Disruption of putative anion channel gene *AtCLC-a* in *Arabidopsis* suggests a role in the regulation of nitrate content

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**Summary**

In animals and yeast, voltage-dependent chloride channels of the CLC family play a role in basic cellular functions such as epithelial transport, plasma membrane excitability, and control of pH and membrane potential in intracellular compartments. To assess the function of CLCs in plants, we searched for CLC insertion mutants in a library of *Arabidopsis* lines transformed by *Agrobacterium tumefaciens* transferred DNA (T-DNA). Using a polymerase chain reaction-based screening procedure, an *Arabidopsis* line that carries a T-DNA insertion within the C-terminus of the *AtCLC-a* coding sequence was identified. Progeny from this plant line, *clca-1*, showed dramatically altered transcription of the *AtCLC-a* gene. Plants homozygous for the *clca-1* mutation exhibited normal development and a morphology indistinguishable from the wild-type. However, their capacity to accumulate nitrate under conditions of nitrate excess was reduced in roots and shoots, by approximately 50%, while chloride, sulphate and phosphate levels were similar to the wild-type. In addition, the herbicide chlorate, an analogue of nitrate, induced a faster and more pronounced chlorosis in mutant plants. Hypersensitivity to chlorate as well as decreased nitrate levels co-segregated with the T-DNA insertion. They were found at various time points of the *clca-1* life cycle, supporting the idea that AtCLC-a has a general role in the control of the nitrate status in *Arabidopsis*. Concordant with such a function, *AtCLC-a* mRNA was found in roots and shoots, and its levels rapidly increased in both tissues upon addition of nitrate but not ammonium to the culture medium. The specificity of *AtCLC-a* function with respect to nitrate is further supported by a similar free amino acid content in wild-type and *clca-1* plants. Although the cellular localization of *AtCLC-a* remains unclear, our results suggest that AtCLC-a plays a role in controlling the intracellular nitrate status.

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**Introduction**

With the aim of applying electrophysiological techniques to plant materials specifically altered in ion channel function, there has been in the past few years an increased interest in the characterization of anion channels from the genetic model system *Arabidopsis thaliana* (Cho and Spalding, 1996; Lew, 1991; Thomine et al., 1995).

The first putative anion channels in plants have been recently isolated via homology-based strategies. The genes, isolated from *A. thaliana* and *Nicotiana tabacum* (Hechenberger et al., 1996; Lurin et al., 1996), belong to the CLC family of voltage-dependent chloride channels (Jentsch et al., 1995). Heterologous expression of tobacco CLC-Nt1 in *Xenopus* oocytes elicited hyper-polarization activated chloride currents (Lurin et al., 1996), but evidence that plant CLCs function as genuine anion channels in native plant membranes is still missing.
In contrast to plants, distinct cellular functions, including plasma membrane excitability and epithelial transport, have been assigned to some of the CLCs found in mammals, on the basis of specific electrophysiological properties and expression patterns (Jentsch et al., 1995). The importance of CLC channels in basic cellular processes has also been underscored by the identification of inheritable diseases linked to CLC genes. Consistent with its presumed function in muscle fibre excitability, mutations in CLC-1 were found to cause several syndromes of myotonia congenita (Steinmeyer et al., 1991). CLC-5 and CLCKb have been linked to Dent’s disease (kidney stones) and Bartter’s syndrome, respectively, two renal pathologies associated with profound disorders in electrolyte homeostasis (Lloyd et al., 1996; Simon et al., 1997). CLC genes have also been recognized in micro-organisms (Fujita et al., 1994; Greene et al., 1993), and mutation in the unique CLC gene of yeast leads to pleiotropic effects including altered iron metabolism and mitochondrial functions (Greene et al., 1993).

In the present work, we have followed a reverse genetic approach to study anion channel function in Arabidopsis. We have identified an Arabidopsis mutant line which carries an Agrobacterium transferred DNA (T-DNA) within the AtCLC-a gene. Phenotypic analysis of mutant and WT plants grown under various culturing conditions revealed an unexpected role for the AtCLC-a gene in regulation of the cellular nitrate status.

Results

Screening for isolation of a CLC T-DNA insertion mutant

An Arabidopsis library of 11 136 lines independently transformed by the Agrobacterium tumefaciens strain MP5-1 (Bechtold et al., 1993; Bouchez et al., 1993) was screened by PCR using a combination of primers specific for CLC genes and T-DNA border sequences, respectively. When this work was initiated, no CLC sequence had been reported in Arabidopsis. Using degenerate primers (Lurin et al., 1996), three distinct PCR fragments of 0.2 kb were obtained. Two of these clones corresponded to the genes AtCLC-a and AtCLC-c (Hechenberger et al., 1996), respectively, and a third represents a yet unidentified CLC-like sequence. Based on the amino acid motif GXGIPEXK conserved in CLCs and the sequences from the cloned CLC fragments, we constructed the degenerate primers 5S, 5A, 11S and 11A (Figure 1a). The presence of CLC::T-DNA insertions within 29 pools, each containing the DNA of 384 mutant Arabidopsis lines, was searched for by separate PCRs using eight possible primer combinations: one of the four CLC primers 5S, 5A, 11S or 11A, with either the LB (left border) (6 Dg) or the RB (right border) (3 Dg) T-DNA primer. Hybridization of PCR products to both an AtCLC-a and a T-DNA probe specifically identified two DNA bands: a 2.6 kb fragment derived from primer combination 11S/6 Dg and a 2.3 kb fragment obtained with primers 5S/6 Dg (Figure 1b, lanes 5 and 6). These products originated from reactions with the same pool (number 4) suggesting that both primer combinations allowed the detection of the same CLC::T-DNA insertion. Sequence analysis of the PCR products (see below) confirmed this and showed that the T-DNA insertion had occurred within AtCLC-a.

Isolation and molecular analysis of the mutant clca-1

By repeating the PCR amplification procedure on eight DNA pools that were subpools of pool 4, a single subpool and finally a single Arabidopsis line, previously named ACS1, was identified. T2 seeds from the ACS1 line showed resistance to kanamycin (Km) which segregated with a 15:1 ratio indicating that this line carried at least two genetically independent T-DNA insertions. After four successive back-crosses, two lines carrying the T-DNA of interest and showing a segregation of 3:1 were selected, and homozygous plants, at this point designated clca-1, were propagated for further study. Primers specific for AtCLC-a (10S and 71A; Figure 1a) were designed to amplify the 5’ and 3’ junctions of the CLC::T-DNA insertion. The 10S/6 Dg combination produced a 620 bp DNA fragment, which hybridized with an AtCLC-a and a T-DNA probe.

Figure 1. PCR analysis of AtCLC-a gene structure in WT and clca-1.
(a) Schematic representation of the AtCLC-a::T-DNA insertion characterized in clca-1. The structure of the AtCLC-a gene and the T-DNA insertion were deduced from PCR analysis, cDNA and genomic sequence analysis.
(b) Agarose gel separation of PCR samples (ethidium bromide) and hybridization analysis (panels II and III). DNA from WT plants (lanes 1–4) and clca-1 (lanes 5–8) was PCR-amplified using the primer combinations 11S/71A (lane 1), 5S/71A (lane 2), 10S/71A (lane 3), 10S/6 Dg (lane 4), 11S/6 Dg (lane 5), 5S/6 Dg (lane 6), 10S/6 Dg (lane 7) and 71A/6 Dg (lane 8). The primer combination 10S/71A used with clca-1 DNA did not give detectable PCR amplification (not shown). The DNA gel blot was hybridized with a radioactive AtCLC-a cDNA probe (panel II) and after stripping re-hybridized with an LB T-DNA probe (panel III). Sizes of the detected bands are indicated in kb.

Disruption of anion channel gene AtCLC-a

WT clca-1 clca-1

R S R S

2.9

1.35

1.0

25S

Figure 3. Northern blot analysis of AtCLC-a expression in WT and clca-1. RNA was isolated from 7-day-old Arabidopsis WT and clca-1 roots (R) and shoots (S). RNA (10 μg) was separated on a formaldehyde 2% agarose gel, blotted to a nylon filter, and hybridized with a radiolabelled AtCLC-a cDNA probe (left panel). The clca-1 RNAs from the same preparation were also hybridized with an LB T-DNA probe (T-DNA LB: right panel). To verify sample loading homogeneity, the 25S ribosomal fraction is shown. Exposure time was overnight, and sizes of the observed bands are given in kb.

The predicted mutant protein is a truncated AtCLC-a whereby the 22 C-terminal amino acids are replaced by six different ones (Figure 2) and a putative phosphorylation site Ser 771 is absent in clca-1. Dots represent omitted T-DNA sequences.

Figure 2. Comparison of genomic WT AtCLC-a and mutant AtCLC-a-1 sequences.

Nucleotide sequences derived from PCRs with chromosomal DNA from WT (upper sequence) and clca-1 (lower sequence) are aligned. Intron sequences (italic) were deduced from comparison with AtCLC-a cDNA sequences not shown here. T-DNA sequences (bold) were found in the clca-1 gene starting at position Leu 754, except for the three terminal bases of the LB sequences being substituted by a single thymidine (Bouchez et al., 1993). As a result, clca-1 contains a premature stop codon (*) and lacks the 22 C-terminal amino acids, which are replaced by six different ones (underlined). Note that the putative phosphorylation site Ser 771 is absent in clca-1. Dots represent omitted T-DNA sequences.

(Figure 1b, lane 7). Surprisingly, the 71A primer also had to be combined with the LB 6Dg primer, and not the RB 3Dg primer, to result in a 460 bp CLC-T-DNA chimeric DNA fragment (Figure 1b, lane 8). The T-DNA insertion therefore probably carries two left arms at its extremities. Inverted tandem T-DNA insertions are commonly observed and offer a straightforward interpretation of this result.

The clca-1 10S/6Dg and 71A/6Dg PCR fragments were sequenced and compared to CLC and T-DNA sequences. In agreement with the hypothesis above, part of each PCR fragment contained LB T-DNA sequences, while the other halves were unambiguously identified as AtCLC-a sequences. In parallel, we analysed the AtCLC-a sequence derived from the 10S/71A 265 bp PCR product, obtained with WT genomic DNA (Figure 1, lane 3). Alignment with the AtCLC-a cDNA sequences revealed the presence of a 73 bp intron between amino acids Gly 741 and Met 742 (Figure 2). The AtCLC-a-1 genomic sequence derived from the 10S/6Dg PCR fragment showed the same intron but deviates from the WT sequence at position Leu 754 (Figure 2). The coding sequence is therefore changed, and a stop codon is introduced 18 bp downstream. Thus, the predicted mutant protein is a truncated AtCLC-a whereby the 22 C-terminal amino acids are replaced by six different ones (Figure 2) and a putative phosphorylation site Ser 771 is absent in clca-1. Dots represent omitted T-DNA sequences.

Transcription of AtCLC-a is altered in clca-1

RNA from WT and mutant seedlings was isolated and analysed by Northern blot. In plants grown in standard light conditions, AtCLC-a showed a spatial regulation with mRNA levels about three times higher in roots than in shoots (Figure 3). In clca-1, a fully sized AtCLC-a transcript was not detected, but a fuzzy 1.35 kb band appeared instead, more abundant in roots than in shoots. In clca-1 roots, additional faint bands appeared at 2.7–2.9 kb, which compare in size to the native AtCLC-a transcripts. Hybridization of the same Northern blot with an LB T-DNA probe revealed two broad bands in clca-1: a 1.35 kb band co-migrating with the major band detected by the
AtCLC-a probe, and a smaller, more intense 1.0 kb band. The relative intensities of these RNAs in the different samples were similar to that found with the AtCLC-a probe, suggesting that the different mRNA species were coordinately transcribed. These results showed that the integrity of the AtCLC-a mRNA was lost in clca-1, and that due to the T-DNA insertion, proper transcription termination and/or mRNA stability were severely disrupted.

(a) seedlings

![Graph showing nitrate-induced expression of AtCLC-a](image)

(b) seedlings

![Graph showing anion content](image)

(c) shoots roots

![Graph showing anion content](image)

**Figure 5.** Nitrate-induced expression of AtCLC-a.
(a) WT seeds were germinated in a liquid, modified ABIS with all nitrate substituted by chloride; 100 mM KNO₃ was added two days after incubation to sustain limited growth. Six days later, 10 mM KNO₃ or 10 mM KCl was added to the cultures and RNA samples were prepared from plants collected at 0.5, 1.5 and 4.5 h after treatment. RNA was separated on a formaldehyde agarose gel, blotted to a nylon filter, and hybridized with an AtCLC-a cDNA probe.
(b) WT seedlings were grown essentially as described above. Seedlings were incubated for 8 days in liquid, modified ABIS without nitrate. KNO₃, NH₄Cl or NH₄NO₃ were added to the liquid culturing medium at a final 10 mM concentration. RNA samples were prepared 3 h after treatment, and analysed as described above.
(c) To separate shoots and roots, WT seedlings were grown essentially as above, except that cultures were on a solid medium. Eight-day-old seedlings were transferred to similar medium containing 10 mM KNO₃. RNA samples were prepared 3 h after treatment, and analysed as described above.

The size of detected bands is indicated in kb, and sample loading was verified for homogeneity by the 25S ribosomal fraction. Exposure time was 4 days.

**Figure 4.** Anion contents of WT and clca-1 as determined by capillary ion analysis (CIA).
(a) Representative CIA chromatogram of WT and clca-1. The peaks were identified in consecutive order as chloride (Cl⁻), sulphate (SO₄²⁻), nitrate (NO₃⁻), citrate (cit), fumarate (fum), malate (mal), and phosphate (PO₄³⁻) using standard solutions.
(b) Anion contents from 14-day-old WT and clca-1 plants grown in vitro on solid ABIS medium. The concentration levels are related to the fresh weight (FW). Data are the mean (± SE) from three independently performed experiments, with a minimum of three samples per experiment.
(c) Anion contents from roots (R) and shoots (S) from 14-day-old WT, clca-1, and heterozygous AtCLC-a/clca-1 seedlings (H) grown in vitro.
clca-1 exhibits an altered nitrate content

In the greenhouse, WT and clca-1 Arabidopsis exhibited similar development and life cycle, although sporadically a slight growth advantage was noted for the mutant. When grown in vitro under standard conditions, both lines were morphologically indistinguishable. Growth on media supplemented with sub-optimal to toxic concentrations of various salts (NaCl, KCl, KNO₃, NaNO₃, Na₂SO₄, NaF, NaI or NaBr) did not reveal any significant phenotypic difference (data not shown). A mutation in AtCLC-a might, however, affect ion transport and thus distribution and storage of anions within the plant. Anions were extracted from Arabidopsis plants grown in vitro or in soil, and separated by capillary ion electrophoresis (CIA). This technique allowed the rapid and quantitative detection of the inorganic anions chloride, sulphate, nitrate and phosphate, together with the organic anions citrate, fumarate and malate. An example of CIA profiles from in vitro grown WT and clca-1 plants is shown in Figure 4(a). Nitrate was the most abundant anion detected. While average concentrations in the WT reached 82.1 ± 3.7 μmol g⁻¹ FW (± SE, n = 5), the levels of nitrate in clca-1 were reduced by 53% (38.7 ± 1.6 μmol g⁻¹ FW; n = 5). A similar decrease in nitrate content was observed in extracts from rosette leaves of mutant plants grown in soil (data not shown). In contrast, no alteration in the concentrations of chloride, sulphate and phosphate was found (Figure 4a,b). The levels of organic acids, citrate, fumarate and malate, were low, in the range of 5–15 μmol g⁻¹ FW in both WT and clca-1 plants (Figure 4b). We noticed, however, a twofold increase in malate content in clca-1.

In another series of experiments, we investigated the organ distribution of anions. Roots contained less nitrate than the shoots; however, a decrease in nitrate content, with chloride and sulphate unchanged, was found in clca-1 (Figure 4c). In all conditions tested, the ion content of heterozygous AtCLC-a/clca-1 Arabidopsis plants was similar to that from the WT (Figure 4c), suggesting that a single copy of the WT AtCLC-a gene was sufficient for normal accumulation of nitrate.

The Cl⁻ phenotype was originally detected in two homozygous isolates from the initial T-DNA line ACS1. A subsequent back-cross of each isolate produced progenies that had lost a second T-DNA-derived Km² marker, but had kept a lowered nitrate content. In the two following back-crosses, this trait co-segregated in each isolate with the

![Figure 6. Growth inhibition by NaClO₃.](image)

Fresh weight (FW) was determined from shoots from 14-day-old seedlings germinated on ABIS without sucrose, in the presence or in the absence of 1 mM NaClO₃. FW was normalized to 100% for WT plants grown without NaClO₃. The results are the mean of three independent experiments (n = 40).

### Table 1. Free amino acid content of WT and clca-1 plants

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>WT (µmol g⁻¹ FW ± SD; n = 3)</th>
<th>clca-1 (µmol g⁻¹ FW ± SD; n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>12.0 ± 0.5</td>
<td>12.2 ± 0.4</td>
</tr>
<tr>
<td>Asn</td>
<td>6.5 ± 0.9</td>
<td>6.3 ± 1.0</td>
</tr>
<tr>
<td>Asp</td>
<td>15.9 ± 2.4</td>
<td>15.2 ± 4.3</td>
</tr>
<tr>
<td>Gin</td>
<td>15.3 ± 2.0</td>
<td>16.3 ± 1.6</td>
</tr>
<tr>
<td>Glu</td>
<td>19.6 ± 0.6</td>
<td>17.7 ± 1.3</td>
</tr>
<tr>
<td>Gly</td>
<td>1.5 ± 0.5</td>
<td>2.9 ± 1.0</td>
</tr>
<tr>
<td>Pro</td>
<td>4.9 ± 1.3</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>Ser</td>
<td>9.0 ± 1.5</td>
<td>8.1 ± 2.3</td>
</tr>
<tr>
<td>Thr</td>
<td>9.6 ± 0.8</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>GABA</td>
<td>6.2 ± 1.7</td>
<td>9.8 ± 1.6</td>
</tr>
</tbody>
</table>

Data from 14-day-old plants grown in vitro in three independent experiments.

In each of the three independent experiments, the composition in terms of major amino acids and γ-aminobutyric acid (GABA) was determined in duplicate. Mean percentage values refer to the total of the 18 species resolved in these analyses. Small or trace amounts (each < 1% of total content) were found for Arg, His, Ile, Leu, Lys, Tyr, Val and Orn.
AtCLC-a:T-DNA insertion, indicating that this mutation was responsible for the observed phenotype.

AtCLC-a transcription is regulated by nitrate

Nitrate uptake and metabolism in plants have been shown to be highly regulated by nitrate itself or its assimilation products (Crawford, 1995). We first examined the expression of AtCLC-a under conditions of changing external nitrate. Arabidopsis seedlings were germinated in a liquid medium with a low nitrate content (100 \muM) in order to achieve starvation. After 7 days, the culture medium was complemented with 10 mM KNO₃, and RNA was extracted at various time points. Northern blot analysis indicated that KNO₃ application resulted in a steady increase in the AtCLC-a mRNA level that was clearly detected after 30 min and was prolonged for up to 4.5 h (Figure 5a). In contrast, plants treated with 10 mM KCl did not show enhanced AtCLC-a expression, indicating that the effects observed with KNO₃ were not due to a change in K⁺ supply or in the osmotic strength of the medium. The treatment of plants with (NH₄)₂SO₄, as an alternative source of inorganic nitrogen, slightly stimulated AtCLC-a expression, but to a much lesser extent than KNO₃ (Figure 5b). When supplied as (NH₄)₂NO₃, and in comparison to KNO₃, ammonium also slightly enhanced the nitrate induction of the gene.

Because AtCLC-a was differentially expressed in roots and shoots (Figure 3), it was interesting to look at AtCLC-a induction by nitrate in the separate tissues. Nitrate-starved plants grown in conditions where only roots were in contact with the solid medium were shifted to a medium supplemented with 10 mM KNO₃ (Figure 5c). Three hours after treatment, AtCLC-a mRNA levels had increased in both roots and shoots, suggesting that nitrate or another signal coming from the roots was rapidly transferred to the shoots to trigger gene activation.

clca-1 is hypersensitive to chlorate

Chlorate, the chlorine analogue of nitrate, can be taken up by plant cells and is reduced, presumably by nitrate reductase, to chlorite which is toxic to plants. When watered with a 1 mM chlorate solution, soil-grown Arabidopsis turned yellow after 2–3 days. We observed, however, that chlorosis occurred earlier in clca-1 than in the WT. In vitro, chlorate inhibited growth more strongly in clca-1 (80%) than in WT (58%) (Figure 6). In either of the growth conditions, greenhouse or in vitro, chlorate concentrations in Arabidopsis WT and clca-1 were very low, just at the detection limit. Therefore, a possible difference between the two lines could not be resolved (data not shown).

Homozygous plants derived from the original ACS1 line, as well as the final, back-crossed derived clca-1 lines, exhibited chlorate hypersensitivity, confirming that this phenotypic trait was linked to the AtCLC-a:T-DNA insertion, in a recessive fashion.

Nitrogen assimilation products in WT and clca-1

Alterations in nitrate storage and/or assimilation, as revealed by an altered nitrate content and chlorate sensitivity in mutant plants, may be linked to deeper alterations in nitrogen metabolism. To investigate these, we characterized the nitrogen status of WT and clca-1 plants. In 14-day-old in vitro grown plants, the total nitrogen contents (in percentage dry weight (DW) ± SE; n=7) were 6.4 ± 0.1 and 5.4 ± 0.1 for WT and clca-1, respectively. Based on a measured DW/FW ratio of 5.0 ± 0.3% (n=3), we calculated that a decrease in NO₃ content of 40 \muM g⁻¹ FW (see Figure 4b) results in a reduction in total nitrogen content of 1.2% DW. This can fully account for the observed difference between WT and clca-1, suggesting that the two plant lines have similar content of reduced nitrogen. Furthermore, the total amino acid content, in the range of 10 \muM g⁻¹ FW, and the amino acid composition were not significantly different in WT and clca-1 plants (Table 1).

Discussion

clca-1 exhibits an altered nitrate status

The Arabidopsis mutant clca-1 was isolated out of 11,136 Agrobacterium transformed lines and shown to carry a T-DNA insertion within the C-terminal part of the AtCLC-a open reading frame. From sequence and Northern analyses, it remains uncertain whether clca-1 is a true AtCLC-a knock-out mutant. However, the T-DNA insertion in clca-1 dramatically altered AtCLC-a mRNA transcription and/or stability, and production of a full-sized mutant protein, if any, may therefore be very low. Furthermore, the clca-1 mutation appeared to be recessive, which, as a simple explanation, may reflect inactivation of the corresponding gene or protein.

Notwithstanding the observation that homozygous clca-1 Arabidopsis plants showed normal growth and development, a careful analysis of these plants revealed an altered anion content. The most striking change was undoubtedly a halving in nitrate content, which resulted in a deficit of up to 40 \muM g⁻¹ FW. In contrast, several anions such as chloride, sulphate, and, as observed in separate experiments, bromide and iodide, showed similar accumulation in WT and clca-1 Arabidopsis (Figure 4 and data not shown). This observation came as a surprise to us, as it has been reported that animal CLCs and plant anion channels are permeable to nitrate but also to halogenic ions (Pusch et al., 1995; Schmidt and Schroeder, 1994;
Thiemann *et al.,* 1992). However, a phenotypic alteration mostly restricted to nitrate accumulation may have been uncovered because, independent of permeation properties in anion channels, nitrate is subject to specialized and highly regulated mechanisms for transport and metabolism in plants (see below). We also observed an increase in the content of organic acids such as malate. Because organic acids can carry several charges when stored in an acidic compartment such as the vacuole (pH 6.0), the modest change in concentration observed may be sufficient to compensate for the charge deficit due the reduction in nitrate content. In agreement with this, the cell sap of WT and *clca-1* plants exhibited very similar osmotic potentials. Thus, the slight alterations in organic acid contents may reflect a metabolic adjustment for cellular charge and osmotic balance.

The notion that the *clca-1* phenotype is primarily linked to an alteration in nitrate status also comes from the observation that *clca-1* was more sensitive to chlorate, an analogue of nitrate that causes chlorosis and subsequent growth inhibition. In contrast, the overall content of reduced nitrogen, and more specifically the content of amino acids such as Glu and Glu were not altered in *clca-1*. The fact that the changes in chlorate sensitivity and nitrate content occurred only in homozygous *clca-1/clca-1* plants, and both co-segregated with the *AtCLC-a:T-DNA insertion in two independent series of four back-crosses, establishes the link between the insertion mutation and the two phenotypes.

The decreased nitrate content in *clca-1* was apparent during different developmental stages, and was not confined to a particular tissue. Nitrate in *clca-1* was normally allocated from roots to shoots, suggesting that transport within the plant was roughly unaffected. Thus, AtCLC-a may serve in a general process for cellular nitrate metabolism and/or partitioning. This conclusion is in good agreement with the ubiquitous expression of AtCLC-a. The *AtCLC-a* gene was rapidly induced in both roots and shoots by nitrate primarily, independently of its assimilation products. The amounts of free nitrate present in plant cells vary dramatically depending on nitrate availability and/or environmental conditions. In our experiments, a reduced nitrate accumulation in *clca-1* was most evident at the high external concentrations (10–50 mm; data not shown). Altogether, these results suggest that AtCLC-a functions in the adaptive response of plants to an excess of nitrate.

**A role for AtCLC-a in the regulation of cellular nitrate status**

The amplitude of alteration in nitrate storage observed in *clca-1* suggests that this mutant is somehow altered in its vacuolar nitrate content since vacuoles comprise the largest pool of free nitrate in plant cells (Martinouia *et al.*, 1981). Although most of the molecular mechanisms involved remain unknown, it is believed that free cellular nitrate is regulated in plant cells as a result of three separate pathways (Crawford, 1995). First, nitrate is taken up by multiple uptake paths (Crawford and Glass, 1998) and can be released in the apoplasms via an efficient efflux system, a mechanism that might control the net nitrate influx (Crawford and Glass, 1998; Jackson *et al.,* 1976). Second, nitrate reductase (NR) converts nitrate to nitrite which is translocated and reduced in the plastids. Thirdly, excess nitrate can be stored in intracellular compartments for future supply. Although mechanistically separate, these pathways are in competition for nitrate availability and each affects the kinetics of the others. In addition, nitrate acts in each pathway both as a substrate and a regulating signal (Crawford, 1995). It may thus be difficult to assess the contribution of each pathway to a change in accumulation.

The mechanistic interpretation of the *clca-1* phenotype very much relies on assessing the cellular localization of AtCLC-a. Work carried out in our laboratory with specific polyclonal antibodies raised against tobacco CLC-N1 showed that this protein is preferentially expressed in an intracellular compartment, identified as the internal membrane of mitochondria (Lurin *et al.,* 2000). However, the specific cellular localization of AtCLC-a remains to be determined. Interestingly, Hechenberger *et al.* (1996) have expressed fusion proteins of AtCLCs with green fluorescent protein (GFP) in yeast. These authors surmised from these experiments that the AtCLC-GFPs were located on internal membranes, different from the tonoplast, which, however, could not be identified in more detail.

Recently, several CLCs in yeast (Gef1p) and mammals (CLC-5, CLC-6) have also been localized in internal cellular membranes (Buyse *et al.,* 1998; Gaxiola *et al.,* 1998; Günther *et al.,* 1998; Schwappach *et al.,* 1998), and mutations of the corresponding genes provoke profound physiological alterations. The yeast *gef1* mutant for instance displays a mitochondrial deficiency (petite) that can be alleviated by supplying an excess of iron (Greene *et al.,* 1993). It was recently demonstrated that Gef1p functions in the late Golgi apparatus to provide chloride for the metellation and/or activity of vesicular enzymes (Davis-Kaplan *et al.,* 1998; Gaxiola *et al.,* 1998). The *gef1* mutation hampers copper loading on Fet3p, a component of the iron uptake system, and the subsequent iron deficiency has pleiotropic effects on mitochondrial functions. Interestingly, AtCLC-c and AtCLC-d but not AtCLC-a complement the *gef1* mutation (Gaxiola *et al.,* 1998; Hechenberger *et al.,* 1996).

In conclusion, the isolation and analysis of the *Arabidopsis* mutant *clca-1* reveals a unique physiological role for CLC channels in plants. The specificity of the phenotype with respect to nitrate and chlorate, together
with the induction of AtCLC-a, suggest a specialized role for AtCLC-a in regulating the nitrate status in plants.

Experimental procedures

Plant material and culture conditions

All experiments were performed using Arabidopsis thaliana ecology Wassilewskija, except for the isolation of the AtCLC-a cDNA clone which was from Arabidopsis thaliana ecology Columbia. Sterilized seeds were grown on standard culture medium (ABIS: KNO₃ 5 mM, MgSO₄ 2 mM, Ca(NO₃)₂ 1 mM, Fe-EDTA 50 μM, sucrose 10 g l⁻¹, agar 0.7 g l⁻¹, micronutrients as described by Murashige and Skoog (1962), MES 1 mM, and K₂HPO₄ + KH₂PO₄ 2.5 mM, pH 6.8). Plants were grown in vertically placed, cleared polystyrene plates (12 × 12 cm) containing 35 ml ABIS at 21°C with 16 h light (120 μE m⁻² s⁻¹) and 8 h dark cycles. Transfer of seedlings to a different medium was performed by using a sheet of sterile ordinary bench paper laid on the culture medium. In the greenhouse, plants were grown in soil at a relative humidity of 40%, and temperature and day/night length were similar to those in vitro. For testing chloride sensitivity, 40–50 plants, grown together in trays, were watered once with 500 ml of a 1 mM NaClO₄ solution.

Screening of an Arabidopsis T-DNA library

The primary PCR screen was performed on pooled chromosomal DNA from independently isolated T-DNA transformed lines (Bechtold et al., 1993; Bouchez et al., 1993). The oligonucleotide primers used to target T-DNA sequences were: 11S, 5'-GGCCGGCTCGGGATCCGATATGGAATTC-3'; 11A, 5'-AGATATGCTTCGATCCGATATGGATATTC-3'; 5S, 5'-CTCTATCTTTTCACTTTTAAGT-3'; 5A, 5'-TCCTATCTTTTAAGATT-3'; where N = A + C + G + T, R = A + G, M = A + C, Y = C + T, K = G + T, and W = A + T. To target T-DNA sequences, we used a left border (LB) primer 6Dg (5'-AAGGGCAATTCGACCTGATTTGACATTTA-3') and a right border (RB) primer 3Dg (5'-ACGCCTTGTCCCACCGAACCCTGATCAAT-3'), that read in opposite directions towards the T-DNA surrounding sequences. Each reaction contained, in 50 μl, 50 ng DNA, 50 pmol T-DNA primer, 100 pmol CLC primer, 1 unit Taq DNA polymerase (Appligene), 5 μl 10 × reaction buffer (Appligene), and 0.2 mM dNTP. The following PCR programme was used: 94°C for 5 min; 55 cycles of 94°C for 45 sec, 55°C for 1 min and 72°C for 2.5 min; 70°C for 5 min. The reactions were separated on 1% agarose gels, blotted to nylon membranes, and hybridized with radiolabelled CLC and T-DNA probes under stringent conditions (Sambrook et al., 1989). The CLC probe was a mix of three Arabidopsis CLC clones isolated by RT-PCR, using the conditions and degenerate primers previously described (Lurin et al., 1996). The T-DNA probe corresponded to two 800 bp HincII fragments from plasmid pGBK8 encompassing both extremities of the T-region (Bouchez et al., 1993).

RNA extraction, DNA extraction, gene isolation and sequence analysis

RNA extraction and hybridization was carried out as described (Kay et al., 1987; Sambrook et al., 1989). DNA preparation from pooled T₂ progeny Arabidopsis plants was done as previously described (Bouchez et al., 1996). PCR fragments were cycle sequenced with an Applied Biosystems 373A automated sequencer (Perkin Elmer). A λ ZapII cDNA library prepared from Arabidopsis shoots was screened, using an AtCLC-a probe. Five full-length cDNAs were isolated and the longest was sequenced (accession number AF044313).

Genetic analysis of clca-1

Plants hetero- or homozygous for the clca-1 locus were distinguished by differential PCR. The WT AtCLC-a gene produced a 285 bp PCR fragment with primers 105/71A, while AtCLC-a-1 gave a 620 bp fragment when the primer combination 105/8D was used (Figure 1). Two homozygous clca-1/clca-1 plants were crossed with the WT, and F₂ progeny grown on selective medium. Lines that segregated for Km₉ as a single locus were selected, and tested by PCR for the presence of the AtCLC-a/T-DNA insertion. This process of back-crossing was repeated four times. Salt stress experiments, and in soil and in vitro culture observations, were done with the original T₂ plants. The results presented in Figure 4 were from plants obtained after the 4th back-cross.

Capillary ion analysis

About 100 mg of rosette leaves from greenhouse-grown plants, or of seedlings grown on a paper-overlaid standard medium, was harvested 6–8 h after the onset of the light period. Samples were immediately freeze-thawed three times in 9 vol Milli-Q purified water (Millipore). Samples were diluted 10–20 times, filtered through a 0.2 μm syringe filter (DynaGard, Microcon) and separated on a 75 μm × 60 cm AccuSep TM capillary column with 10 mm sodium chromate plus 0.5 mM OFM Anion-RT (Waters) as the electrolyte buffer. Sample injection was done by gravity (30 sec), and migration was at ~20 kV. Ions were detected with a fixed-wavelength UV detector at 254 nm. Quantification was achieved by freshly made standard solutions, using the ion analyser Millennium 2010 software (Waters).

Nitrogen metabolism analysis

Nitrogen assimilation product analyses were performed on 14-day-old plants grown in vitro. Total nitrogen content was measured using a Fisons/isochrom EA mass spectrometer at the Unité d’Agronomie of INRA, Laon-Peronne, France. Seven samples from three independent experiments were analysed for both WT and clca-1 plants. For amino acid analysis, samples of 1–2.5 g of fresh material were lyophilized, and homogenized in 6 ml of 2% sulphosalicylic acid. The free amino acid content in each sample was estimated by a colorimetric method (Rosen, 1957). Amino acid composition was determined using a Biotronik LC5001 analyser according to the manufacturer’s specifications.

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