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The plant cDNA *LCT1* mediates the uptake of calcium and cadmium in yeast

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ABSTRACT **Nonessential metal ions such as cadmium are most likely transported across plant membranes via transporters for essential cations. To identify possible pathways for Cd^{2+} transport we tested putative plant cation transporters for Cd^{2+} uptake activity by expressing cDNAs in *Saccharomyces cerevisiae* and found that expression of one clone, *LCT1*, renders the growth of yeast more sensitive to cadmium. Ion flux assays showed that Cd^{2+} sensitivity is correlated with an increase in Cd^{2+} uptake. *LCT1*-dependent Cd^{2+} uptake is saturable, lies in the high-affinity range (apparent K_M for $Cd^{2+} = 33$ μM) and is sensitive to block by La^{3+} and Ca^{2+}. Growth assays demonstrated a sensitivity of *LCT1*-expressing yeast cells to extracellular millimolar Ca^{2+} concentrations. *LCT1*-dependent increase in Ca^{2+} uptake correlated with the observed phenotype. Furthermore, *LCT1* complements a yeast disruption mutant in the *MID1* gene, a non-*LCT1*-homologous yeast gene encoding a membrane Ca^{2+} influx system required for recovery from the mating response. We conclude that *LCT1* mediates the uptake of Ca^{2+} and Cd^{2+} in yeast and may therefore represent a first plant cDNA encoding a plant Ca^{2+} uptake or an organellar Ca^{2+} transport pathway in plants and may contribute to transport of the toxic metal Cd^{2+} across plant membranes.**

Calcium is an important nutrient for plant growth and Ca^{2+} influx and Ca^{2+} release from organelles play important roles in many plant signaling cascades (1, 2). However, plant cDNAs that mediate calcium transport into the cytosol of plant cells have not yet been identified even though in recent years molecular approaches, in particular the complementation of yeast mutants, have allowed the identification of a number of plant transporters, for nutrients including sucrose (3), potassium (4–6), sulfate (7), phosphate (8–10), iron (11), and copper (12).

Cation transporters offer potential transport pathways for phytotoxic metals. For example, certain potassium channels and transporters are permeable to Na^+ (13–15). Nonessential heavy metals such as cadmium are also most likely taken up via plant nutrient transporters or channels that are not completely selective. In animals voltage-gated Ca^{2+} channels are implicated in Cd^{2+} uptake (16). Voltage-dependent Ca^{2+} influx activities have been reported from plant plasma membranes (17–20). No plant transporter has yet been shown to mediate Cd^{2+} uptake into the cell. Vacuolar transporters have been cloned recently from yeast that mediate uptake of Cd-phytochelatin complexes into vacuoles (21–23) and homologous genes have been identified in *Arabidopsis* (24).

Understanding the mechanism of heavy metal transport across plant membranes has been proposed to aid in engineering plants with enhanced or decreased uptake (25). The uptake of heavy metal ions, including Cd^{2+}, by agricultural plants is a major cause for the accumulation of these toxic cations in the human body (26, 27). On the other hand, plants that hyperaccumulate heavy metals might be useful for phytoremediation, i.e., the removal of toxic materials from soils and water (25, 28, 29). Physiological studies suggest that an increased metal uptake activity could be among several essential components required for metal hyperaccumulation (30). Therefore, genes encoding proteins that are involved in transport represent promising targets for multigene engineering of plant heavy metal accumulation.

We used the expression of plant cDNAs in *Saccharomyces cerevisiae* to test putative and known plant cation transporters and channels for their Cd^{2+} uptake capacity. Here we report the characterization of the recently identified wheat cDNA *LCT1* that was shown to induce low-affinity Na^+ and Rb^+ uptake in yeast (31), as a protein mediating the uptake of Ca^{2+} and Cd^{2+} into yeast cells.

MATERIALS AND METHODS

Yeast Culture, Expression of Plant cDNAs in *S. cerevisiae* and Growth Assays. INVSc1 cells (Invitrogen) were transformed with wheat cDNAs inserted into pYES2 (6) by using standard procedures and grown on yeast nitrogen base minimal medium lacking uracil. All growth assays with transformed yeast were carried out by using arginine-phosphate medium (32) containing 1 mM K^+, 1% sucrose, 1% galactose. For plate assays 0.8% ultra-pure agarose (GIBCO) was added. Cells were grown overnight and either streaked out on plates or diluted into 4 ml of fresh liquid medium containing different $CdCl_2$ or $CaCl_2$ concentrations. Cells to be used in uptake experiments were also grown in arginine-phosphate medium.

Uptake Assays. We found that the INVSc1 yeast line (Invitrogen) generates larger *LCT1*-dependent metal uptake rates than the *gal*$^-$ CY162 strain (4, 31). Therefore INVSc1 cells were used for uptake assays here. The isotopes $^{109}Cd^{2+}$ and $^{45}Ca^{2+}$ were used for uptake assays. Uptake rates were always measured in parallel for freshly transformed *LCT1*-expressing cells and control cells harboring the empty pYES2 plasmid. Cells were grown to an OD_{600} of ≈0.2, washed with H_2O and resuspended in uptake solution (10 mM Hepes·Tris, pH 6.0/100 μM $MgCl_2$/1% sucrose/1% galactose) to an

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OD_{600} of 0.5. After a 5 min preincubation at 30°C, uptake was started by adding either $CaCl_2$ or $CdCl_2$ containing 0.2–2 μCi (1 Ci = 37 GBq) of the respective isotope to the cells. Other metals used in competition experiments and inhibitors were added to the uptake assay at the beginning of the preincubation period. Total assay volume was 3.5 ml. Aliquots were taken in intervals of 2–6 min, harvested on nylon membranes (0.8 μm) and washed twice with 5 ml of 100 mM $CaCl_2$. Radioactivity on the membranes was counted in the presence of liquid scintillation mixture (Ecoscint). *LCT1*-mediated Cd^{2+} uptake was also confirmed in pilot atomic absorption spectroscopy measurements (data not shown).

Disruption of the MID1 Locus. The selectable marker *LEU2* was inserted into the coding sequence of the *MID1* gene in yeast strain C699–5 (*MATa ade2 can1 his3 leu2 trp1 ura3 bar1:HisG*) as described in the following. A 1,979-bp fragment of the *MID1* gene was amplified from C699–5 genomic DNA by PCR using the following primers 5′-CGACCGGCTGACGTACCGT and 5′-ACTGCTAACGCCGAAGAACA. This product, containing the entire *MID1* coding sequence, was cloned into the TA cloning site of pCRII (Invitrogen) to generate plasmid pMID1. The yeast *LEU2* gene was obtained as a 1,732-bp fragment cloned into the *Eco*RV site of pBluescript. A 2,178-bp *Pvu*II fragment containing the yeast *LEU2* gene was isolated and cloned into the unique *Nde*I site of pMID1 to make pMID1-LEU2. The yeast strain C699–5 was transformed with a 5,864-bp *Bgl*I fragment of pMID1-LEU2 containing the interrupted *MID1* gene. Transformants were selected on media lacking leucine and tested for reduced viability in the presence of α-factor by using the methylene blue liquid assay (33). Disruptions in *MID1* were confirmed by using PCR and one strain (C699–5Δmid1) was selected for further experiments.

Complementation of the mid1 Mutant Phenotype. Yeast strains C699–5 and C699–5Δmid1 were each transformed with either the pYES2 expression vector or pYES2 containing *LCT1*. Freshly grown colonies were picked from plates without glucose containing 2% galactose and 2% raffinose. For C699–5 the media lacked uracil and for C699–5Δmid1 the media lacked uracil and leucine. The cells were suspended in microtiter wells containing 100 μl of media [yeast nitrogen base (Difco)/2% galactose/2% raffinose/0.005% methylene blue/0.8 g $liter^{-1}$ of drop-out mixture without uracil (Bio101) with and without 3 μM of the yeast peptide pheromone α-factor (Nova Biochem)]. The cells were incubated at 30°C for 3 hr, and viability was determined microscopically (33).

RESULTS

LCT1 Expression Leads to an Increase in Cd^{2+} Uptake. Because Cd^{2+} is a nonessential element for plants (34), it is likely that plant cells do not express specific Cd^{2+} transporters. Certain metal uptake transporters in plants are relatively nonselective, such that both metal nutrients and toxic metals are taken up (15, 18). We therefore conducted a secondary screen among K^+ uptake complementing cDNAs for those that might be involved in the transport of divalent heavy metal cations. Wheat cDNAs were expressed in the *S. cerevisiae* wild-type strain INVSc1 and the effects of media containing different Cd^{2+} concentrations on yeast growth were determined. Yeast expressing known transporters including KAT1 and HKT1 (4, 6) did not cause shifts in the Cd^{2+} sensitivity of yeast (data not shown). However, *LCT1* (31) expression produced a dramatic increase in the Cd^{2+} sensitivity of yeast growth on plates. *LCT1* expressing cells (YLCT1) did not grow in an arginine-phosphate medium containing 50 μM Cd^{2+}, whereas control cells carrying the empty pYES2 plasmid were able to grow in the presence of 50 μM Cd^{2+} on plates (Fig. 1).

The initial characterization of *LCT1* had shown that it encodes a protein with 6–8 putative membrane-spanning

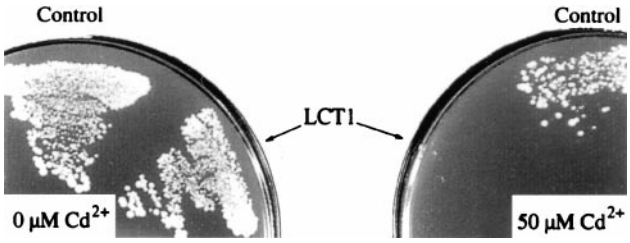

FIG. 1. Effect of *LCT1* expression on cadmium sensitivity of yeast. INVSc1 cells transformed with either the empty pYES2 plasmid (Control) or *LCT1* in pYES2 were grown on arginine-phosphate medium without Cd^{2+} (*Left*) or with 50 μM Cd^{2+} (*Right*).

domains and mediates Na^+ and low rates of Rb^+ uptake and perhaps also Ca^{2+} transport in yeast (31). We pursued uptake assays for Cd^{2+} by using $^{109}Cd^{2+}$ to determine whether the observed effect of Cd^{2+} on the growth of *LCT1*-expressing cells is accompanied by an increased transport rate for Cd^{2+}. The expression of *LCT1* produced an increase in Cd^{2+} uptake by yeast cells of ≈75% at an external concentration of 30 μM (Fig. 2, ●). To determine whether cell wall binding or uptake accounted for the Cd^{2+} accumulation, control experiments were carried out at 0°C demonstrating that the measured amounts of Cd^{2+} were transported into the cells dependent on protein activity (Fig. 2, ■, □) and not simply by temperature-independent adsorption to cell walls (34). We therefore conclude, that the observed increase in Cd^{2+} sensitivity upon expression of *LCT1* may be caused by elevated Cd^{2+} uptake into the yeast cells. *LCT1*-mediated uptake was linear for at least 20 min at 30°C.

The affinity of *LCT1* for Cd^{2+} was studied by measuring the difference in Cd^{2+} uptake between control cells and YLCT1 at different external Cd^{2+} concentrations (Fig. 3). *LCT1*-mediated cadmium uptake could be described by Michaelis-Menten kinetics with an apparent K_M of ≈33 μM. Thus, expression of *LCT1* in yeast induced an increase in Cd^{2+} uptake that showed saturation characteristics and an apparent affinity in the high-affinity uptake range (Fig. 3). *LCT1*-dependent Cd^{2+} uptake displayed a mild pH dependence. Uptake rates were highest at a pH of 6 and ≈30% higher at pH

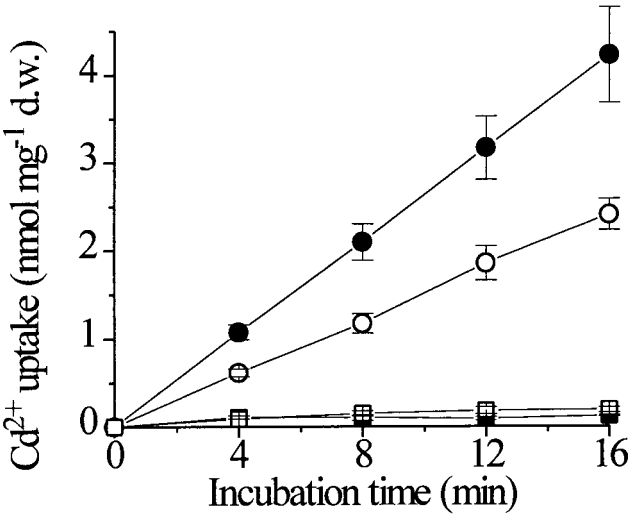

FIG. 2. Effect of *LCT1* expression on cadmium uptake in yeast. INVSc1 cells, transformed with *LCT1* in pYES2 (■, ●) and with the empty control pYES2 plasmid (□, ○) were grown in arginine-phosphate medium to an OD of ≈0.2 and assayed for cadmium uptake with ^{109}Cd. Cells were incubated in uptake solution, containing 30 μM Cd^{2+}, at 30°C (●, ○) or 0°C (■, □). Aliquots were taken at different time points and radioactivity was measured. Bars = SE; n = 8 for each data point.

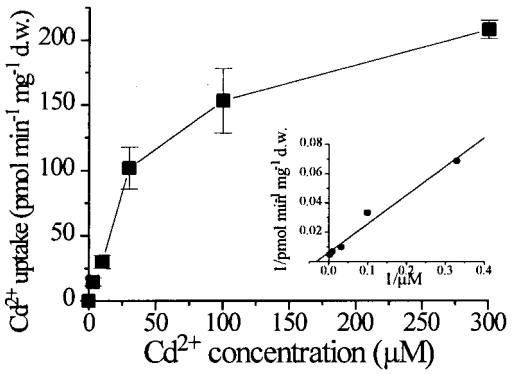

FIG. 3. Concentration-dependence of *LCT1*-dependent uptake. Cd^{2+} uptake rates of INVSc1 control cells (carrying the empty pYES2 plasmid) were subtracted from Cd^{2+} uptake rates of *LCT1*-expressing cells to determine the *LCT1*-dependent Cd^{2+} uptake rate at different Cd^{2+} concentrations. Bars = SE; $n = 3$. The inset shows a Lineweaver–Burke plot of the uptake data. $K_M = 32.9$ μM.

6 than at pH 5 and ≈10% higher at pH 6 than at pH 7 ($n = 3$, data not shown).

LCT1-Mediated Ca^{2+} Uptake. Studies in animal cells have led to the hypothesis that Cd^{2+} uptake is, at least in part, mediated by Ca^{2+} transporters (16). In addition, previous work on *LCT1* had indicated a possible Ca^{2+} transport activity (31). We therefore tested whether *LCT1* also mediates uptake of the physiological metal Ca^{2+}. We first analyzed the competition of Mg^{2+} and Ca^{2+} ions with *LCT1*-dependent Cd^{2+} uptake (Fig. 4 *A* and *B*). The addition of Mg^{2+} to the uptake solution containing 30 μM Cd^{2+} mildly inhibited the *LCT1*-mediated accumulation of Cd^{2+} at concentrations exceeding 100 μM (Fig. 4*A*, ■). However, Ca^{2+} ions interfered strongly with the uptake of Cd^{2+}. At concentrations of Ca^{2+} over 100 μM, *LCT1*-dependent Cd^{2+} uptake was strongly blocked (Fig. 4*B*, ■). The calculated values for half-maximal inhibition of *LCT1*-mediated Cd^{2+} uptake were 600 μM for Mg^{2+} and 25 μM for Ca^{2+}. The native background Cd^{2+} transport activity in yeast was also affected by Ca^{2+}, but in a different fashion. Native Cd^{2+} uptake was less sensitive to block by Ca^{2+} than the *LCT1*-dependent Cd^{2+} uptake. Half-maximal inhibition in controls occurred at a Ca^{2+} concentration of ≈700 μM (Fig. 4*B*, ○). Furthermore, *LCT1*-dependent Cd^{2+} uptake was sensitive to the channel blocker La^{3+}. 10 μM La^{3+} in the uptake assay almost completely abolished the effect of *LCT1* expression on Cd^{2+} accumulation ($n = 2$, data not shown). The addition of 3 μM La^{3+} produced an inhibition by ≈70% ($n = 2$). The *LCT1*-induced Cd^{2+} uptake was more sensitive to block than the native Cd^{2+} uptake in yeast cells that was inhibited by ≈40% and 60% at 3 μM and 10 μM La^{3+}, respectively ($n = 2$).

Because the Ca^{2+} competition data and the block by Ca^{2+} and La^{3+} indicated a possible Ca^{2+} transport activity of *LCT1*, we investigated the effects of different Ca^{2+} concentrations in the medium on yeast growth. Excess Ca^{2+} accumulation in yeast is toxic (36). Thus, the unregulated overexpression of a Ca^{2+}-transporting protein should render the growth of yeast cells sensitive to elevated external Ca^{2+} concentrations. At 10 μM Ca^{2+} in argininine-phosphate medium no significant differences were observed in growth of control and *LCT1*-expressing yeast cells (Fig. 5, ○, ●). However, at 3 mM Ca^{2+} growth of *LCT1*-expressing cells was severely inhibited (Fig. 5, ■) whereas control cells tolerated this concentration with only a minor reduction in growth rate (Fig. 5, □). Half-maximal inhibition of growth occurred at a Ca^{2+} concentration of 1.7 mM for *LCT1*-expressing cells. This finding provides an indication for *LCT1*-mediated enhancement of Ca^{2+} uptake. This hypothesis was directly tested by measuring Ca^{2+} uptake rates. Uptake assays using $^{45}Ca^{2+}$ demonstrated an *LCT1*-dependent increase in Ca^{2+} uptake (Fig. 6). At an external Ca^{2+} concentration of 100 μM YLCT1 took up approximately twice as much Ca^{2+} as control cells (Fig. 6, ●). Uptake assays at 0°C showed that protein-dependent uptake into the cells and not adsorption to the cell wall accounted for the observed accumulation of Ca^{2+} (Fig. 6, ■, □). *LCT1*-dependent Ca^{2+} uptake was linear up to a concentration of at least 3 mM ($n = 5$, data not shown).

To further test whether *LCT1*-mediated Ca^{2+} uptake can be distinguished from native uptake in control cells, effects of other heavy metals on *LCT1*-mediated Ca^{2+} uptake were studied. The addition of 10 μM Pb^{2+} or 100 μM Zn^{2+} abolished *LCT1*-mediated Ca^{2+} uptake almost completely (Fig. 7, ■). Mn^{2+} (100 μM) and Cd^{2+} (100 μM) also inhibited *LCT1*-mediated Ca^{2+} uptake significantly whereas Co^{2+} had only a mild effect and Ni^{2+} addition led to a small increase in uptake. The competition of Cu^{2+} could not be determined because Cu^{2+} stimulated the native Ca^{2+} uptake system in yeast cells leading to a several-fold increase in Ca^{2+} accumu-

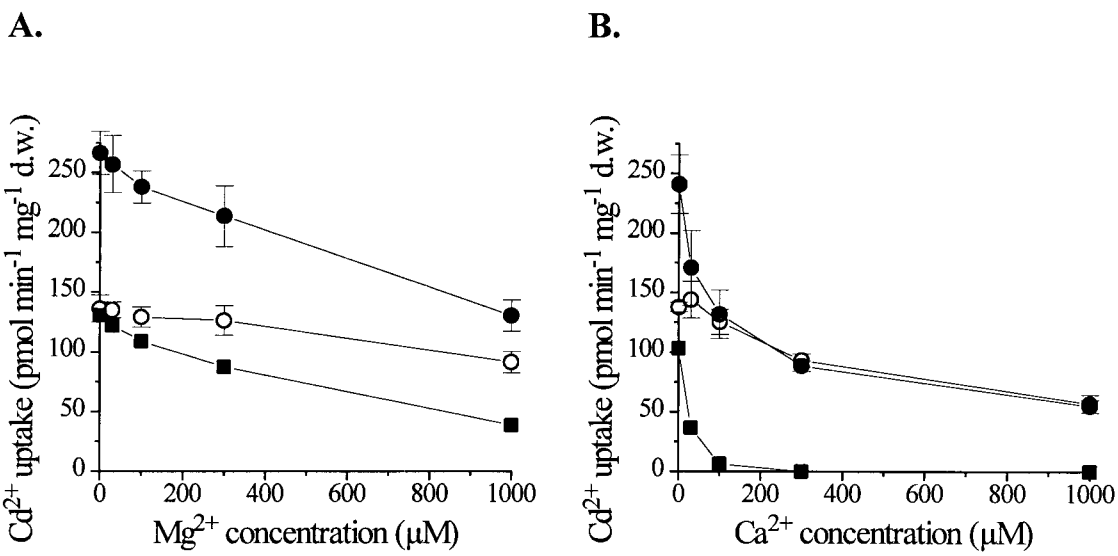

FIG. 4. Competition of Ca^{2+} and Mg^{2+} with *LCT1*-dependent Cd^{2+} uptake. Cd^{2+} uptake rates were determined for INVSc1 controls (○) and *LCT1*-expressing cells (●) in the presence of different Mg^{2+} (*A*) and Ca^{2+} (*B*) concentrations. *LCT1*-dependent Cd^{2+} uptake (■) was determined by subtracting the rates of control cells from the rates of *LCT1*-expressing cells. Bars = SE; $n = 4$.

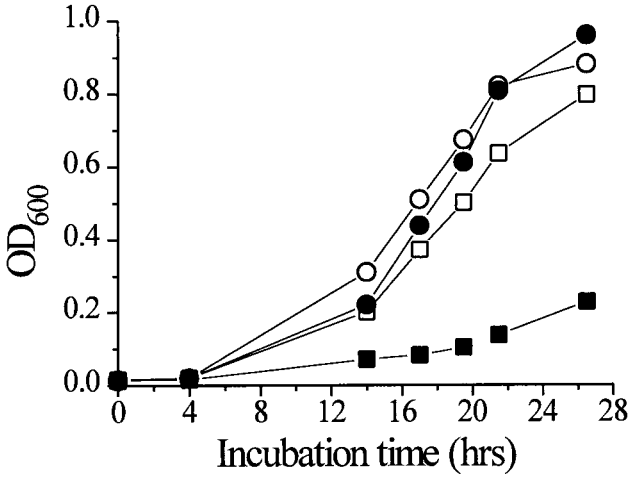

FIG. 5. Ca^{2+} sensitivity of *LCT1*-expressing cells. *LCT1*-expressing cells (■, ●) and INVSc1 control cells (□, ○) were grown in arginine-phosphate medium containing either 10 μM Ca^{2+} (●, ○) or 3 mM Ca^{2+} (■, □).

lation that masked the effect of *LCT1* expression (n = 3, data not shown). As found above for the Ca^{2+} and La^{3+} sensitivity of Cd^{2+} uptake (Fig. 4*B*), the native background Ca^{2+} uptake was less sensitive to some of the cations tested (Fig. 7, □). In particular, addition of 100 μM Zn^{2+} caused only a 45% reduction in Ca^{2+} accumulation and 10 μM Pb^{2+} a reduction by 64%. A concentration of 100 μM Cd^{2+} did not affect the wild-type uptake rate (Fig. 7).

LCT1 Complements a *mid1* Knock-Out. To further test our hypothesis that *LCT1* encodes a Ca^{2+} transporter, we analyzed its ability to complement a *S. cerevisiae* strain with a disruption of the *MID1* gene. *MID1* encodes an integral plasma membrane protein mediating Ca^{2+} influx required for recovery from the yeast mating response (33, 37). The C699–5 yeast strain was selected for disruption of the *MID1* gene and complementation experiments. This strain is hypersensitive to

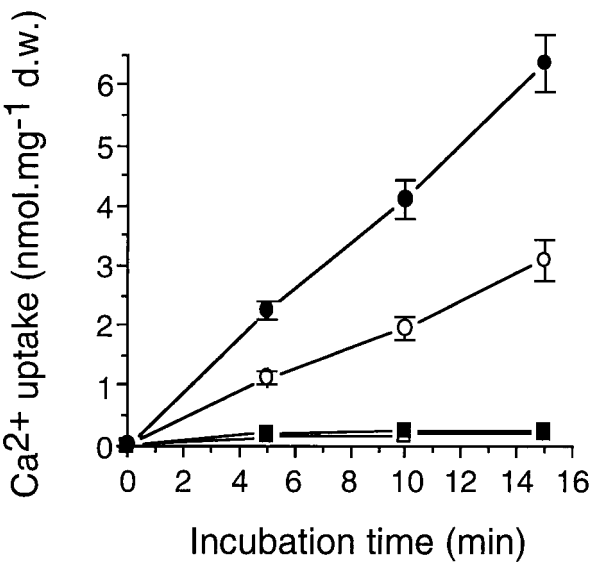

FIG. 6. Effect of *LCT1* expression on calcium uptake in yeast. INVSc1 cells transformed with *LCT1* in pYES2 (■, ●) and with the empty pYES2 control plasmid (□, ○) were grown in arginine-phosphate medium to an OD of ≈0.2 and assayed for calcium uptake with $^{45}Ca^{2+}$. Cells were incubated in uptake solution, containing 100 μM Ca^{2+}, at 30°C (●, ○) or 0°C (■, □). Aliquots were taken at different time points and radioactivity was measured. Bars = SE; n = 7 for each data point.

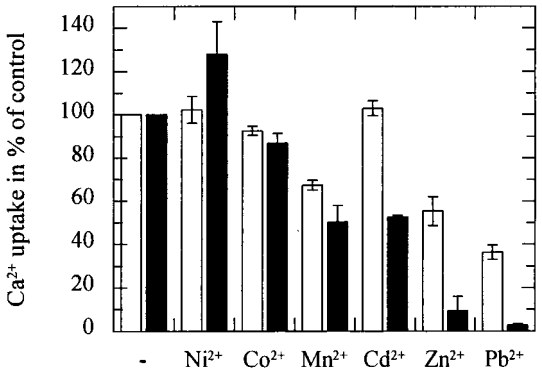

FIG. 7. Effect of different divalent cations on *LCT1*-dependent Ca^{2+} uptake. Ca^{2+} uptake of *LCT1*-expressing cells (■) and INVSc1 control cells (□) was measured in the presence of other cations. The external Ca^{2+} concentration was 100 μM, the concentration of Pb^{2+} was 10 μM, the concentration of the other cations was 100 μM. The uptake rates are shown as % of controls that had no competing metal added to the uptake assay. Native background Ca^{2+} uptake (□) is compared with *LCT1*-dependent Ca^{2+} uptake (■). *LCT1*-dependent Ca^{2+} uptake was determined by subtracting the rate of control cells from the rate determined for *LCT1*-expressing cells. The average Ca^{2+} uptake rate in these experiments was 213.0 (±20.1) pmol·min^{-1}·mg^{-1} d.w. for control cells and 444.0 (±26.4) pmol·min^{-1}·mg^{-1} dry weight for *LCT1*-expressing cells. Error bars represent SE, n = 3 for each condition.

α-factor due to the disruption of the *BAR1* gene that encodes a protease that degrades α-factor (38). In the present experiments, 54 potential *mid1* knock-out mutants were selected by growth on media without leucine. Of these, nine transformants were found to display reduced viability in the presence of α-factor as reported for *mid1* mutants (33). All of these were found to contain the expected complete knock-outs in the *MID1* gene.

A typical morphological response to α-factor, the change into cells with one or more projections on the cell surface ("shmoos," ref. 37), was observed for both yeast strains. The control yeast strain C699–5, containing either pYES2 or pYES2-LCT1 displayed no reduction in viability after treatment with α-factor for 3 hr (Fig. 8, open bar, striped bar). However, viability of the C699–5Δmid1 yeast containing pYES2 decreased to 58% within 3 hr (Fig. 8, ▨). This decrease

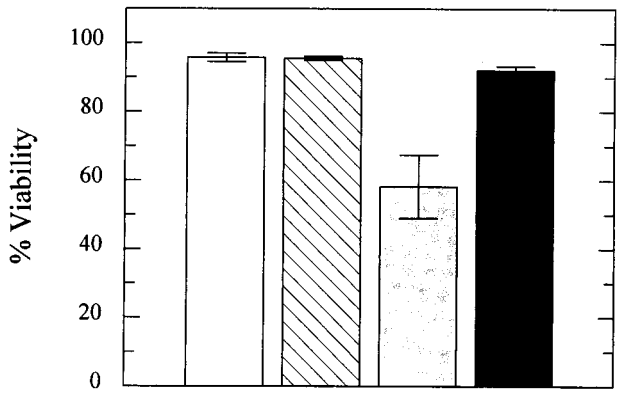

FIG. 8. Expression of *LCT1* in the *mid1* mutant prevented cell death in response to mating pheromone compared with non-*LCT1*-expressing *mid1* cells. Yeast viability was determined by using the methylene blue liquid method. Cells were incubated for 3 hr in the presence of 3 μM α-factor. Four hundred yeast cells were scored for uptake of methylene blue by microscopic observation for each strain-plasmid combination [□, C699–5 (pYES2); ▧, C699–5 (*LCT1*); ▨, C699–5Δmid1 (pYES2); ■, C699–5Δmid1 (*LCT1*)] in this experiment and two independent transformants were tested for each with the same result. Bars = SD.

is similar to the level of decrease in viability reported previously in *mid1* mutants under the same conditions (33). Expression of *LCT1* in the *mid1* mutant prevented this reduced viability, only 8% of the yeast cells displayed methylene blue uptake (Fig. 8, ■). Therefore, *LCT1* complemented the *mid1* mutation.

DISCUSSION

The complementation of a *mid1* knock-out mutant by *LCT1* together with the effects of *LCT1*-expression on yeast growth sensitivity to Ca^{2+} and Cd^{2+} (Figs. 1 and 5) and on yeast uptake of Ca^{2+} and Cd^{2+} (Figs. 2 and 6) support the hypothesis that *LCT1* encodes a transport activity mediating the uptake of Ca^{2+} and Cd^{2+} across the plasma membrane of yeast.

LCT1 Mediates Cd^{2+} and Ca^{2+} Uptake in Yeast. We found that overexpression of the wheat cDNA *LCT1* (31) dramatically increased the Cd^{2+} sensitivity of *S. cerevisiae* growth on plates. This effect is less pronounced in liquid culture, possibly due to different external conditions, such as removal of local external ion gradients in liquid culture. Cd^{2+} uptake assays confirmed that the observed phenotype correlated with higher Cd^{2+} uptake rates of *LCT1*-expressing cells with distinct properties to native Cd^{2+} uptake (Figs. 2, 3, and 4*B*). In contrast to a number of studies on plant ion transporters expressed in yeast, we were not able to use a control yeast mutant deficient in the respective uptake activity (3, 4, 11). This explains the considerable background uptake of controls. The rates for *LCT1*-dependent Cd^{2+} uptake at different external Cd^{2+} concentrations showed a component with an apparent K_M of 33 μM for Cd^{2+}.

The uptake of nonessential and phytotoxic Cd^{2+} ions suggests that transport of beneficial cations may be the natural function of *LCT1*. Schachtman *et al.* (31) demonstrated an increase in Rb^+ uptake of *LCT1*-expressing cells. The low rates and the low affinity, however, led to the conclusion that *LCT1* does not contribute significantly to potassium nutrition (31) and a physiological role of *LCT1* therefore remained to be determined. Cd^{2+} and other heavy metal cations are known to interact with Ca^{2+} channels, Ca^{2+} transporters and Ca^{2+} binding proteins (39–41). Furthermore, studies in animal cells suggested Cd^{2+} uptake via voltage-gated Ca^{2+} channels (16). Plants have to take up calcium from the soil via the root system. Huang *et al.* (18) described depolarization-activated Ca^{2+} influx into wheat root vesicles. Several different pathways for calcium uptake are likely to exist in plants that can play diverse roles in signal transduction and Ca^{2+} nutrition. Hyperpolarization and elicitor-activated channels in tomato cells (42), elicitor-activated Ca^{2+} permeable channels in parsley cells (43), and ABA-activated nonselective Ca^{2+} permeable channels in guard cells (44) have been characterized. We note that in plant cells, *LCT1* may be targeted to organellar membranes or to the plasma membrane. In either case, to our knowledge no plant cDNA has yet been shown to mediate Ca^{2+} influx or organellar Ca^{2+} release and *LCT1* is a first putative candidate contributing to one of these functions.

The differential effect of Mg^{2+} and Ca^{2+} on *LCT1*-dependent Cd^{2+} uptake (Fig. 4 *A* and *B*) and the La^{3+} sensitivity indicated that *LCT1* might function as a Ca^{2+} transport system. Growth assays and uptake experiments demonstrated that *LCT1* expression induced a significant protein-dependent increase in Ca^{2+} uptake of yeast cells. A K_M for Ca^{2+} could not be determined because *LCT1*-dependent Ca^{2+} influx does not show saturation up to a concentration of at least 3 mM. The growth inhibition of *LCT1* expressing cells at different Ca^{2+} concentrations indicated an apparent K_M in the millimolar range. This seems to contradict the high-affinity binding of Ca^{2+} indicated by the strong block of Cd^{2+} uptake (Fig. 4*B*.). However, it has been often found that Ca^{2+} channels possess multiple ion binding sites and show a high affinity binding of Ca^{2+} but a low affinity for Ca^{2+} conductance (45).

We propose from these data that *LCT1* may function as a Ca^{2+} transport system, possibly contributing to either Ca^{2+} influx across the plasma membrane or Ca^{2+} transport across organelle membranes in plants. *LCT1*-mediated cation uptake is nonselective allowing permeation of both Cd^{2+} (Fig. 2) and Na^+ (31). Low-affinity Na^+ uptake mediated by *LCT1* has led to the suggestion that *LCT1* could provide one of the pathways for Na^+ transport into the cytosol of plant cells (31). *LCT1*-mediated Ca^{2+} and Na^+ transport are consistent with the observation in plants that Ca^{2+} reduces transport of Na^+ (46). Note that intracellular Ca^{2+} regulation, as a second messenger, also contributes to Ca^{2+} regulation of Na^+ transport.

Experiments testing the effect of other divalent cations on *LCT1*-induced Ca^{2+} uptake showed the expected inhibition by Cd^{2+}. Zn^{2+}, and Pb^{2+} blocked Ca^{2+} uptake even more efficiently. Zn^{2+} was shown by Schachtman *et al.* (31) to be a potent blocker of Rb^+ uptake that is not transported by *LCT1*. Further uptake studies are necessary to test whether Pb^{2+} is a transported substrate for *LCT1*. The low selectivity of *LCT1* indicates that *LCT1* may mediate the transport of additional cations.

LCT1 Complements a *mid1* Knock-Out. The *MID1*-mediated influx of Ca^{2+} in yeast after mating response is a regulated process (33, 37). However, the finding that elevated Ca^{2+} in the medium prevents cell death in Δ*mid1* mutants (33) led us to hypothesize that heterologously expressed transporters, such as *LCT1* that allow Ca^{2+} influx in yeast, but are not expected to respond to the mating signal transduction pathway might complement this mutant. We decided to construct a *mid1*-knock-out strain to further test the hypothesis that *LCT1* functions as a Ca^{2+} influx system in yeast. Analysis of the mating factor response of control cells, the Δ*mid1* knock-out strain and the Δ*mid1* knock-out strain expressing *LCT1* clearly demonstrated that *LCT1* complements the Δ*mid1* disruption. These data provide additional strong evidence that *LCT1* represents a Ca^{2+} transporter. In addition, the results of these experiments indicate that the utilization of *mid1* mutants could provide a means to test Ca^{2+} uptake by other transporters from plants or animals.

Does LCT1 Encode the Transporter Itself? The *LCT1*-dependent increases in Ca^{2+} and Cd^{2+} uptake rates could theoretically be explained as an activation of yeast uptake systems by *LCT1*. However, there are no homologous sequences in the completely sequenced *S. cerevisiae* genome (47) and the amino acid sequence of *LCT1* displays the hydrophobic domains reminiscent of a membrane protein (31). Furthermore, *LCT1*-dependent Ca^{2+} and Cd^{2+} uptake displayed some characteristics different from the native background uptake. *LCT1* induced a Cd^{2+} influx component that is significantly more sensitive to external Ca^{2+} and La^{3+} than Cd^{2+} influx measured in control cells (Fig. 4*B*). Cd^{2+}, Mn^{2+}, Zn^{2+}, and Pb^{2+} inhibited the *LCT1*-dependent Ca^{2+} uptake more strongly than the wild-type Ca^{2+} uptake (Fig. 7). In addition, Δ*mid1* complementation demonstrated that *LCT1* can replace a yeast component of a plasma membrane Ca^{2+} influx system.

This evidence supports the hypothesis that *LCT1* encodes a membrane protein permeable to Ca^{2+} and Cd^{2+}. The activation of an endogenous transport activity by *LCT1*, however, cannot be entirely ruled out. Whether the *LCT1* protein is targeted to the plasma membrane or organellar membranes in plants will be addressed in future studies. Although Ca^{2+} influx into cells does not require active transport, the question whether *LCT1* encodes a Ca^{2+}-permeable channel or a carrier mechanism remains to be addressed.

A Role for LCT1 in Plant Heavy Metal Transport? To our knowledge, at present no plant cDNA has been shown to mediate the influx of Cd^{2+} or other nonessential, phytotoxic heavy metals. Different pathways for the entry of cadmium

into the cytosol should exist. For instance, iron transport mediated by IRT1 is sensitive to Cd^{2+} block (11) and Cd^{2+} uptake remains to be analyzed. The presented effect of *LCT1*-expression on Cd^{2+} uptake in yeast suggests that *LCT1* might contribute to cadmium transport in plants. Taking the Ca^{2+} sensitivity of *LCT1*-dependent Cd^{2+} uptake into account, however, the Cd^{2+} permeability of *LCT1* is probably not relevant in soils with high Ca^{2+} concentrations unless local Ca^{2+} depletion occurs.

Heavy metal uptake is one of the essential components of heavy metal hyperaccumulation. It is, however, not clear whether uptake rates are a limiting factor. Studies on the hyperaccumulating plant *Thlaspi caerulescens* and the nonhyperaccumulating plant *Thlaspi arvense* show higher Zn^{2+} uptake rates for the hyperaccumulating species (30). Therefore, modification of uptake transporters may represent a target for multigenic enhancement of heavy metal hyperaccumulation.

In conclusion, we have shown that expression of the wheat cDNA *LCT1* in *S. cerevisiae* leads to an increase in Ca^{2+} and Cd^{2+} uptake with a high affinity for Cd^{2+}. Physiologically *LCT1* may function in plant cell Ca^{2+} influx or organellar Ca^{2+} transport. The membrane targeting and tissue localization of *LCT1* in plants remains to be determined. Ca^{2+} transport mediated by *LCT1* was demonstrated by measuring the Ca^{2+} sensitivity and Ca^{2+} uptake of *LCT1*-expressing yeast cells, by characterizing competition of cations with Cd^{2+} and Ca^{2+} and by complementing the knock-out of *MID1*, a yeast plasma membrane Ca^{2+}-influx system. To our knowledge, this is the first report of a plant cDNA that mediates both Ca^{2+} and Cd^{2+} transport into the cytosol of cells.

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