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CHL1 Encodes a Component of the Low-Affinity Nitrate Uptake System in Arabidopsis and Shows Cell Type–Specific Expression in Roots

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The Arabidopsis *CHL1* (*AtNRT1*) gene confers sensitivity to the herbicide chlorate and encodes a nitrate-regulated nitrate transporter. However, how CHL1 participates in nitrate uptake in plants is not yet clear. In this study, we examined the in vivo function of CHL1 with in vivo uptake measurements and in situ hybridization experiments. Under most conditions tested, the amount of nitrate uptake by a *chl1* deletion mutant was found to be significantly less than that of the wild type. This uptake deficiency was reversed when a *CHL1* cDNA clone driven by the cauliflower mosaic virus 35S promoter was expressed in transgenic *chl1* plants. Furthermore, tissue-specific expression patterns showed that near the root tip, *CHL1* mRNA is found primarily in the epidermis, but further from the root tip, the mRNA is found in the cortex or endodermis. These results are consistent with the involvement of *CHL1* in nitrate uptake at different stages of root cell development. A functional analysis in Xenopus oocytes indicated that CHL1 is a low-affinity nitrate transporter with a K_m value of ~8.5 mM for nitrate. This finding is consistent with the chlorate resistance phenotype of *chl1* mutants. However, these results do not fit the current model of a single, constitutive component for the low-affinity uptake system. To reconcile this discrepancy and the complex uptake behavior observed, we propose a "two-gene" model for the low-affinity nitrate uptake system of Arabidopsis.

INTRODUCTION

Most plants can satisfy their nitrogen requirements by assimilating nitrate. Therefore, nitrate uptake and transport are vital processes in plant growth. However, soil nitrate concentrations available to plants can vary by four orders of magnitude (Crawford, 1995). To adapt to such environmental variation, plants have evolved highly regulated and multiphasic nitrate uptake systems. In several plant species, nitrate uptake has been shown to be biphasic with a high-affinity transport system (HATS; K_m between 5 and 300 μM) and a low-affinity transport system (LATS; showing linear kinetics or K_m values >0.5 mM) (Doddema and Telkamp, 1979; Goyal and Huffaker, 1986; Siddiqi et al., 1990; Glass et al., 1992). Both systems employ electrogenic H^+/NO_3^- symporters (McClure et al., 1990; Ullrich and Novacky, 1990; Ruiz-Cristin and Briskin, 1991; Glass et al., 1992) and are subject to different modes of regulation (Behl et al., 1988; Warner and Huffaker, 1989; Siddiqi et al., 1990; Aslam et al., 1992; Glass et al., 1992; Kronzucker et al., 1995). In several plant species, including maize (Hole et al., 1990), barley (Aslam et al., 1992), and spruce (Kronzucker et al., 1995), HATS has constitutive and nitrate-inducible components (reviewed in Glass and Siddiqi, 1995). In contrast, LATS has thus far been shown to display only constitutive activity (Siddiqi et al., 1990; Glass et al., 1992; Kronzucker et al., 1995). Based on these results, the current model for nitrate uptake in plants is that there are three components, a constitutive high-affinity system, an inducible high-affinity system, and a constitutive low-affinity system (Glass and Siddiqi, 1995).

Structural genes can be identified and used to probe the intricate process of nitrate uptake and transport to test this model. *CHL1* (*AtNRT1*) is a nitrate transporter gene identified in Arabidopsis (Tsay et al., 1993). It was isolated from a T-DNA–tagged, chlorate-resistant mutant (Tsay et al., 1993). Chlorate is a nitrate analog that can be taken up by plants and then reduced by nitrate reductase to chlorite, which is toxic (Åberg, 1947). Most chlorate-resistant plants are defective in nitrate reductase activity, but *chl1* is an exception (Doddema et al., 1978; Braaksma and Feenstra, 1982; Crawford and Arst, 1993; Hoff et al., 1994; Crawford, 1995). *chl1* mutants were shown to be defective in nitrate/chlorate uptake in the low-affinity range (concentrations >1 mM; Doddema and Telkamp, 1979). Cloning of the *CHL1* gene revealed that it encodes a hydrophobic protein with 12 putative transmembrane segments, which is a topology found in many cotransporters (Saier, 1994). Injection

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of *CHL1* mRNA into Xenopus oocytes resulted in a nitrate- and pH-dependent current, indicating that *CHL1* encodes an electrogenic, proton-coupled nitrate transporter. *CHL1* mRNA is found predominantly in roots, and interestingly, its level is transiently induced by nitrate. Low levels of *CHL1* mRNA are found in plants grown with ammonium as the sole nitrogen source, but when the ammonium-grown plants are exposed to nitrate, the level of *CHL1* mRNA begins to rise within 30 min and peaks at 2 hr. After 4 hr of nitrate exposure, the level declines, suggesting negative feedback regulation (Tsay et al., 1993).

Taken together, these data indicate that CHL1 is a nitrate-inducible, proton-coupled nitrate transporter (Tsay et al., 1993). However, in plants, the evidence for direct involvement of *CHL1* in nitrate uptake comes from a single study by Doddema and Telkamp (1979). In addition, if *CHL1* indeed is a component of the LATS system, as suggested from the phenotype of the *chl1* mutants, this finding seems to conflict with the current model that the low-affinity transporter is constitutively expressed. To address these issues, we performed a more detailed characterization of the biological function of *CHL1*. Based on the results obtained, we propose a "two-gene" model for the LATS nitrate uptake system for Arabidopsis.

RESULTS

Tissue-Specific Expression Pattern of the *CHL1* Gene Is Consistent with a Role in Uptake

Because RNA analysis had shown that *CHL1* is predominantly expressed in roots (Tsay et al., 1993), our study focuses on the expression pattern of *CHL1* in root tissues. Spatial distribution patterns of *CHL1* mRNA in different root tissues were analyzed by in situ hybridization. Growth conditions to maximize *CHL1* mRNA levels were used as established previously (Tsay et al., 1993). Plants were cultivated on vertical agar plates with ammonium as the sole nitrogen source for 10 days and then shifted to a nitrate-containing medium for 2 hr. Roots of the plants were then fixed and sectioned. These sections were hybridized with ^{35}S-labeled sense and antisense transcripts of the *CHL1* cDNA.

From the exterior to the core, Arabidopsis roots contain one layer of epidermal cells, followed by one to two layers of cortical cells, one layer of endodermal cells, and then the vascular cylinder (Dolan et al., 1993). As shown in Figures 1A and 1B, 1E and 1F, and 1I and 1J, a high density of silver grains was found in the epidermal cells close to the root tip and in both cross (Figure 1B) and longitudinal (Figures 1F and 1J) sections. As a control, similar sections were hybridized with the sense probe that showed only a background level of signal in all layers of the root (Figures 1C and 1D, and 1G and 1H). These results show that in newly differentiated cells (root diameters of 0.13 mm), *CHL1* mRNA accumulation occurs primarily in the epidermal tissue (Figures 1A and 1B, 1E and 1F, 1I and 1J, and 2A and 2B). However, in more mature sections of the root (with diameters between 0.17 and 0.22 mm),

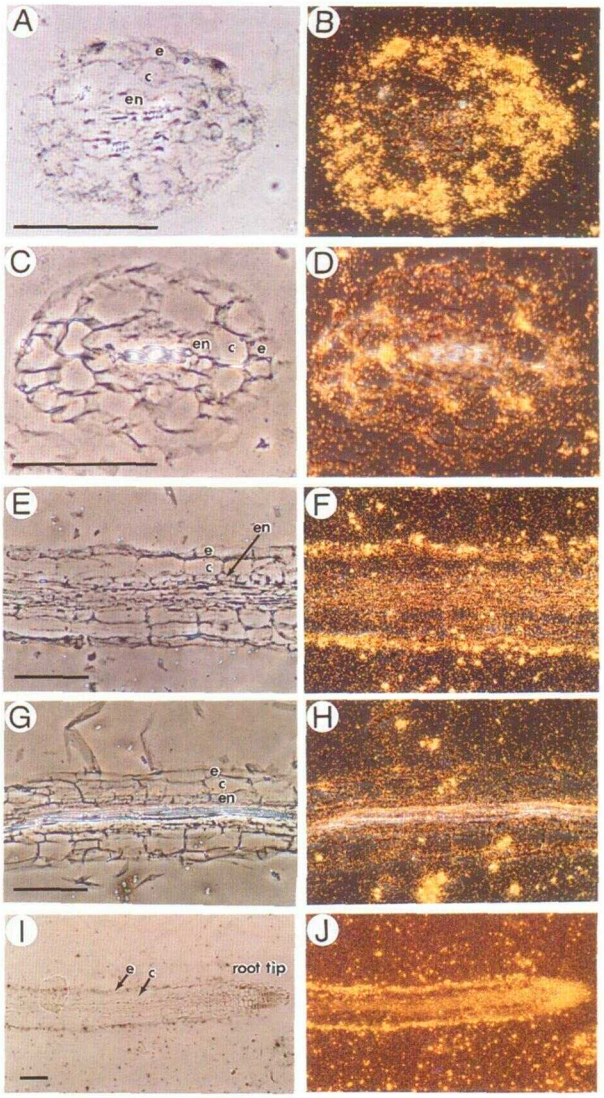

Figure 1. *CHL1* mRNA Accumulates to High Levels in Epidermal Cells of Young Roots.

In situ hybridization of antisense (**[A]** and **[B]**, **[E]** and **[F]**, and **[I]** and **[J]**) and sense (**[C]** and **[D]**, and **[G]** and **[H]**) *CHL1* probes to cross-sections (**[A]** to **[D]**) and longitudinal sections (**[E]** to **[J]**) of Arabidopsis root tissues close to the root tip is shown. Bright-field microscopy is shown in **(A)**, **(C)**, **(E)**, **(G)**, and **(I)**. Double exposures using a colored filter for the dark-field exposure cause the *CHL1* signals to appear yellow in **(B)**, **(D)**, **(F)**, **(H)**, and **(J)**. c, cortical cells; e, epidermal cells; en, endodermal cells.

(A) and **(B)** In the cross-section of the root tissue close to the root tip, *CHL1* mRNA accumulates in epidermal cells.

(C) and **(D)** As a control for **(A)** and **(B)**, only a background level of signal was found in similar sections hybridized with a sense probe.

(E) and **(F)** In the longitudinal section of the root tissue close to the root tip, *CHL1* mRNA was found in epidermal cells.

(G) and **(H)** A sense probe was used as a control for **(E)** and **(F)**.

(I) and **(J)** A lower magnification of **(E)** and **(F)** is shown. In the region close to the root tip, *CHL1* mRNA is expressed primarily in epidermal cells.

Bars in **(A)**, **(C)**, **(E)**, **(G)**, and **(I)** = 100 μm.

CHL1 mRNA accumulation is seen primarily in the cortex (Figures 2C and 2D) or endodermis (Figures 2E and 2F, and 2G and 2H) but not in the epidermis. Control experiments on sections of similar diameters also showed only a background level of signal in all layers of the cells (data not shown). Therefore, the layer of cells in which *CHL1* mRNA accumulates appears to shift inward as one moves upward along the root axis. In the sections that we examined, no *CHL1* mRNA signal was found within the root central cylinder. These results support the model that *CHL1* is directly involved in nitrate uptake. Furthermore, the tissue-specific expression patterns of *CHL1* depend on the developmental stage of the root section.

Nitrate Uptake of Wild-Type, *chl1-5*, and Transgenic Plants Containing 35S-*CHL1*

To elucidate the role of *CHL1* in nitrate uptake, both long-term (Figure 3) and short-term (Figure 4) measurements of nitrate uptake activity by the wild type, the *chl1-5* deletion mutant (Tsay et al., 1993), and transgenic plants containing the cauliflower mosaic virus (CaMV) 35S-*CHL1* cDNA were compared under different growth conditions. The CaMV 35S promoter drives gene expression at high levels in plants (Odell et al., 1985). The transgenic plants used here were constructed by introducing 35S-*CHL1* into *chl1-5*. For long-term measurements, 30 seeds for each of the three Arabidopsis plants were sown in 25 mL of a nutrient solution containing either KNO_3 or NH_4NO_3 as the nitrogen source. After 18 days of growth in 16- to 8-hr day–night cycles, the amount of nitrate depleted from the medium was determined by HPLC (Thayer and Huffaker, 1980).

As shown in Figure 3, nitrate depletion by wild-type plants is essentially the same in either solution. However, depletion by the deletion mutant and transgenic plants was greater in the medium with NA_4NO_3 than with KNO_3. The amount of nitrate depletion by *chl1-5* mutants was only approximately one-eighth that of the wild type when plants were grown in KNO_3, but when plants were grown in NH_4NO_3, the difference was 40%. Expression of the *CHL1* cDNA with the 35S promoter reversed the uptake defect in the deletion mutant so that in transgenic plants, higher depletion levels were observed.

For the short-term measurements, plants were grown in continuous light to avoid the effect of circadian rhythms. Fourteen-day-old plants that had been grown in either 25 mM KNO_3 (Figure 4A) or 25 mM NH_4NO_3 (Figure 4B) were transferred to a medium containing 8 mM KNO_3. In addition, some of the cultures were given a pretreatment with 2 days of nitrogen starvation (Figures 4C and 4D) or 2 days of growth with ammonium as sole nitrogen source (Figures 4E and 4F), before being shifted to the medium containing 8 mM KNO_3. The amount of nitrate depleted from the medium was then monitored for 24 hr, and the results are shown in Figure 4.

Under all of the conditions tested, with the exception of one, wild-type plants depleted significantly more nitrate from the medium than did the *chl1-5* mutant, although the difference between these two lines fluctuated depending on the nitrogen

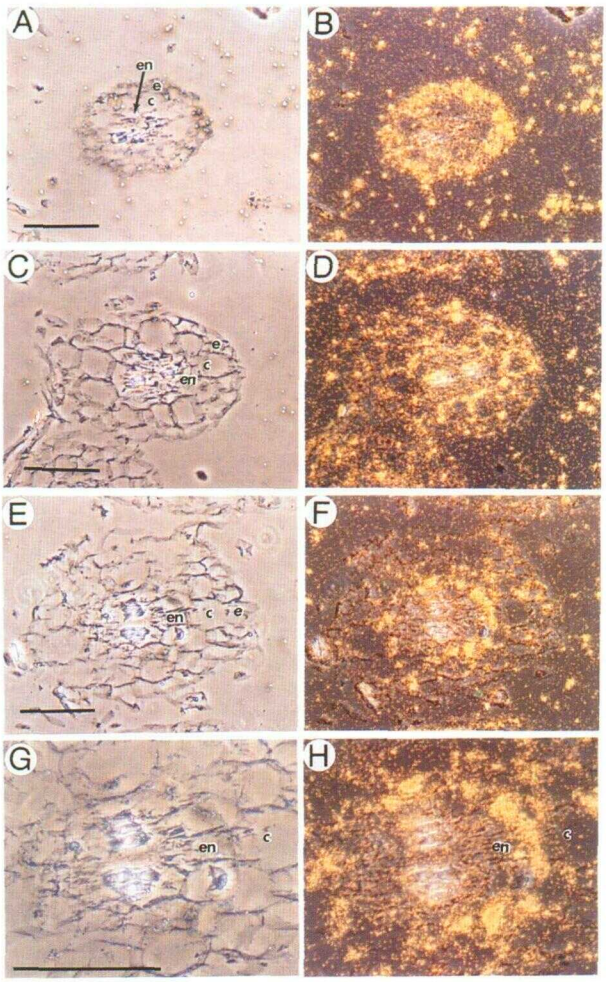

Figure 2. *CHL1* mRNA Accumulation in Older Root Tissues.

In situ hybridization of *CHL1* probes to cross-sections of different regions of Arabidopsis root tissues is shown. The diameters of sections **(A)** and **(B)**, **(C)** and **(D)**, and **(E)** and **(F)** are 0.13, 0.17, and 0.22 mm, respectively. Bright-field microscopy is shown in **(A)**, **(C)**, **(E)**, and **(G)**. Double exposures using a colored filter for the dark-field exposure cause the *CHL1* signals to appear yellow in **(B)**, **(D)**, **(F)**, and **(H)**. c, cortical cells; e, epidermal cells; en, endodermal cells.

(A) and **(B)** In the cross-section with a diameter of 0.13 mm, *CHL1* mRNA accumulates in epidermal cells.

(C) and **(D)** In the cross-section with a diameter of 0.17 mm, *CHL1* mRNA accumulates in the cortex.

(E) and **(F)** In the cross-section with a diameter of 0.22 mm, *CHL1* mRNA accumulates in endodermal cells.

(G) and **(H)** A higher magnification of **(E)** and **(F)** is shown. *CHL1* mRNA signals appear to cluster on the outer tangential side of the endodermal cells.

Bars in **(A)**, **(C)**, **(E)**, and **(G)** = 100 μm.

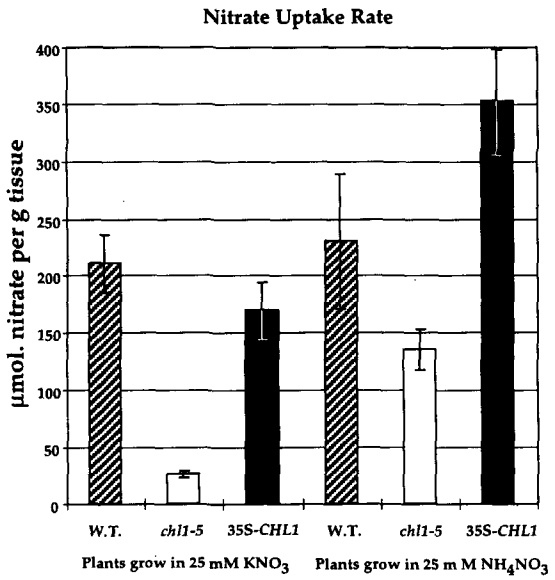

Figure 3. Long-Term (18 Day) Nitrate Uptake Studies of Wild-Type, *chl1-5*, and Transgenic Plants Containing 35S–*CHL1*.

Plants were germinated in a liquid medium with 25 mM potassium nitrate or 25 mM ammonia nitrate as the nitrogen source and grown in 16-hr-day and 8-hr-night cycles. Eighteen days after germination, the amount of nitrate depleted from the medium was measured by HPLC analysis. Values are in micromoles of nitrate per gram fresh weight of plant tissues. When plants were grown in 25 mM potassium nitrate, the fresh weights of *chl1-5* and transgenic plants were ~70 and 92% that of the wild type. Plants grew much better in ammonia nitrate, and there was no significant difference in the fresh weight among these three plants. The average root/shoot ratio of the wild type, *chl1-5*, and transgenic plants was 0.48, 0.56, and 0.29, respectively, for plants grown in potassium nitrate, and 0.23, 0.34, and 0.14 for plants grown in ammonia nitrate, respectively. For plants grown in potassium nitrate, the nitrate uptake rates of *chl1-5* and the transgenic plants were ~13 and 81%, respectively, that of the wild type. However, the same percentage increased to 59 and 153%, respectively, if plants were grown in ammonia nitrate. W.T., wild type.

status of the plant. The one exception was KNO_3-grown plants subjected to 2 days of nitrogen starvation; under this condition, the depletion rate of the wild type and mutants was similar (Figure 4C). The 35S–*CHL1* transgenic plants depleted as much (Figures 4A, 4C, and 4D) or more (Figures 4B, 4E, and 4F) nitrate than did the wild-type plants and significantly more than did the deletion mutants in all cases (Figures 4A and 4B, and 4D to 4F) except one (Figure 4C). Thus, expression of the *CHL1* cDNA in the *chl1-5* mutants reversed the uptake defect.

Kinetic Studies Indicate that CHL1 Is a Low-Affinity Transporter

To determine the nitrate affinity of CHL1, we injected in vitro–synthesized *CHL1* complementary RNA into stage IV and V Xenopus oocytes. Two to 3 days after injection, each whole oocyte cell was bathed in a mannitol solution, pH 7.4, as described previously (Tsay et al., 1993), voltage clamped at −60 mV, and then exposed to nitrate at pH 5.5. As shown in Figure 5 and Table 1, the amplitudes of the inward positive currents elicited by nitrate at pH 5.5 are concentration dependent. For most batches of oocytes, little or no current was elicited by nitrate at pH 5.5 in the water-injected controls (Figure 5). However, for some batches, even in the absence of nitrate, elicitation of a small inward current (<5 nA) was observed in both *CHL1*-injected or water-injected oocytes when the solution was shifted from pH 7.4 to pH 5.5. In these cases, this small current was subtracted from that elicited by nitrate at the same pH value of 5.5. The average K_m value resulting from fitting these nitrate-elicited currents into a Lineweaver-Burk plot is 8.5 mM (Table 1). This result is consistent with the data obtained in the experiments of Doddema and Telkamp (1979) in which the *chl1* mutant was found to be defective in low-affinity nitrate uptake but typical in high-affinity uptake. Thus, *CHL1* serves as a low-affinity nitrate transporter gene in Arabidopsis.

These measurements provide strong evidence that *CHL1* is involved in low-affinity nitrate uptake in vivo. Furthermore, they reveal complex kinetics of nitrate uptake in Arabidopsis that are influenced by the nutrition environment, as has been shown for other plants (reviewed in Glass and Siddiqi, 1995).

DISCUSSION

Our in vivo uptake measurements show that under most of the growth conditions tested, the amount of nitrate taken up by *chl1* mutants was less than that in wild-type plants and this uptake deficiency was effectively corrected in 35S–*CHL1* transgenic plants of the *chl1* mutant. Together with the functional assay in Xenopus oocytes and the mRNA localization data, the collective evidence strongly indicates that *CHL1* (*AtNRT1*) encodes a transporter involved in low-affinity nitrate uptake in plants.

It is clear from the observed mRNA accumulation patterns of *CHL1* that its mRNA levels change along the axis of the root. In regions close to the root tip (at least 0.5 cm away from the tip), *CHL1* mRNA is found primarily in the epidermal cells. However, in sections with more developed stele and xylem (thus with a bigger section diameter), mRNA is found in cells beyond the epidermis but never reaching the vascular cylinder.

Enstone and Peterson (1992) had found that in mature roots of several plant species, including broad bean, pea, corn, onion, and sunflower, the walls of the epidermis and cortex up to the Casparian band of the endodermis are permeable, but the apoplastic permeability of regions near root tips is greatly restricted. Therefore, it is possible that in young root sections (such as regions close to the root tip), where horizontal transport of nitrate into xylem stream may be mediated by the symplastic pathway, nitrate is taken up by CHL1 in the epidermal cells, whereas in older root sections, *CHL1* is expressed mainly in the cortex or endodermis, where the apoplastic

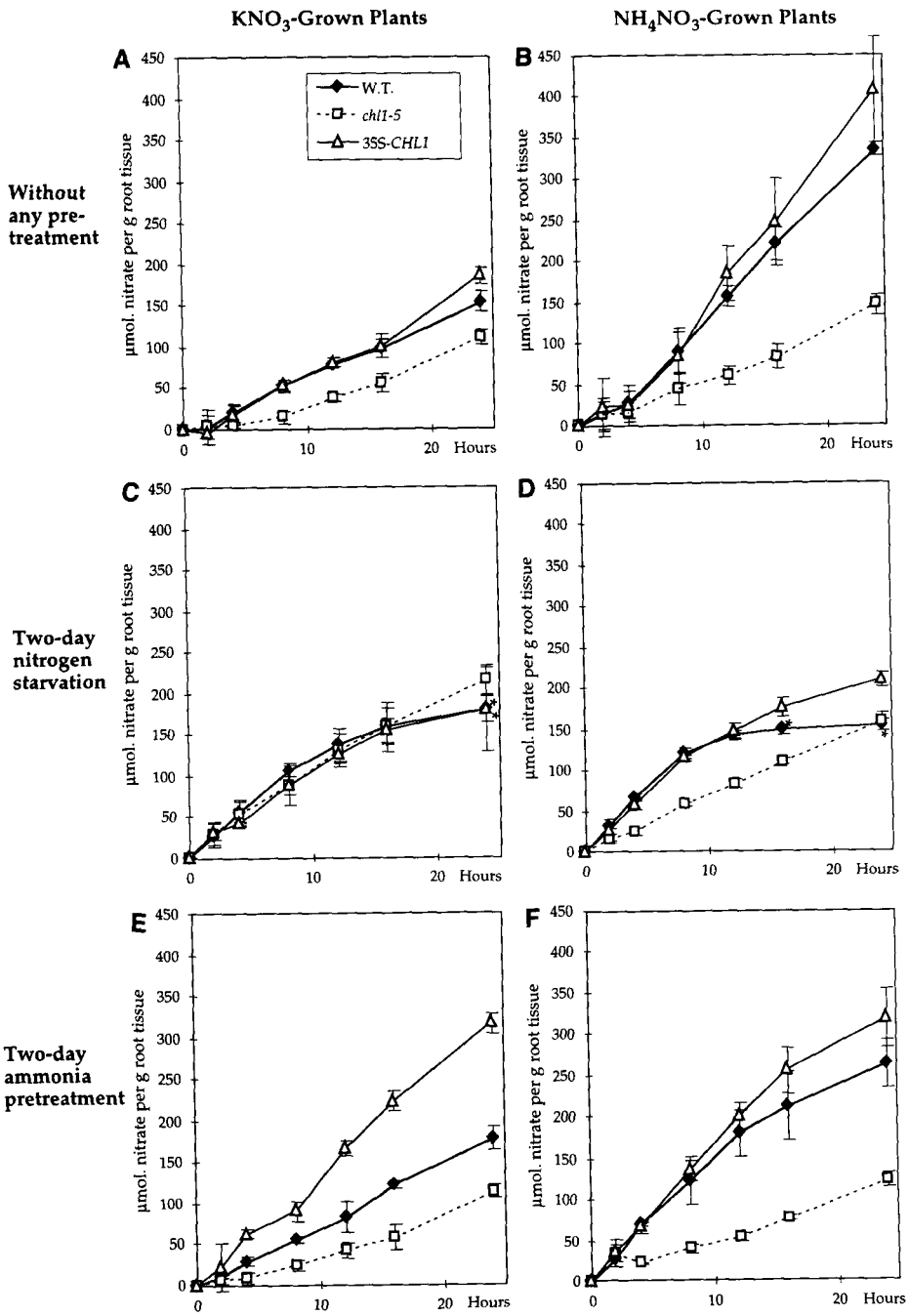

Figure 4. Short-Term (24 hr) Nitrate Uptake Studies of Wild-Type, *chl1-5*, and Transgenic Plants Containing 35S-*CHL1*.

KNO_3-grown plants were grown in 25 mM KNO_3 with 24 hr of illumination. NH_4NO_3-grown plants were grown in 25 mM NH_4NO_3 with 24 hr of illumination. Uptake studies were done with 8 mM KNO_3 as the nitrogen source, and the amount of nitrate depleted from the medium was monitored for 24 hr. Values are in micromoles of nitrate per gram fresh weight of root tissues. The experiments were performed in triplicate; their average and standard deviation are shown. The asterisks indicate that the uptake leveling off is due to a nearly complete depletion of nitrate in the medium. W.T., wild type.

(A) and **(B)** Fourteen-day-old plants were shifted directly from the growth medium to 8 mM KNO_3 for nitrate uptake measurement.

(C) and **(D)** Twelve-day-old plants were nitrogen starved for 2 days before the uptake measurement.

(E) and **(F)** Twelve-day-old plants were shifted to 12.5 mM $(NH_4)_2$ succinate (i.e., ammonium is the sole nitrogen source) for 2 days before the uptake measurement.

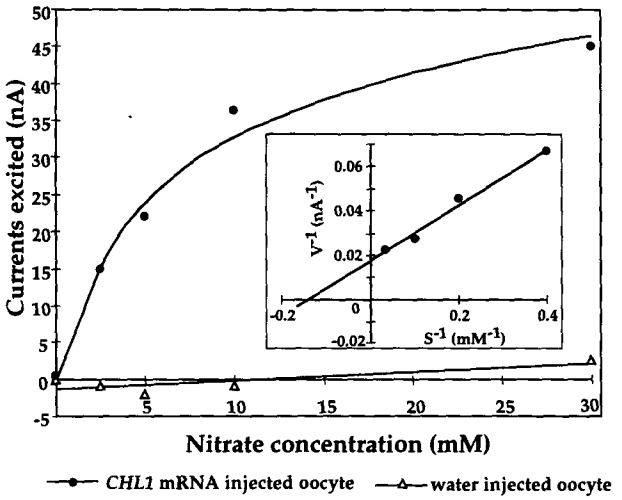

Figure 5. Kinetics of Nitrate Uptake by CHL1 as a Function of Concentration.

The currents (nA) elicited in a single *CHL1* mRNA-injected oocyte (●) and a water-injected control oocyte (△) were plotted as a function of nitrate concentration (at 2.5, 5, 10, and 30 mM). The insert provides a Linewear-Burk plot. The Linewear-Burk equation is $1/V = K_m/V_{max}[S] + 1/V_{max}$, where $[S]$ is the nitrate concentration in mM, and V represents the current elicited in nanoamperes. The K_m value calculated by using the Linewear-Burk plot for this particular experiment is 7.4 mM.

pathway may be less restricted. Immunolocalization of CHL1 protein will be needed to substantiate this hypothesis.

Another interesting observation can be made from these tissue-specific expression patterns. When moving basipetally, cells in which *CHL1* mRNA accumulates at high levels are located closer to the vascular cylinder: first, in the cortex alone; then, in the cortex plus endodermis; and finally, in the endodermis alone (Figure 2). A similar but not identical pattern of gene expression along the root axis has also been described for an Arabidopsis potassium channel gene, *AKT1*. Using β-glucuronidase (*GUS*) as a reporter gene fused to the *AKT1* promoter, Lagarde et al. (1996) found that in young mature roots, the epidermis, cortex, and endodermis were stained; however, in older roots grown in soil, only inner cortical cells and endodermal cells were stained. Therefore, it might be a general phenomenon that the location of cells important for ion uptake becomes more internal as root cells mature.

These in situ hybridization data also show that when *CHL1* is expressed in the epidermal or cortical cells, *CHL1* mRNA is distributed throughout the whole cell; however, when it is expressed in the endodermis, it is more localized in a portion of the outer rim of the cell on the cortex side (Figures 2E and 2F, and 2G and 2H). These results may indicate an intracellular localization of *CHL1* mRNA, analogous to the several examples reported in animal systems (reviewed in Johnston, 1995). More precise localization experiments are needed to verify these findings.

The conclusion that *CHL1* is an inducible LATS deviates from the current model for nitrate uptake that proposes that LATS has only a constitutive component. However, the complex behavior of the low-affinity nitrate uptake observed in this study argues for a more complicated model involving multiple transporters. In an analogy to the HATS system (Hole et al., 1990; Aslam et al., 1992; Glass and Siddiqi, 1995; Kronzucker et al., 1995), we propose that the LATS system of Arabidopsis is also comprised of both inducible and constitutive elements. These components of LATS may be encoded by multiple genes; however, for simplicity we propose a two-gene model for the present. The two-gene model can explain the observed fluctuation in the nitrate uptake activity of *chl1* mutant and wild-type plants, which appears to depend on the nitrogen status of the plant. Namely, the expression levels of the two genes can vary, depending on the growth condition. Thus, in addition to nitrogen source, pH is another factor that can affect the expression of *CHL1* (Tsay et al., 1993). With this hypothesis, it follows that only when *CHL1* gene expression dominates over the second gene will a significant difference in the uptake activity between the *chl1* mutant and the wild type be evident. The two-component model is consistent with the observation that *chl1* mutants are resistant to 2 mM chlorate but sensitive to 8 mM chlorate in the presence of 2 mM nitrate (N.-C. Huang and Y.-F. Tsay, unpublished data).

The use of chlorate as a valid tracer for measuring nitrate uptake and selecting defective nitrate transporters in the low-affinity range has been controversial. In tomato root, chlorate has not been found to be a useful analog for high-affinity nitrate uptake experiments (Kosola and Bloom, 1996). In a tobacco suspension cell study, Guy et al. (1988) showed that chlorate uptake in the LATS range showed only minor inhibi-

Table 1. Currents Elicited by Nitrate Uptake and Resulting K_m Values Measured in *CHL1*-Injected Oocytes

	Current Excited by Nitrate[a]					
Experiment No.	2.5 mM (nA)	5 mM (nA)	10 mM (nA)	20 mM (nA)	30 mM (nA)	K_m[b] (mM)
Oocyte 1	15.0	22.0	36.5		45.0	7.4
Oocyte 2	17.0	18.5	25.5		58.5	7.7
Oocyte 3	8.0	13.0	24.3		36.5	15.6
Oocyte 4	13.6	16.5	21.5		24.5	2.4
Oocyte 5	15.0		37.0		53.3	9.3
Oocyte 6			55.0		80.0	8.8
Oocyte 7			30.0	39.0		8.6
Oocyte 8			17.0	22.0		8.3
Oocyte 9		22.0	31.0			6.9
Oocyte 10		12.0	18.0			10.0

[a] Ten *CHL1*-injected oocytes from six independent donor frogs were voltage clamped at −60 mV and then perfused with different concentrations of nitrate. Blank areas indicate that the currents at those nitrate concentrations were not recorded.

[b] Currents excited at different nitrate concentrations were recorded and then fit by the Linewear-Burk plot for K_m value calculation. The average K_m value is 8.5 ± 3.1 mM.

tion by external nitrate even at 15 mM. However, the tobacco suspension cells used in that study were derived from pith cells, which might not be relevant to nitrate uptake in root tissues. In fact, in a study using plasma membrane vesicles of maize roots, chlorate uptake activities were found to be inhibited by external nitrate in both ranges of HATS and LATS (Ruiz-Cristin and Briskin, 1991). The fact that one can obtain nitrate uptake mutants using chlorate selections in Arabidopsis indicates that chlorate can be useful at least in the initial identification of mutants.

The two-gene model would be strengthened by the identification of a second LATS gene involved in nitrate uptake. In addition to two peptide transporter genes, *AtPTR2-A* and *AtPTR2-B* (Steiner et al., 1994; Song et al., 1996), there are at least nine homologs of *CHL1* in the Arabidopsis expressed sequence tag data base; some of these homologs may be involved in nitrate uptake or long-distance transport. Indeed, we have found that properties of a *CHL1* homolog, *NTL1*, fit the criteria of a second, constitutive LATS. For example, functional analysis in Xenopus oocytes indicated that it also encodes a nitrate transporter, and transgenic plants containing antisense *NTL1* in the *chl1* deletion background are more resistant to chlorate treatment than is the *chl1* mutant (N.-C. Huang and Y.-F. Tsay, unpublished data).

Recently, two *CHL1* homologs from tomato, *LeNrt1-1* and *LeNrt1-2*, have been reported (Lauter et al., 1996). Like the two Arabidopsis genes *CHL1* and *NTL1*, both *LeNrt1-1* and *LeNrt1-2* express in root tissues, and interestingly, the expression of *LeNrt1-2* is regulated by nitrate but that of *LeNrt1-1* is not. However, it was not shown to which uptake system (LATS or HATS) these genes belong. If *LeNrt1-1* and *LeNrt1-2* also encode low-affinity nitrate transporters that are directly involved in nitrate uptake, then our two-gene model may also apply to tomato.

In conclusion, our studies as well as those of others have revealed an elaborate mechanism of nitrate uptake in higher plants. The complexity may have evolved to counter the harsh environment in which plants have survived for millions of years. Characterization of *CHL1* and *CHL1* homologs by using both classic and advanced molecular genetic approaches as well as physiology should provide us with clues to determine the versatile mechanism for nitrate uptake.

METHODS

In Situ Hybridization

Plants were grown vertically on agarose plates with 12.5 mM $(NH_4)_2$ succinate for 12 days, as described by Tsay et al. (1993), and then shifted to medium containing 25 mM KNO_3 for 2 hr before harvesting the root tissue for fixation. We have tried several protocols to obtain intact sections of *Arabidopsis thaliana* roots and found the following procedure to work best in our experiments. The fixative contained 0.05 M Pipes buffer, pH 7.3, 4% paraformaldehyde, and 0.25% glutaraldehyde. Immediately before use, the fixative was mixed with an equal volume of *n*-heptane for 1 min, after which the phases were allowed to separate. The *n*-heptane phase was transferred to Petri dishes. Plant material was immersed in the *n*-heptane phase for 10 min at room temperature and cut into pieces <5 mm long while in heptane. The 5-mm-long root pieces were transferred to the fixative for 2 hr at room temperature. The specimens were washed three times with 0.05 M Pipes buffer, dehydrated, and embedded in paraplast through the following steps: 25% ethanol, three times for 30 min; 50% ethanol, three times for 30 min; 70% ethanol, three times for 30 min (standing overnight was optional); 95% ethanol, three times for 30 min; 100% ethanol, three times for 30 min; 100% xylene, three times for 15 min; xylene/paraplast (1:1 [v/v]) at 42°C, overnight; paraplast at 60°C, three times for 1 hr. After being embedded in paraplast in the desired orientation, the tissue was cut into 8-μm sections. Before hybridization, the sections were treated as described by Gustafson-Brown et al. (1994) to reduce background with acetylation reaction using triethanolamine and acetic anhydride and to increase tissue permeability by proteinase K digestion.

^{35}S-labeled *CHL1* antisense (or sense) mRNA was synthesized using T3 (or T7) RNA polymerase from XbaI- (or HindIII)-linearized pKS-1.8 RI*CHL1* (pBluescript II KS+ [Stratagene, La Jolla, CA] containing the 1.8-kb EcoRI fragment of the *CHL1* cDNA without the 3′ untranslated region and poly[A] tail). Root sections were hybridized with hydrolyzed RNA probes and washed as described previously (Jackson, 1991; McKhann and Hirsch, 1993). Slides were exposed for 2 months.

Plant Transformation

To overexpress *CHL1* in Arabidopsis, we fused the *CHL1* cDNA to the cauliflower mosaic virus (CaMV) 35S promoter. Then 35S–*CHL1* was inserted into binary vector pBIN19 (Bevan, 1984) and introduced into *Agrobacterium tumefaciens* AGL1 (AGL1 was a gift of Dr. R. Ludwig, University of California–Santa Cruz). To avoid cosuppression caused by duplicated genes, we used this strain to infect root explants of *chl1-5* plants (not the wild type) by the method of Valvekens et al. (1988). Progeny from six independent transgenic plants were obtained and tested for chlorate sensitivity. All six of the transgenic plants, like the wild type, were chlorate sensitive. This indicates that the chimeric construct does encode a functional CHL1 protein. The copy numbers of insertion were determined by genomic DNA gel blot analysis and the kanamycin segregation ratio. Two of the six transgenic plants have been tested for their nitrate uptake activity, and both of them have restored the uptake deficiency of *chl1*. The one with two copies of closely linked inserts was used in this study for more detailed analysis.

Nitrate Uptake Assay of Arabidopsis Plants

Arabidopsis seed were surface sterilized and sown in 125-mL flasks with 25 mL, pH 5.5, of a solution containing 10 mM K_2HPO_4-KH_2PO_4, 2 mM $MgSO_4$, 0.1 mM $FeSO_4$-EDTA, 1 mM $CaCl_2$, 50 μM H_3BO_3, 12 μM $MnSO_4 \cdot H_2O$, 1 μM $ZnCl_2$, 1 μM $CuSO_4 \cdot 5H_2O$, 0.2 μM $Na_2MoO_4 \cdot 2H_2O$, 1 g/L 2-(*N*-morpholino)ethane-sulfonic acid, 1% sucrose, 1 mg/L thiamine, 100 mg/L inositol, 0.5 mg/L pyridoxine, 0.5 mg/L nicotinic acid, plus the nitrogen source (25 mM KNO_3 or 25 mM NH_4NO_3). The flasks were exposed to 16-hr-light and 8-hr-dark cycles and rotated at 80 rpm at 24°C. After 18 days, the amount of nitrate left in the medium was determined by injecting 25 μL of 1:20 diluted medium into a PARTISIL 10 SAX (strong anion exchange) column (Whatman, Clifton, NJ) and analyzed by HPLC (Thayer and Huffaker, 1980).

For short-term uptake measurements, plants were sown in 25 mL of nutrient solution, as described above, and exposed to continuous illumination. For the nitrogen starvation pretreatment, plants were

washed twice with 5 mM K_2HPO_4-KH_2PO_4, pH 5.5, and then shifted to the 25-mL nutrient solution, as described above, but without any nitrogen source for 2 days. For the ammonium pretreatment, plants were washed twice with 5 mM K_2HPO_4-KH_2PO_4, pH 5.5, and then shifted to the 25-mL nutrient solution with 25 mM $(NH_4)_2$ succinate as the sole nitrogen source. Twelve-day-old plants with or without pretreatment were then washed again, as given above, and shifted to the 25-mL nutrient solution with 8 mM KNO_3. A solution (0.7 mL) was taken out of the flask at different time points and subsequently analyzed by HPLC.

Functional Expression of *CHL1* in Xenopus Oocytes

Full-length cDNAs of *CHL1* were subcloned into the transcription vector pBluescriptII KS+. Capped mRNA was transcribed from the linearized plasmid in vitro using mMESSAGE mMACHINE kits (Ambion, Austin, Texas). Oocytes were isolated and injected with 50 ng of complementary RNA, as described by Tsay et al. (1993). Measurements were made in a solution containing 230 mM mannitol, 0.15 mM $CaCl_2$, and 10 mM Hepes (pH 7.4; the pH was adjusted with CsOH) and then perfused with 220 mM mannitol, 0.15 mM $CaCl_2$, 10 mM Hepes (pH 5.5 or 7.4; the pH was adjusted with CsOH) plus $CsNO_3$ at the concentration indicated. For the current measurements, oocytes were voltage clamped to −60 mV, using two microelectrodes as described by Cao et al. (1992). Measurements were recorded by a 486-based personal computer using the AXOTAPE or pCLAMP6 program (Axon Instruments, Foster City, CA).

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REFERENCES

Åberg, B. (1947). On the mechanism of the toxic action of chlorates and some related substances upon young wheat plants. Ann. R. Agric. Coll. Swed. **15,** 37–107.

Aslam, M., Travis, R.L., and Huffaker, R.C. (1992). Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley (*Hordeum vulgare* L.) seedlings. Plant Physiol. **99,** 1124–1133.

Behl, R., Tichner, R., and Raschke, K. (1988). Induction of a high-capacity nitrate-uptake mechanism in barley roots prompted by nitrate uptake through a constitutive low-capacity mechanism. Planta **176,** 235–240.

Bevan, M.W. (1984). Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. **12,** 8711–8721.

Braaksma, F.J., and Feenstra, W.J. (1982). Isolation and characterization of nitrate reductase–deficient mutants of *Arabidopsis thaliana*. Theor. Appl. Genet. **64,** 83–90.

Cao, Y., Anderova, M., Crawford, N.M., and Schroeder, J.I. (1992). Expression of an outward-rectifying potassium channel from maize mRNA and complementary RNA in Xenopus oocytes. Plant Cell **4,** 961–969.

Crawford, N.M. (1995). Nitrate: Nutrient and signal for plant growth. Plant Cell **7,** 859–868.

Crawford, N.M., and Arst, H.N.J. (1993). The molecular genetics of nitrate assimilation in fungi and plants. Annu. Rev. Genet. **27,** 115–146.

Doddema, H., and Telkamp, G.P. (1979). Uptake of nitrate by mutants of *Arabidopsis thaliana* disturbed in uptake or reduction of nitrate. II. Kinetics. Physiol. Plant. **45,** 332–338.

Doddema, H., Hofstra, J., and Feenstra, W. (1978). Uptake of nitrate by mutants of *Arabidopsis thaliana* disturbed in uptake or reduction of nitrate. I. Effect of nitrogen source during growth on uptake of nitrate and chlorate. Physiol. Plant. **43,** 343–350.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., and Scheres, B. (1993). Cellular organization of the Arabidopsis root. Development **119,** 71–84.

Enstone, D.E., and Peterson, C.A. (1992). The apoplastic permeability of root apices. Can. J. Bot. **70,** 1502–1512.

Glass, A.D.M., and Siddiqi, M.Y. (1995). Nitrogen absorption by plant roots. In Nitrogen Nutrition in Higher Plants, H.S. Srivastava and R.P. Singh, eds (New Delhi: Associated Publishing Co.), pp. 21–56.

Glass, A.D.M., Shaff, J.E., and Kochian, L.V. (1992). Studies of the uptake of nitrate in barley. Plant Physiol. **99,** 456–463.

Goyal, S.S., and Huffaker, R.C. (1986). A novel approach and a fully automated microcomputer-based system to study kinetics of NO_3^-, NO_2^-, and NH_4^+ transport simultaneously by intact wheat seedlings. Plant Cell Environ. **9,** 209–215.

Gustafson-Brown, C., Savidge, B., and Yanofsky, M.F. (1994). Regulation of the Arabidopsis floral homeotic gene *APETALA1*. Cell **76,** 131–143.

Guy, M., Zabala, G., and Filner, P. (1988). The kinetics of chlorate uptake by XD tobacco cells. Plant Physiol. **86,** 817–821.

Hoff, T., Truong, H.N., and Caboche, M. (1994). The use of mutants and transgenic plants to study nitrate assimilation. Plant Cell Environ. **17,** 489–506.

Hole, D.J., Emran, A.M., Fares, Y., and Drew, M. (1990). Induction of nitrate transport in maize roots and kinetics of influx measured with nitrogen-13. Plant Physiol. **93,** 642–647.

Jackson, D. (1991). *In situ* hybridization in plants. In Molecular Plant Pathology: A Practical Approach, D.J. Bowles, S.J. Gurr, and M. McPhereson, eds (Oxford, UK: Oxford University Press), pp. 163–174.

Johnston, D.S. (1995). The intracellular location of messenger RNAs. Cell **81,** 161–170.

Kosola, K.R., and Bloom, A.J. (1996). Chlorate as a transport analog for nitrate absorption by roots of tomato. Plant Physiol. **110,** 1293–1299.

Kronzucker, H.J., Siddiqi, M.Y., and Glass, A.D.M. (1995). Kinetics of NO_3^- influx in spruce. Plant Physiol. **109,** 319–326.

Lagarde, D., Basset, M., Lepetit, M., Conejero, G., Gaymard, F., Astruc, S., and Grignon, C. (1996). Tissue-specific expression of Arabidopsis *AKT1* gene is consistent with a role in K^+ nutrition. Plant J. **9,** 195–203.

Lauter, F.-R., Ninnemann, O., Bucher, M., Riesmeier, J.W., and Frommer, W.B. (1996). Preferential expression of an ammonium transporter and of two putative nitrate transporters in root hairs of tomato. Proc. Natl. Acad. Sci. USA **93,** 8139–8144.

McClure, P.R., Kochian, L.V., Spanswick, R.M., and Shaff, J.E. (1990). Evidence for cotransporter of nitrate and protons in maize roots. Plant Physiol. **93,** 281–289.

McKhann, H.I., and Hirsch, A.M. (1993). *In situ* localization of specific mRNAs in plant tissues. In Methods in Plant Molecular Biology and Biotechnology, B.R. Glick and J.E. Thompson, eds (Boca Raton, FL: CRC Press), pp. 179–205.

Odell, J.T., Nagy, F., and Chua, N.-H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313,** 810–812.

Ruiz-Cristin, J., and Briskin, D.P. (1991). Characterization of a H^+/NO_3^- symport associated with plasma membrane vesicles of maize roots using $^{36}ClO_3^-$ as a radiotracer analog. Arch. Biochem. Biophys. **285,** 74–82.

Saier, M.H. (1994). Computer-aided analyses of transport protein sequences: Gleaning evidence concerning function, structure, biogenesis, and evolution. Microbiol. Rev. **58,** 71–93.

Siddiqi, M.Y., Glass, A.D.M., Ruth, T.J., and Rufty, T.W., Jr. (1990). Studies of the uptake of nitrate in barley. Plant Physiol. **93,** 1426–1432.

Song, W., Steiner, H.-Y., Zhang, L., Naider, F., Stacey, G., and Becker, J.M. (1996). Cloning of a second Arabidopsis peptide transport gene. Plant Physiol. **110,** 171–178.

Steiner, H.-Y., Song, W., Zhang, L., Naider, F., Becker, J.M., and Stacey, G. (1994). An Arabidopsis peptide transporter is a member of a new class of membrane transport proteins. Plant Cell **6,** 1289–1299.

Thayer, J.R., and Huffaker, R.C. (1980). Determination of nitrate and nitrite by high-pressure liquid chromatography: Comparison with other methods for nitrate determination. Anal. Biochem. **102,** 110–119.

Tsay, Y., Schroeder, J.I., Feldmann, K.A., and Crawford, N.M. (1993). The herbicide sensitivity gene *CHL1* of Arabidopsis encodes a nitrate-inducible nitrate transporter. Cell **72,** 705–713.

Ullrich, C.I., and Novacky, A.J. (1990). Extra- and intracellular pH and membrane potential changes induced by K^+, Cl^-, $H_2PO_4^-$, and NO_3^- uptake and fusicoccin in root hairs of *Limnobium stoloniferum*. Plant Physiol. **94,** 1561–1567.

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*–mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA **85,** 5536–5540.

Warner, R.L., and Huffaker, R.C. (1989). Nitrate transport is independent of NADH and NAD(P)H nitrate reductases in barley seedlings. Plant Physiol. **91,** 947–953.