and Ca<sup>2+</sup> Transport through LCT1

High affinity block ( $K_i = 0.3$  mM) of <sup>22</sup>Na<sup>+</sup> uptake by external Ca<sup>2+</sup> has been reported for wheat roots (Davenport et al., 1997; see below). Rb<sup>+</sup> and Cd<sup>2+</sup> uptake through LCT1 has also been found to be blocked by very low external Ca<sup>2+</sup> concentrations (Schachtman et al., 1997; Clemens et al., 1998). However, since Rb<sup>+</sup> and Cd<sup>2+</sup> themselves were used in low concentrations, these experiments could not distinguish between high-affinity block and competitive inhibition. In our study, LCT1-induced salt hypersensitivity and Na<sup>+</sup> uptake at high external Na<sup>+</sup> concentrations (20–50 mM) were not affected by external Ca<sup>2+</sup> concentrations below 10 mM, indicating that Ca<sup>2+</sup> inhibition of cation transport through LCT1 does not represent a high-affinity block, but is most probably due to competition.

High concentrations of external Ca<sup>2+</sup> are toxic for yeast and it has been shown that LCT1 induces hy-



**Figure 8.** Combined effects of Na<sup>+</sup> toxicity, Ca<sup>2+</sup> rescue, and Ca<sup>2+</sup> toxicity on growth of EV- and LCT1-transformed G19 cells. Growth of G19 cells transformed with EV and LCT1 on plates containing AP medium with different concentrations of NaCl and or CaCl<sub>2</sub>. Bars are qualitative measures for colony density on the plates (see box for description). Each transformant was streaked twice in the same conditions (compare with Fig. 5). Growth was identical for both replica in all conditions. Note hypersensitivity of LCT1 transformants to Na<sup>+</sup> and Ca<sup>2+</sup>, lack of effect of 2 mM Ca<sup>2+</sup> on Na<sup>+</sup> sensitivity, and rescue of LCT1 and control cultures on high Na<sup>+</sup> by 20 mM Ca<sup>2+</sup>.

persensitivity toward Ca<sup>2+</sup> in the yeast strain INVSc1 (Clemens et al., 1998). In G19 we also observed an increase of Ca<sup>2+</sup> sensitivity after transformation with LCT1, but much higher Ca<sup>2+</sup> concentrations than in the previous study were needed to provoke growth reduction. Figure 8 summarizes three major findings concerning the effects of Na<sup>+</sup> and Ca<sup>2+</sup> on transformed G19: LCT1 increases the Na<sup>+</sup> sensitivity of G19, LCT1 increases the Ca<sup>2+</sup> sensitivity of G19, and external Ca<sup>2+</sup> alleviates the Na<sup>+</sup> sensitivity only when present in high (but sublethal) concentrations. Induction of Na<sup>+</sup> and Ca<sup>2+</sup> hypersensitivity are specifically linked to the presence of LCT1, whereas the beneficial influence of Ca<sup>2+</sup> on Na<sup>+</sup> sensitivity is a general phenomenon observed in EV- and LCT1-transformed cells.

### Physiological Relevance of LCT1-Mediated Na<sup>+</sup> Uptake

Salt stress in plants is alleviated by increasing the external Ca<sup>2+</sup> concentration (LeHaye and Epstein, 1969). The role of external Ca<sup>2+</sup> in salt tolerance has been linked to Ca<sup>2+</sup> dependence of cytoplasmic signaling components (Liu and Zhu, 1998), as well as to inhibition of toxic Na<sup>+</sup> uptake (Davenport and Tester, 2000). <sup>22</sup>Na<sup>+</sup> uptake by wheat roots is inhibited by external Ca<sup>2+</sup> with relatively high affinity, i.e. a  $K_1$  of around 0.3 mM (Ca<sup>2+</sup> activity, Davenport et al., 1997). The fact that a non-selective cation channel (NSC) in the wheat plasma membrane is blocked by external Ca<sup>2+</sup> with a similar  $K_1$  is a good indication that the NSC is the major component of Na<sup>+</sup> uptake (Tyerman et al., 1997; Davenport and Tester, 2000).

By contrast, Ca<sup>2+</sup> inhibition of LCT1-mediated Na<sup>+</sup> uptake at high external Na<sup>+</sup> levels (20–100 mM) will only become physiologically important if high levels of Ca<sup>2+</sup> (above 10 mM) prevail in the soil. However, this does not exclude a function of LCT1 in Na<sup>+</sup> uptake in agriculturally relevant situations. The aforementioned high-affinity Ca<sup>2+</sup> block affects only about 40% of the Na<sup>+</sup> influx in wheat, resulting in a considerable amount of Na<sup>+</sup> uptake that is insensitive to external Ca<sup>2+</sup> concentrations between 3 and 10 mM (Davenport et al., 1997). Since typical Ca<sup>2+</sup> concentrations of saline soils fall into this range, it is the Ca<sup>2+</sup>-insensitive component of Na<sup>+</sup> influx into wheat roots that has to be targeted in the engineering of salt tolerance (Schachtman and Liu, 1999). Currents through the NSC measured in lipid bilayers display a Ca<sup>2+</sup>-independent component, but unlike Na<sup>+</sup> influx into roots, saturate at Na<sup>+</sup> concentrations above 7 mM (Davenport and Tester, 2000). By contrast, as shown in our study, LCT1-mediated Na<sup>+</sup> sensitivity in transformed yeast cells sharply increases between 10 and 50 mM Na<sup>+</sup> (Fig. 1), indicating an important increase in LCT1-mediated Na<sup>+</sup> influx over this concentration range (note that the Na<sup>+</sup> sensitivity of control cells does not significantly increase over the same range). Attempts to determine ion fluxes through LCT1 directly by measuring electrical currents have been unsuccessful (D. Schachtman and S. Roberts, unpublished data). Voltage-clamp experiments on *Xenopus* oocytes injected with LCT1 mRNA and patch-clamp experiments on LCT1-transformed JKmc yeast cells did not resolve any currents that could be linked to LCT1. This was probably due to insufficient translation or membrane targeting in the oocytes and small size of membrane surface (and therefore of currents) in yeast.

### Role of the N Terminus in LCT1 Function

We have started to assess putative functional domains of LCT1. The N terminus of LCT1 is hydrophobic and contains a highly significant PEST sequence (Schachtman et al., 1997). PEST sequences have been found in rapidly degraded proteins and it

has been suggested that such sequences tag proteins for degradation or modification (Rogers et al., 1986; Rechsteiner and Rogers, 1996). At least two membrane proteins have been found to contain PEST sequences that play a role in modulating protein function (Shevchenko et al., 1998; Decatur and Portnoy, 2000). The skeletal muscle ryanodine receptor contains a PEST sequence in the N-terminal region that is cleaved by a specific calpain-like protease (Shevchenko et al., 1998). Application of the purified protease to the ryanodine receptor in lipid bilayers reduces receptor channel activity, but not its ability to bind ryanodine. The pathogenic bacterium *Listeria monocytogenes* produces a pore-forming protein called Listeriolysin O that provides a release route for the bacterium from the macrophage vacuole into the cytoplasm where it can replicate. Any pore-forming protein produced in the cytoplasm is targeted for degradation to avoid destruction of the macrophage's outer membrane before the bacterium has had time to replicate inside its host (Decatur and Portnoy, 2000). The degradation signal is encoded by a PEST-like sequence at the N terminus of the protein. Post-translational modification of LCT1 may occur in planta and not in yeast. We, therefore, mimicked N-terminal cleavage in LCT1 by creating a set of 5'-cDNA truncations. These truncations were then expressed in yeast. In accordance with the expectations based on the original hypothesis (Rogers et al., 1986) and recent reports (Shevchenko et al., 1998; Decatur and Portnoy, 2000) we found that cleavage of the N terminus of LCT1 at the PEST sequence diminished LCT1-induced phenotypes in yeast, i.e. Ca<sup>2+</sup> influx and Na<sup>+</sup> sensitivity. Removal of the entire N terminus, including the PEST sequence, abolished the activity of the protein as determined by an inability to complement the CY162 yeast mutant (data not shown).

### Outlook

Our results suggest that LCT1 is a potentially important pathway for Na<sup>+</sup> and Cs<sup>+</sup> uptake in wheat and thus a potential target for molecular engineering of salt tolerance or exclusion of radioactive caesium in this crop. Its physiological role, however, remains to be established in planta. The possibility that LCT1 plays a role in Ca<sup>2+</sup> uptake has to be taken into account if the goal is to produce salt-tolerant or caesium-excluding wheat plants by modification of this gene. Any positive effect of reduced Na<sup>+</sup> or Cs<sup>+</sup> uptake by simple knock-out of LCT1 or overexpression of non-functional mutations (such as N-terminal truncated protein) may be impaired by insufficient Ca<sup>2+</sup> uptake (see Fig. 7). Molecular identity of Ca<sup>2+</sup> and Na<sup>+</sup> transport pathways could explain the limited success in the breeding of salt tolerant varieties. Thus, to avoid a potentially damaging loss of Ca<sup>2+</sup> uptake capacity, modification of LCT1 would have to



be more specific, i.e. target its  $\text{Na}^+/\text{Ca}^{2+}$  or  $\text{Cs}^+/\text{Ca}^{2+}$  selectivity. Detailed structure-function analysis of LCT1 will be a necessary undertaking for further progress in this matter.

## MATERIAL AND METHODS

### Yeast Strains

The *Saccharomyces cerevisiae* strain G19 (*MAT $\alpha$  leu2-3, 2-112, trp1-1, ura3-3, ade2-1, his3-11 can1-100, 15( $\phi$ ) ena1::HIS3::ena4*, Quintero et al., 1996) was used for all experiments except  $^{45}\text{Ca}$  uptake measurements, which were performed with a strain produced in our laboratory (here called JKmc: *MAT $\alpha$  leu2-3, 112, his4, trp1, ura3-52, rme1, mid1::KAN, cch1::KAN*, Fischer et al., 1997). Cells of both strains were transformed with the pYES2 plasmid alone (empty vector, EV), or pYES2 containing full-length LCT1 or one of the N-terminally truncated LCT1 cDNAs (*LCT1-115*, *LCT1-126*, see below). Transformants were isolated on SD medium (Sherman, 1991) supplemented with amino acids. Presence of LCT1 and its mutated versions in the transformed cell lines was confirmed on Southern blots.

### Growth Experiments

G19 transformants were adapted to growth on AP medium (Rodríguez-Navarro and Ramos, 1984) containing 1 mM KCl, 2 mM  $\text{MgSO}_4$ , 0.2 mM  $\text{CaCl}_2$ , and 3% (w/v) Gal as the sole carbon source. For phenotypic assessment, AP medium was supplemented with various concentrations of alkali cations or  $\text{Ca}^{2+}$ , all added as their chloride salts. Growth of the cultures was monitored on 2.5% (w/v) agar plates or in liquid media at 30°C. Doubling times in liquid media were calculated from growth rates during the exponential phase. Numbers of cells were deduced from the  $\text{OD}_{600}$  of liquid cultures according to a calibration curve based on representative cell counts.

### Measurements of Ion Contents

For the determination of intracellular  $\text{Na}^+$  and  $\text{K}^+$  contents, cultures were grown in  $\text{Na}^+$ -free liquid AP medium at 30°C to the early stationary phase ( $\text{OD}_{600}$  around 1.2). Fifty millimolar NaCl (in some experiments together with other salts) was added and 5-mL samples were taken from the culture after various incubation times. Cells were collected on a nitrocellulose filter, washed twice with ice-cold 100 mM  $\text{MgCl}_2$ , rinsed onto a new filter, and washed once more. Ions were acid-extracted in 5 mL of 100 mM HCl and 100 mM  $\text{MgCl}_2$  and concentrations of  $\text{Na}^+$  and  $\text{K}^+$  were determined by atomic absorption spectroscopy. In an alternative experiment, cultures were grown in AP medium supplemented with 10 or 20 mM NaCl and harvested as above in the stationary phase for determination of  $\text{Na}^+$  contents. To check the viability of the cells during salt exposure, methylene-blue staining was performed on cell samples. Intracellular ion contents are expressed in nanomoles per  $10^6$  cells where the number of cells was derived from  $\text{OD}_{600}$ .

### Uptake of $^{45}\text{Ca}$

JKmc cells were grown in SD medium with 2% (w/v) Gal, modified according to Iida et al. (1994) to obtain a concentration of 100  $\mu\text{M}$   $\text{Ca}^{2+}$ . Exponentially growing cells (liquid culture at 30°C) were harvested and re-suspended to a density of  $10^8$  cells/mL in 5 mM HEPES-choline buffer (pH 7) containing 2% (w/v) Gal and 100  $\mu\text{M}$   $\text{CaCl}_2$ . Cells were incubated at room temperature with 185 kBq  $^{45}\text{CaCl}_2$  per mL (1.8 kBq/nmol). At various incubation times samples were taken, filtered through nitrocellulose membrane filters pre-soaked in 5 mM  $\text{CaCl}_2$ , and washed with 25 mL of 5 mM  $\text{CaCl}_2$ . Filters were dried and radioactivity counted by liquid scintillation.

### Construction of N-Terminal Deletion Mutants of LCT1

Truncations of the N-terminal region of LCT1 were made using PCR. Primers were designed that contained a *Bam*HI restriction site and a start codon (ATG). These primers annealed to different regions of the LCT1 cDNA and were constructed to maintain the same open reading frame as the predicted protein. Expand High Fidelity PCR System (Boehringer Mannheim, Basel) and a PCR protocol with a low cycle number (20) were used to minimize mistakes introduced by the PCR process. After PCR the products were cloned in pYES2 (Invitrogen, Carlsbad, CA) and sequenced at the 5' end to ensure that the correct truncations and reading frames were present in the cDNA clone. Clones that contained the introduced ATG and were in the correct reading frame were transformed into the CY162 yeast strain (Anderson et al., 1992) to test for complementation of the  $\text{K}^+$  uptake-deficient phenotype (Schachtman et al., 1997). Deletion of the entire N terminus up to the first transmembrane domain (amino acid residues 1–168) resulted in non-functional LCT1. Two mutated LCT1 cDNAs that did functionally complement CY162 were chosen for transformation of the yeast strains G19 and JKmc. These were LCT1-115, deleted in the residues 1 to 115, and LCT1-126, deleted in the residues 1 to 126. The position of the deletions in the LCT1 sequence is shown in Figure 6a.

### RNA Extraction and Northern Blotting

Total RNA of exponentially growing G19 cells was extracted with the RNeasy Plant Mini kit (Qiagen, Valencia, CA). Total RNA was separated by electrophoresis in denaturing conditions on a 1.2% (w/v) agarose-formaldehyde gel and blotted onto a Zetaprobe nitrocellulose membrane (Bio-Rad, Hercules, CA). A 670-bp PshAI fragment within the LCT1 open reading frame was labeled with [ $\alpha$ - $^{32}\text{P}$ ]dATP and used to probe the nitrocellulose filter. The filter was later stripped according to the manufacturer's instructions and rehybridized with a 760-bp control actin probe labeled with [ $\alpha$ - $^{32}\text{P}$ ]dATP.

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