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Overexpression of *LCT1* in tobacco enhances the protective action of calcium against cadmium toxicity

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"Capsule": *This is the first report of LCT1 in Ca acquisition and regulation of Cd-toxicity by Ca.*

Abstract

Wheat cDNA LCT1, a nonspecific transporter for Ca^{2+}, Cd^{2+}, Na^{+} and K^{+}, was overexpressed in tobacco. Transformants were tested for their sensitivity to a range of Ca^{2+}-concentrations [0.01–10 mM $Ca(NO_3)_2$] with or without the presence of 0.05 mM $Cd(NO_3)_2$. Calcium and cadmium accumulation was also determined. *LCT1*-transformed plants expressed a phenotype distinct from controls only under conditions of low calcium (0.01–1 mM Ca^{2+}). They grew significantly better and had slightly higher shoot calcium concentration. Transformants subjected to 0.05 mM $Cd(NO_3)_2$ in the presence of 1 mM Ca^{2+} displayed a substantially higher level of tolerance to cadmium and accumulated less Cd in roots. These results are the first to demonstrate the involvement of *LCT1* in calcium acquisition and in the regulation of amelioration of Cd-toxicity by calcium.

Keywords: Ca-dependent Cd-tolerance; Transgenic; Tobacco; LCT1

1. Introduction

Depending on their concentration in the environment, heavy metals, including micronutrients like Zn, Ni, Co, Cu necessary for plant growth and those such as Cd, Pb or Hg, for which no function in living organisms has yet been demonstrated, exert a toxic influence on plant metabolism. Numerous studies report an ameliorating effect of calcium on heavy-metal toxicity (Simon, 1978; Karataglis, 1981). For example, calcium involvement in zinc uptake and detoxification was studied in Zn^{2+}-tolerant and non-tolerant populations of *Silene maritima With*. Increasing calcium concentrations reduced Zn-toxicity, and led to a higher level of zinc accumulation by the roots of the tolerant plants, but decreased transport to the shoots of both types (Baker, 1978). A higher calcium concentration in a medium was also reported to abolish the toxic efects of both Cd^{2+} (Skórzyńska-Polit et al., 1998; Skórzyńska-Polit and Baszyński, 2000) and Pb^{2+} (Rashid and Popovic, 1990) on the activity of photosystem II. In addition, high Ca-status and a high level of tolerance to Ca-deficit accompanied enhanced Zn, Pb, Cu and Al tolerance (Rengel, 1992; Antosiewicz, 1993, 1995).

The underlying mechanism of Ca^{2+}-dependent heavy metal toxicity has not been elucidated yet. The regulation of heavy-metal uptake and internal transport constitutes part of the basis of plant resistance to their toxicity. The mechanism, however, accounting for the transport of heavy metals across membranes in plants and its regulation is far from understood. The general view is that non-essential metals usually cross plasmalemma and internal membranes through cation transporters with a broad substrate specificity, or use pathways reserved for other ions (Williams et al., 2000; Clemens, 2001). For example, cadmium and lead were shown to be transported through pathways for calcium ions. Thus, it has been demonstrated that lead entered mammalian cells through voltage-activated Ca^{2+}-channels (Simonis and Pocock, 1987; Tomsig and Suszkiw, 1991). It has been suggested that in plants, putative tonoplast Ca^{2+}/H^{+} antiporters encoded by CAX1 and

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CAX2 from *Arabidopsis* are involved in the transport of cadmium from the cytoplasm to the vacuole (Fox and Guerinot, 1998). In turn, LCT1 cloned from wheat roots (however expressed in both roots and leaves), a non-selective transmembrane transporter for Na^+, K^+ (Schachtman et al., 1997; Amtman et al., 2001) and for Ca^{2+}, also appeared to mediate Cd^{2+} transport to the cell (Clemens et al., 1998). Such toxic metal ions as Cd^{2+} or Pb^{2+} are known as very effective substituents of Ca^{2+}, e.g. in calmodulin, consequently seriously interfering with the role of this cation in a number of metabolic processes (Habermann et al., 1983; Cheung, 1984; Richardt et al., 1986). In this context it seems possible that the regulation of heavy-metal uptake/transport in a plant by the presence of calcium in various concentrations in the medium could in part contribute to an ameliorative effect of calcium on heavy-metal toxicity.

To gain insight into the molecular mechanism of Ca^{2+}-dependent Cd^{2+}-tolerance, tobacco was transformed with wheat cDNA LCT1, the first cloned plant influx system mediating the uptake of both Ca^{2+} and Cd^{2+} ions into a cell (Clemens et al., 1998). Transformants were tested for the possible involvement of *LCT1* in diminishing Cd-toxicity with the enhancement of Ca^{2+} concentration in the medium.

Here we demonstrate that *LCT1* is involved in calcium acquisition and in the alleviation of toxic effects of Cd^{2+} by enhanced external Ca^{2+} concentration.

2. Material and methods

2.1. Construction of transgenic plants

The coding region of the wheat low-affinity cation transporter gene (LCT1) (Schachtman et al., 1997; Clemens et al., 1998) was excised from the pYES/LCT1 plasmid with *Eco*RI and *Xba*I. The 1982 bp fragment was cloned into vector pFF19 (Timmermans et al., 1990), digested with *Bam*HI/*Xba*I and then the enhanced CaMV 35S promoter/LCT1/3′NOS expression cassette was cloned as an *Eco*RI–*Cla*I fragment into the binary vector pGA482 and transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Leaf discs of *N. tabacum* cv. Burley LA21 were transformed and kanamycin-resistant plants regenerated on MS medium according to standard methods (Murashige and Skoog, 1962; Horsch et al., 1985). The presence of the transgene was checked by PCR with *LCT1*-specific primers (5′-TTTAGCG-CATCCAAGTCCAAGGTC-3′)/5′-GCATCCG-TAGCCCCCTTCTTCTCT-3′). *LCT1* expression in T^0 plants was confirmed by the Northern technique according to the standard procedure (Sambrook et al., 1989). T^0 plants expressing *LCT1* were grown to maturity and self-pollinated. T^1 seeds were germinated on plates containing Knop's medium, 2% sucrose, 200 μg/ml kanamycin and 1.2% agar to determine the segregation ratios of kanamycin-resistant (Km^r) to kanamycin-sensitive seedlings. Km^r T^1 plants were cultivated further in pots in a growth chamber, 23/16 °C day/night, 16 h photoperiod and quantum flux density (PAR) 250 μmol $m^{-2}s^-$ fluorescent Flora tubes and tested for *LCT1* presence by PCR.

Km^r T^1 plants were used exclusively for the determination of the phenotye of *LCT1*-transformants. As a control, plants transformed with vector pGA482 were used (the absence of LCT1 was confirmed by PCR).

2.2. Tests for Ca^{2+} and Cd^{2+} sensitivity and accumulation

2.2.1. Short-term experiments

Five-day-old Km^r T^1 seedlings were transferred to plates containing 1/4 Knop's medium, 2% sucrose, 1.2% agar and a range of tested concentrations of $Ca(NO_3)_2$: (0.01, 1, 3, 10 mM) and $Cd(NO_3)_2$ (0 and 0.05 mM). From yeast experiments it is known that Cd^{2+}-uptake is saturable in the high-affinity range with $K_M = 0.033$ mM (Clemens et al., 1998). They were cultivated for a further 3 days, in some cases up to 8 days. Sensitivity to external calcium and cadmium was assessed based on measurements of the root growth rate (Wilkins, 1978). A "tolerance index" (TI) was calculated from the ratio:

$$\mathrm{TI} = \frac{\text{root growth increment in(+Cd) treatment}}{\text{root growth increment in control(−Cd)}} \times 100$$

2.2.2. Long-term experiments

Three-week-old plants grown on perlit on Knop's basal medium with the addition of 200 μg/ml kanamycin were transferred to a hydroponic culture on a medium of the same composition without the antibiotic. After 10 days of adjustment to the new growth conditions, plants of equal size (5-leaf stage) were chosen for further experiments. They were cultivated for the next 7 days in constantly aerated pre-treatment solutions containing basal medium with modified $Ca(NO_3)_2$ concentrations: 1 and 3 mM. Afterwards the medium was replaced with a fresh one supplemented with $Cd(NO_3)$ (0 and 0.05 mM) and the plants were grown for a further 7 days. During pre-treatment and the incubation the medium was changed every day to maintain the ion concentration at the same level.

To determine calcium and cadmium concentrations in plant tissues, roots were rinsed briefly with deionized water, roots and shoots separated and dried in a 55 °C oven for 2 days. Wet ashing was performed in 65%

HNO_3 and 39% H_2O_2 (9:1) at 180 °C for 15 min in a closed system microwave mineralizator (Milestone ETHOS plus). Cadmium and calcium content was determined by flame atomic absorption spectrophotometry (TJA Solution Solar M).

3. Results

3.1. LCT1 tobacco transformation and expression

Out of seven T^0 independent *LCT1*-positive lines (PCR analysis, data not shown) *LCT1* expression was confirmed in two of them (Fig. 1). Germination of T^1 seeds in the presence of kanamycin showed different segregation ratios in both lines: 78% ± 5 of T^1 seedlings of line no. 1 were K^r, whereas in the case of line B7 21% ± 6 . Thirty T^1 Km^r plants from line no. 1 and 30 from no. B7 were screened by PCR for the presence of *LCT1* and all of them contained the gene.

All experiments aimed at determining the transgenic plant phenotype were performed on Km^r plants of line no. 1.

3.2. LCT1 improved plant performance at low external calcium

The difference in the growth responses of *LCT1*-transformed and control plants to the range of external Ca^{2+} concentrations (0.01; 1, 3 and 10 mM), determined on agar plates is shown in Fig. 2. Root elongation of control plants in the presence of 0.01 and 1 mM Ca^{2+} was substantially lower in comparison with *LCT1*-transformed ones, which clearly demonstrates that the expression of *LCT1* improved tobacco performance under these lower calcium concentrations. However, both plant lines developed equally well on plates containing 3 and 10 mM Ca^{2+}.

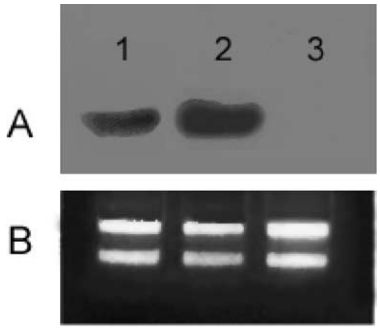

Fig. 1. A: Confirmation of *LCT1* gene expression in two transgenic lines. The presence of *LCT1* mRNA was detected by Northern blotting (10 μg of total RNA were loaded per lane). Lanes 1–2: transgenic lines respectively—no. 1; B7; lane 3—control RNA isolated from *N. tabaccum* Xanthi. B: Ethidium-bromide-stained total RNA present on the original gels.

3.3. LCT1 contributes to Ca^{2+}-dependent Cd^{2+}-tolerance of plants

For experiments pursued to check whether *LCT1*-expression would influence a Ca^{2+}-protective effect against the harmful action of cadmium, two external calcium concentrations (chosen as a result of experiments described in the previous section) were employed: 1 mM (at which the phenotype of *LCT1* transformed plants was different from controls), and 3 mM (no difference in the response of both plant lines). The results of experiments performed on agar plates on small seedlings are shown in Fig. 3. Calcium appeared to alleviate the toxicity of Cd^{2+} to both control and *LCT1*-transformed plants, but to a different degree. The

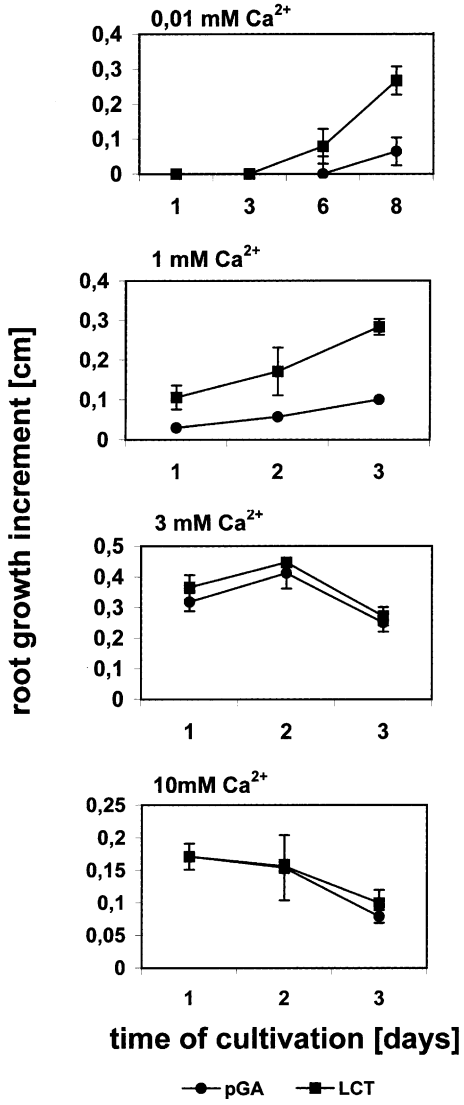

Fig. 2. Root growth increment of two lines of tobacco plants: control (pGA—transformed with the empty plasmid pGA482) and *LCT1*-transformed (LCT—transformed with pGA482/LCT1) cultivated on medium solidified with agar [basal medium supplemented with the range of Ca^{2+} concentrations: 0.01; 1; 3; 10 mM $Ca(NO_3)_2$]. Error bars represent S.D. at $P \leqslant 0.05$ ($n = 30$).

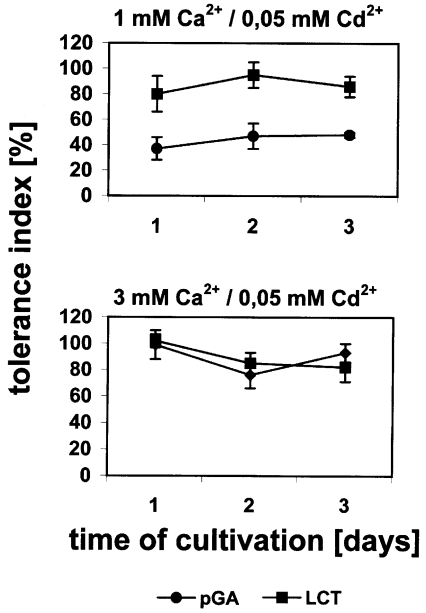

Fig. 3. Tolerance Index (growth of experimental roots expressed as percentage of controls) during 3 days of exposure to 0.05 mM $Cd(NO_3)_2$ administered in the presence of two calcium concentrations: 1 and 3 mM $Ca(NO_3)_2$. Seedlings cultivated on medium solidified with agar. PGA—control line transformed with the empty plasmid pGA482; LCT—plants transformed with pGA482/LCT1. Error bars represent S.D. at $P \leqslant 0.05$ ($n = 30$).

administration of 0.05 mM Cd^{2+} in the presence of 1 mM Ca^{2+} was highly toxic for control plants and caused a reduction of root elongation by 50–60%, while added together with 3 mM Ca^{2+} it did not change root growth in comparison with plants that were not exposed to cadmium. In turn, the presence of *LCT1* in transformed plants protected them from cadmium toxicity, thus the Tolerance Index (TI) measured for those subjected to 1 mM Ca^{2+}/0.05 mM Cd^{2+} was close to 100% (i.e. much higher than for controls). However, upon addition of 0.05 mM Cd^{2+} to the media containing 3 mM Ca^{2+} there was no difference detected in the TI value between transformed and non-transformed plants, and for both lines the TI was close to 100%, manifesting almost no growth inhibition due to the presence of Cd^{2+}.

3.4. *LCT1* increased calcium and decreased cadmium accumulation in transformed plants

The administration of 0.05 mM Cd^{2+} to the hydroponic culture for 7 days to 6-week-old plants precultured for 1 week under two calcium regimes (1 and 3 mM), confirmed the results obtained from experiments performed on agar plates.

There were almost no toxic effects of Cd^{2+} on both control and transformed plants when the metal was administered in the medium containing 3 mM Ca^{2+}. Leaves were green with only a few spots of dead cells (Fig. 4A and B). The adverse effects of cadmium were visible in the presence of 1 mM Ca^{2+}, but control plants were much more severely damaged than the *LCT1*-transformed ones. As shown in Fig. 4C, the oldest leaf of the control plant had large wilted areas and numerous spots of dead cells, whereas that from a transformed plant had only smaller and less numerous areas of dead cells (Fig. 4D). It is worth noting that this difference was accompanied by the same level of cadmium concentration in shoots (Fig. 5), indicating more efficient Cd-detoxification by transformants. Interestingly, in both plant lines cultivated at 1 mM Ca^{2+}, the cadmium concentration in shoots was at the same level, but less cadmium was detected in the roots of transformed plants (Fig. 5).

Regarding calcium accumulation, the difference between *LCT1*-expressing and control plants was detected only at 1 mM of external Ca^{2+} (Fig. 6). Transformed plants grown without cadmium accumulated slightly more calcium in shoots than control ones. However, when 0.05 mM Cd^{2+} was added to the medium containing 1 mM Ca^{2+} the picture reversed and the calcium concentration slightly increased in roots of *LCT1*-transformed plants, whereas its transport to shoots was restricted, leading to decreased shoot calcium accumulation in both plant lines in comparison with the conditions without cadmium (Fig. 6).

Both cadmium and calcium concentrations in experimental plants were at the same level when 3 mM Ca^{2+} was present in the medium (Figs. 5 and 6).

4. Discussion

For the time being there is no data on which the molecular mechanism underlying the phenomenon of Ca^{2+}-dependent heavy metal tolerance can be elucidated. In this study, LCT1, the putative plasmalemma non specific transporter for Ca^{2+}, Cd^{2+}, Na^+ and K^+ (Schachtman et al., 1997; Clemens et al., 1998; Amtmann et al., 2001) was used for tobacco transformation in order to check whether the toxicity of cadmium administered in a range of Ca^{2+} concentrations to control and *LCT1*-transformed plants would be different. From yeast experiments (Clemens et al., 1998) it is known that the uptake of Ca^{2+} and Cd^{2+} by *LCT1*-expressing cells was increased, as was their sensitivity to extracellular calcium and cadmium concentrations. It was shown that the *LCT1*-dependent Ca^{2+}-uptake was linear up to the concentration of 3 mM. In turn, Cd^{2+}-uptake was saturable in the high-affinity range with $K_M = 0.033$ mM. It was also demonstrated that *LCT1*-mediated Ca^{2+} and Cd^{2+} uptake was mutually blocked by these ions. At 0.03 mM Cd^{2+} in the uptake solution, half-maximal inhibition of *LCT1*-mediated Cd^{+2} uptake was 0.025 mM for Ca^{2+}. Regarding cadmium, 0.1 mM Cd^{2+} blocked *LCT1*-dependent

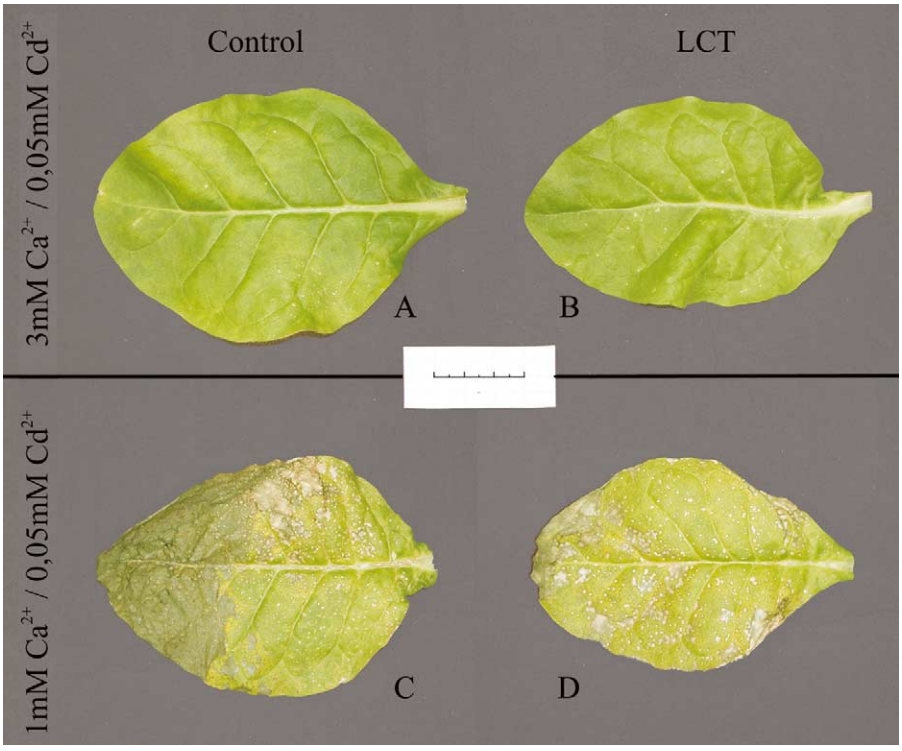

Fig. 4. Appearance of the oldest leaf of the 6-week-old control (transformed with empty plasmid pGA482) and *LCT1*-transformed plants cultivated in hydroponic culture [7 days of preculture in two calcium regimes—1 and 3 mM $Ca(NO_3)_2$ were followed by 7 days of treatment with 0.05 mM $Cd(NO_3)_2$ administered in the presence of 1 and 3 mM Ca^{2+}].

Ca^{2+}-uptake by around 50% when the Ca^{+2} concentration was also 0.1 mM. The competition of Ca^{2+} and Cd^{2+} ions for binding sites was also implicated in other papers (Cheung, 1984; Kahn et al., 1984; Richardt et al., 1986). Since the amelioration of heavy metal toxicity by calcium often involves changes in the uptake of the metal or its accumulation (Baker, 1978; Simon, 1978) the fact that Ca^{2+} and Cd^{2+} ions compete for the *LCT1*-mediated transport made *LCT1* a candidate for a gene potentially involved in the regulation of the phenomenon of Ca^{2+}-dependent Cd^{2+}-toxicity at the uptake level.

4.1. LCT1 improved plant performance at low external calcium

LCT1 expression in tobacco improved the growth of plants only within a limited range of calcium concentrations: 0.01–1.0 mM Ca^{2+} but importantly, they fit the range of soil solution calcium concentrations (Piñeros and Tester, 1995). This indicates that the LCT1-encoded protein may contribute to plant calcium acquisition. In the presence of 3 and 10 mM Ca^{2+}, the performance of both plant lines (Fig. 2) as well as calcium accumulation (Fig. 6) did not differ significantly. In contrast, growth of *LCT1*-expressing yeast cells at 3 mM Ca^{2+} was strongly reduced, while in the presence of 0.01 mM Ca^{2+}, control and *LCT1*-expressing cells grew equally well (Clemens et al., 1998). Such a conflicting outcome might result from the different calcium requirement of unicellular yeast and higher plants. It is highly likely that the activity of an introduced Ca^{2+} transport system resulting from the expression of *LCT1* and allowing calcium ions to enter cells, stimulates the activity of the system maintaining a low nM cytosol Ca^{2+} concentration, as was already described when cytosol Ca^{2+} increases (Evans and Williams, 1998; Geisler et al., 2000a,b). Possibly, the unicellular yeast cultivated at 3 mM Ca^{2+} was not able to readjust the cytosol calcium concentration which had been enhanced as a result of *LCT1* activity, back to low nM levels. In turn, plants take up ions to maintain the growth of a whole, multicellular body, therefore the need for calcium is higher than for a single yeast cell. Ca^{2+} ions absorbed by an epidermal or cortical cell of the root are transported radially across the root tissues through apoplastic and symplastic pathways to be finally delivered into the conductive tissue, and then further up to the shoot.

The improvement of the performance of transformants growing at 1 mM Ca^{2+} was accompanied by an unchanged root calcium level as compared with pGA control plants, and a small increase of the shoot Ca concentration (Fig. 6), indicating enhanced Ca^{2+} uptake. This small excess of calcium was not retained in roots but transported to shoots. Such results could be ascribed to the activity of *LCT1*, not only in roots but also in shoots, likely contributing to more efficient

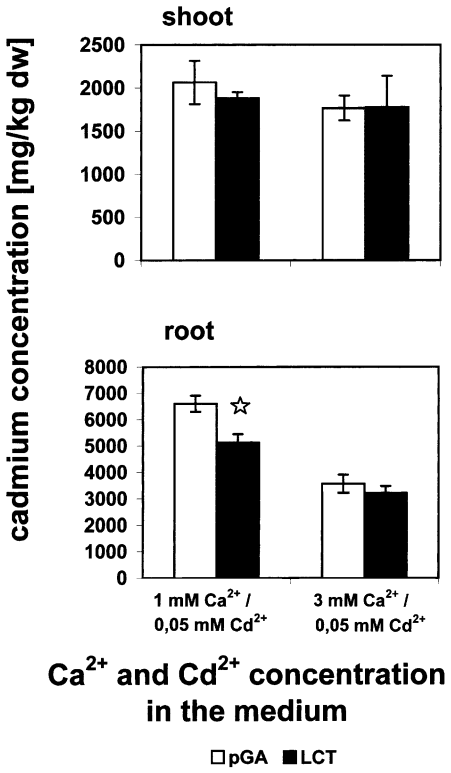

Fig. 5. Cadmium concentration in the shoot and root of control and LCT1-transformed plants cultivated in hydroponic culture [7 days of preculture in two calcium regimen—1 and 3 mM $Ca(NO_3)_2$ were followed by 7 days of treatment with 0.05 mM $Cd(NO_3)_2$ administered in the presence of 1 mM and 3 mM Ca^{2+}]. PGA—control line transformed with the empty plasmid pGA482; LCT—plants transformed with pGA482/LCT1. Error bars represent S.D. at $P \leqslant 0.05$ ($n = 3$). ☆—significantly different from the corresponding control (Student's t-test at $P \leqslant 0.05$). As standards for the control of the elemental analysis, Virginia tobacco leaves (CTA-VTL-2) were used and the results fit the range of certified recommended values).

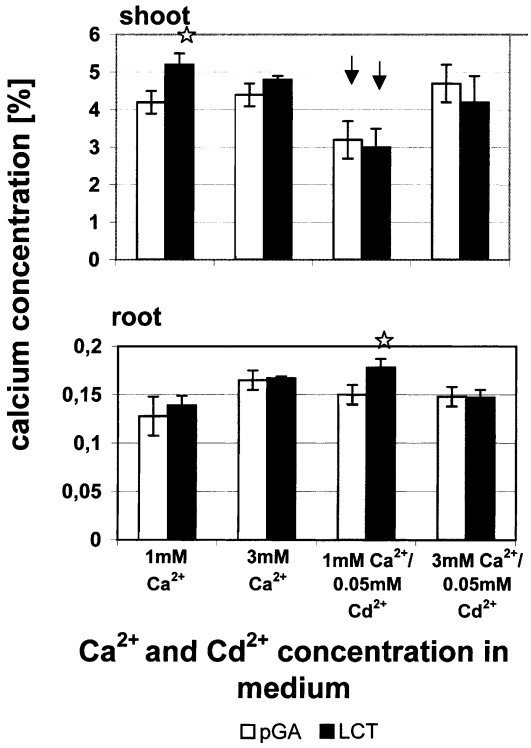

Fig. 6. Calcium concentration in the shoot and root of 6-week-old control and LCT1-transformed plants cultivated in hydroponic culture [7 days of preculture in two calcium regimes—1 and 3 mM $Ca(NO_3)_2$ were followed by 7 days of treatment with 0.05 mM $Cd(NO_3)_2$ administered in the presence of 1 and 3 mM Ca^{+2}]. PGA—control line transformed with the empty plasmid pGA482; LCT—plants transformed with pGA482/LCT1. Error bars represent S.D. at $P \leqslant 0.05$ ($n = 3$). ☆—significantly different from the corresponding control (Student's t-test at $P \leqslant 0.05$). →—significantly different from the corresponding values for calcium concentrations in shoots of plants cultivated at 1 mM Ca^{2+}. As standards for the control of the elemental analysis, Virginia tobacco leaves (CTA-VTL-2) were used and results fit the range of certified recommended values).

calcium movement within a plant body. Schachtman et al. (1997) found that *LCT1* undergoes expression in both roots and leaves of *Triticum aestivum* (other organs were not studied), so the above suggestion seems probable. Considering the role of LCT1 in transport across the plasmalemma, the question still remains open whether its protein localises to the plasmalemma (delivering calcium to the cytosol) or to other internal membranes. The results of Clemens et al. (1998) demonstrating *LCT1*-mediated calcium and cadmium uptake by yeast cells and the current ones showing higher calcium shoot accumulation and better performance on low calcium of *LCT1*-transformed plants (Figs. 2, 4 and 6) makes the plasmalemma localisation highly likely. However, further study specifically addressing this question are required.

4.2. *What could be the possible contribution of* LCT1 *to* Ca^{2+}*-dependent* Cd^{2+}*-tolerance?*

Numerous authors have described the phenomenon of calcium mitigating heavy metal toxicity (Baker, 1978; Simon, 1978; Karataglis, 1981; Rashid and Popovic, 1990; Skórzyńska-Polit et al., 1998; Skórzyńska-Polit and Baszyński, 2000). The reported amelioration constitutes a part of the whole-plant defence system that includes both uptake/transport and detoxification/sequestration. Based on the known broad spectrum of the role of Ca^{2+} in the regulation of metabolic processes (Bush, 1995), calcium might be an important factor in any out of these components. It is not known whether the observed reduced toxicity might result from less cadmium uptake or from more efficient detoxification. For example Choi et al. (2001) demonstrated the contribution of calcium to the protection mechanism by immobilization of the metal as co-precipitates with calcium and phosphorous. Reduction of cadmium uptake and accumulation by calcium was also reported for several plant species (John, 1976; Hardiman and Jacoby, 1984; Godbold, 1991) as was the opposite effect—inhibition of the accumulation of calcium by cadmium, leading to calcium deficiency (Burzyński,

1987). Because Ca^{2+} and Cd^{2+} ions compete for binding sites, their concentration in the uptake solution could be decisive for the resulting transport across a membrane (Habermann et al., 1983; Cheung, 1984; Richardt et al., 1986; Tester, 1990; Godbold, 1991). Since *LCT1* is permeable, among others, to both Ca^{2+} and Cd^{2+}, plant cadmium sensitivity was studied at two Ca^{2+} concentrations—1 mM, at which *LCT1* expression increased plant performance, and at 3 mM, at this calcium concentration both types of plants grew alike (Fig. 2).

Our results revealed that the control tobacco plants used for experiments possess their own system regulating Ca^{2+}-dependent Cd^{2+}-tolerance. This was manifested by the considerable alleviation of cadmium toxicity in the presence of 3 mM of external Ca^{2+} in comparison with 1 mM Ca^{2+} (Fig. 3). Such a result was not a surprise, since this phenomenon has already been reported for many plants, and not only for cadmium but also for other heavy metals like lead, zinc (Baker, 1978; Simon, 1978; Karataglis, 1981; Rashid and Popovic, 1990; Skórzyńska-Polit et al., 1998), aluminium (Rengel, 1992) as well as for Na^{+}-toxicity (Epstein, 1998). However, in tobacco the protective effect of calcium was enhanced at a lower (1 mM Ca^{2+}) concentration as the result of transformation with *LCT1* (Figs. 3 and 4C, D), suggesting a contribution of *LCT1* to the control of calcium-dependent cadmium-tolerance. The enhanced level of Cd-tolerance at 1 mM Ca^{2+} due to *LCT1*-expression was accompanied by a decreased root cadmium concentration (Fig. 5), although this decrease was not proportionate to the rise of TI (Fig. 3). However, it is well known that there is the general lack of a proportion between the level of tolerance to a metal and the amount of it accumulated in plant tissues (Baker and Walker, 1989; Wierzbicka, 1999). Since this decrease was not dramatic and has been detected only in roots, while in shoots with the same cadmium concentration the extent of leaf impairment was visibly lower in transgenic plants (Fig. 4C, D), the implication is that *LCT1* could also be involved there in more efficient cadmium detoxification. The fact that *LCT1* undergoes expression in both roots and leaves in *Triticum aestivum* (Schachtman et al., 1997) gives additional support to the idea that the gene functions in both above-mentioned plant parts. In general, *LCT1*-transformed and control plants accumulated very high amount of cadmium (Fig. 5). To compare, Matthews and Thornton (1982) found that Cd concentration in shoots of pasture plants inhabiting soil containing 3 mg Cd/kg was 2–4 mg Cd/kg d.w. [according to Lagerwerf and Specht (1970) many agricultural soils contain approximately 1 mg Cd/kg over-dry soil]. Most of cadmium taken up by plants is retained in roots (Baker and Walker, 1989). However, tobacco is known from its particular efficiency in accumulating this pollutant into leaves. Studies of Wagner and Yeargan (1986) demonstrated that *Nicotiana tabaccum* var KY14 grown in gravel culture containing 3 μM Cd^{2+} accumulated respectively 172 and 110 mg Cd/kg d.w. in shoots and roots. Further detailed studies will be conducted to elaborate more on the role of *LCT1* in the amelioration of cadmium-toxicity by calcium, including a detailed study of the relationship between the amount of accumulated cadmium and the sensitivity of *LCT1*-transformed plants to the metal.

Having found that *LCT1* mediated transport of both competing Ca^{2+} and Cd^{2+} ions across the plasmalemma in yeast (Clemens et al., 1998) it seemed likely that the encoded protein could function as a non-specific Ca^{2+} transporter as well as a Ca^{2+}-dependent entry path for cadmium ions in planta. Indeed, *LCT1* overexpression improved plant growth on 0.01–1 mM Ca^{2+} and increased the calcium concentration in shoots. However, at 1 mM Ca^{2+}/0.05 mM Cd^{2+} transformants had a higher Tolerance Index (were less susceptible to cadmium) and unexpectedly accumulated less cadmium than pGA control plants. Such a result could be ascribed to the block of tobacco constitutive cadmium transport as a result of *LCT1* activity. Taken together, the theoretical hypothesis could be forwarded that due to *LCT1*-expression, upon exposure to 1 mM Ca^{2+}/0.05 mM Cd^{2+}, additional calcium transport takes place, which could interfere with calcium-mediated signalling, leading to a partial block of constitutive cadmium uptake and lowering its accumulation in the roots of transformed plants. Thus, LCT1 does not seem to be a major uptake pathway for cadmium.

Recent works report the direct involvement of intracellular calcium signalling that mediates a similar phenomenon of Ca^{2+}-dependent salt-tolerance, i.e. the amelioration of the toxic effects of salt on plants with an increase in the Ca^{2+} concentration (Epstein, 1998). Several possible Ca^{2+}-signalling pathways have been suggested (Geisler et al., 2000a,b; Pandey et al., 2002). The study of the possible involvement of LCT1 in the regulation of Ca^{2+}-dependent Na^{+}-toxicity performed on *LCT1* expressing G19 yeast strain did not give a positive answer (Amtmann et al., 2001). The phenomenon of the beneficial influence of Ca^{2+} on Na^{+} toxicity was detected as a general one observed in both control and *LCT1*-transformed cells. The involvement of *LCT1* in the amelioration of cadmium toxicity by calcium demonstrated in this paper will be subjected to further detailed study to elaborate more on the mechanism of this relationship.

In summary, we have demonstrated the improvement of growth of *LCT1*-transformed tobacco on low, physiological calcium concentrations (0.01–1 mM Ca^{2+}) and a small increase in calcium accumulation in shoots, which points to a role of LCT1 in calcium acquisition. In addition, *LCT1*-expression enhanced Cd^{2+}-tolerance when 1 mM Ca^{2+} was present in the medium and led to

a decreased root cadmium concentration. These results indicate that due to *LCT1* expression, constitutive Cd^{2+} transport was blocked, leading to lower cadmium accumulation, likely by involvement in the signalling processes. At 3 mM of medium Ca^{2+}, no difference was detected between both plant lines in their responses to external cadmium and calcium under the applied experimental conditions. LCT1 is the first gene reported to be involved in the regulation of Ca^{2+}-dependent Cd^{2+}-tolerance.

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