A Family of Putative Chloride Channels from Arabidopsis and Functional Complementation of a Yeast Strain with a CLC Gene Disruption*

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We have cloned four novel members of the CLC family of chloride channels from Arabidopsis thaliana. The four plant genes are homologous to a recently isolated chloride channel gene from tobacco (CLC-N1; Lurin, C., Geelen, D., Barbier-Brygoo, H., Guern, J., and Maurel, C. (1996) Plant Cell 8, 701–711) and are about 30% identical in sequence to the most closely related CLC-6 and CLC-7 putative chloride channels from mammalia. AtCLC transcripts are broadly expressed in the plant. Similarly, antibodies against the AtCLC-d protein detected the protein in all tissues, but predominantly in the siliques. AtCLC-a and AtCLC-b are highly homologous to each other (~87% identity), while being ~50% identical to either AtCLC-c or AtCLC-d. None of the four cDNAs elicted chloride currents when expressed in Xenopus oocytes, either singly or in combination. Among these genes, only AtCLC-d could functionally substitute for the single yeast CLC protein, restoring iron-limited growth of a strain disrupted for this gene. Introduction of disease causing mutations, identified in human CLC genes, abolished this capacity. Consistent with a similar function of both proteins, the green fluorescent protein-tagged AtCLC-d protein showed the identical localization pattern as the yeast ScCLC protein. This suggests that in Arabidopsis AtCLC-d functions as an intracellular chloride channel.

Chloride channels are passive anion transport proteins involved in functions common to all cells, such as regulation of cell volume and intracellular pH. In animals, chloride channels are important for transepithelial transport and regulation of excitability of muscle and nerve, as demonstrated by several diseases which result from their genetic alteration.

In plants, chloride channels contribute to a number of plant-specific functions, such as regulation of turgor, stomatal movement, nutrient transport, and metal tolerance (for reviews, see Refs. 1–3). In contrast to the situation in animals, they are also responsible for the generation of action potentials (reviewed in Ref. 4). In recent years, various plant chloride channels have been characterized biophysically, both in plasma membranes and in membranes of different organelles. The best documented examples are chloride channels of guard cells (5–7), which control opening and closing of stomata. Activity of these channels is subject to extensive regulation by extracellular factors such as hormones and photosynthetic metabolites, as well as by intracellular Ca2+ and nucleotides (8–10), and provide a major mechanism for the control of gas and water exchange. Plant chloride channels are also believed to play a prominent role in signal perception and transduction since a variety of signals such as light, hormones, and pathogen-derived elicitors cause membrane depolarization by stimulating anion efflux (11, 12). Being the most abundant anion in higher plants, chloride is important for plant nutrition and osmoregulation. The vacuole as the major storage site for ions and nutrients plays a crucial role in turgor formation and in intracellular degradation of proteins. Experimental evidence has accumulated that tonoplast anion channels participate in these functions (13) and therefore are important for plant cell growth and development.

Despite their key roles in various functions, little is known about the molecular structure of plant chloride channels. Very recently, and in parallel with our work, a putative chloride channel has been identified from tobacco (14) by homology to the CLC family of voltage-gated chloride channels. This gene family, originally established by expression cloning of CLC-0 (15), the voltage-gated chloride channel from Torpedo electric organ, comprises members in bacteria (16), yeast (17) and mammals. Nine CLC genes have been discovered so far in a single mammalian species, which based on homology can be divided into three subfamilies (reviewed in Ref. 18). The first branch includes the CLC-1, CLC-2, and the CLC-K chloride channels. CLC-1 as the major skeletal muscle chloride channel (19) controls the excitability of the muscle fiber. Mutational inactivation of CLC-1 leads to myotonia (20, 21), an inherited disease both in animals and in humans. The ubiquitously expressed CLC-2 (22) can be activated by cell swelling in oocytes (23) and possibly plays a role in the regulation of cell volume. Rabbit CLC-2 was suggested to be stimulated by protein kinase A, but the ion selectivity of the reported single channel currents (24) differed from those of macroscopic rat CLC-2 currents (22, 25). The CLC-K1 and CLC-K2 channels are exclusively expressed in the kidney (26–28), where they may play a role in urinary concentration. CLC-3, -4, and -5 constitute another subfamily of this gene family. Whereas the function of CLC-3 (29) and -4 (30) is not clear, mutations in the CLC-5 gene, which is expressed mainly in the kidney (31–33), result in several kid-

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ney stone disorders (34). The single CLC homologue in yeast, ScCLC (originally termed GET1), is most closely related to these genes. Since disruption of this yeast gene results in sensitivity to low iron levels in the growth medium (17), ScCLC is somehow involved in iron metabolism. The most recent addition to the CLC family, namely the ubiquitously expressed CLC-6 and CLC-7 (35), represents a further branch. As is the case with several other members of the CLC family their physiological role is not known at present.

To further increase our knowledge of this important gene family of chloride channels, we have now cloned and analyzed novel plant CLC cDNAs. Starting from expressed sequence tags (ESTs) we have cloned four putative chloride channels from Arabidopsis thaliana. As described previously (36). Prior to cDNA synthesis, total RNA was isolated from different organs of Arabidopsis thaliana libraries (SuperscriptII, LifeTechnologies, Inc.) in 50 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM Hepes, pH 7.4) after 2–4 days at 18°C. They were then investigated by two-electrode voltage clamping using a Dagan amplifier and cPLAMP software (Axon Instruments). Recordings were performed at room temperature in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 5 mM MgCl2, 5 mM Hepes, pH 7.4). Several voltage-clamp protocols (spanning a voltage-range between –160 and +100 mV) were used. In ion substitution experiments 80 mM chloride was replaced by isoosmotic concentrations of malate and nitrate. To increase intracellular CAMP oocytes were perfused with a mixture containing 200 μM chlorophyllin-cAMP, 12 μM forskolin, and 500 μM 3-iso-buty1-1-methyIxanthine. Similar concentrations lead to a robust increase in cys tic fibrosis transmembrane conductance regulator currents (not shown).

Production of Antiserum—For generation of antiserum the prokaryotic pRSET expression system (Invitrogen) was used. A Xhol/EcoRI frag ment of cDNA clone AtCLC-d, coding for the last 192 C-terminal amino acid residues was cloned into the vector pRSET-C, and XL-1Blue bacteria were transformed. For induction of fusion protein cells at 0.3 A600 were treated with isoprophe1-β-thiogalactopyranoside and in feced in the absence of M13 helper virus. The corresponding truncated protein sequence (772-1192 amino acids) continues at position 788 with RSVQ. We could not detect any functional differences either in the yeast complementation assay or in the oocyte system between both forms. The results presented were obtained with the cDNA construct containing the longer open reading frame. The sequences were deposited in the GenBankTM/EMBL data base, accession nos. Z71445, Z71446, and Z71450.

RT-PCR Analysis—Total RNA was isolated from different organs of A. thaliana as described previously (36). Prior to cDNA synthesis, total RNA samples were treated with DNase I (RNase-free) to remove possible traces of genomic DNA. 5 μg total RNA per tissue were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (SuperscriptII, LifeTechnologies, Inc.) in 50 mM KCl, pH 9.2, 200 μM dNTP, 2 mM MgCl2, and 2.5 units of Tqpl plus polymerase (Stratagene). The following primers were used at 1 μM concentration: ATCLC-a, 5’-GGATGATGCACAGGGCAAG-3’; ATCLC-b, 5’-ACCATGGCACACTACATGCAG-3’; ATCLC-c, 5’-CCCATGCGGCGCAGAAGT-3’; ATCLC-d, 5’-CTTCCAGTGCCTATCCAG-3’; ATCLC-e, 5’-GCCATTAGCGCTTGAG-3’; ATCLC-f, 5’-TTCTGCATTCGTCTTATG-3’; ATCLC-g, 5’-ACCAGATCAGGAAGG-3’; ATCLC-h, 5’-CAAGATCAAGTTATATG-3’; ATCLC-i, 5’-ATGATGATGATACTGAG-3’; ATCLC-j, 5’-AAGTGGGCAAGTTTATG-3’; ATCLC-k, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-l, 5’-GGGAGAGGCAAGTTTATG-3’; ATCLC-m, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-n, 5’-GGGAGAGGCAAGTTTATG-3’; ATCLC-o, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-p, 5’-GGGAGAGGCAAGTTTATG-3’; ATCLC-q, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-r, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-s, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-t, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-u, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-v, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-w, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-x, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-y, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-z, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-aa, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ab, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ac, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ad, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ae, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-af, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ag, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ah, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ai, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-aj, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ak, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-al, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-am, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-an, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ap, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-aq, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ar, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-as, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-at, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-au, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-av, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-aw, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ax, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-ay, 5’-GACCTGGGCAAGTTTATG-3’; ATCLC-az, 5’-GACCTGGGCAAGTTTATG-3’.
AtCLC proteins expressed in yeast were analyzed as described previously (44). Briefly, 15 A_{mM} units of yeast cells were harvested and resuspended in 100 μl of TEA (7.5 g/liter triethanolamine; 0.38 g/liter EDTA, pH 8.9, supplemented with “complete” protease inhibitor (Boehringer Mannheim, Germany) and 1 mM diisopropyl fluorophosphate. Cells were lysed by 5 × 30 s vortexing cycles with glass beads added to the suspension. The equivalent of 1 A_{mM} was loaded per lane and resolved on a 10% SDS-gel.

Crude membranes were prepared from different Arabidopsis tissues as described previously (45), in the presence of 1 mM phenylmethylsulfonyl fluoride, 2.5 mM p-aminobenzamide, and with complete protease inhibitor; 20 μg of protein were analyzed on a 8.5% SDS-gel. Gels were blotted on nitrocellulose. Antiserum against AtCLC-d was used at a 1:1000 dilution, and anti-GFP antiserum (Clontech) was used at a 1:2000 dilution. Horseradish peroxidase-coupled protein A and the DuPont Renaissance reagents were used for chemiluminescent detection of bound antibody.

Site-directed Mutagenesis—Point mutations P471L, I180R, and P471L, I180R were introduced into the AtCLC-d cDNA by recombinant PCR. Briefly, two fragments were amplified with primers containing the desired mutation in a short overlapping region, joined by recombinant PCR, digested with appropriate restriction endonucleases, and ligated into the cDNA. PCR-derived fragments and restriction sites used for ligation were sequenced.

Generation of GFP-tagged AtCLC Constructs—To detect expression of AtCLC proteins in Xenopus oocytes and in yeast cells, fusion proteins of AtCLC-a and the GFP (46) were generated by ligating a cDNA encoding the GFP in frame to the C terminus of the AtCLC dRNAs. For expression in oocytes, fusion protein constructs were cloned into the pTTL vector and into pDR46 for expression in yeast.

Fluorescence Microscopy Studies in Yeast Cells—Early log-phase yeast cells, expressing the different AtCLC-GFP fusion proteins, were fixed in formaldehyde/paraformaldehyde (47). Spheroplasts were prepared as described (47) and mounted onto poly-L-lysine-coated glass slides. GFP fluorescence was analyzed in UV light using a Zeiss Axio-phot microscope, equipped with a fluorescein isothiocyanate filter set.

RESULTS

Cloning and Sequence Analysis of Arabidopsis CLC Chloride Channels—Search of the GenBankTM/EMBL data base using the TBLASTN algorithm revealed several partial cDNA sequences from Arabidopsis (T44914, T44764, T44555, T76902, and T04412) with significant homology to the CLC family of chloride channels. Using this sequence information we isolated overlapping cDNA clones covering the coding sequences of four distinct Arabidopsis mRNAs. The initiator methionine of each cDNA was assigned to the first ATG in frame. In AtCLC-a and AtCLC-d the start codon is preceded by stop codons in frame. We did not locate an upstream stop codon in AtCLC-b and -c, although the ATGs are surrounded by sequences corresponding to a Kozak translation-initiation site. The open reading frames of the respective cDNA clones code for proteins consisting of 775 (AtCLC-a), 780 (AtCLC-b), 779 (AtCLC-c), and 792 (AtCLC-d) amino acids, respectively, with similar predicted molecular masses of ~85 kDa. The protein sequence of AtCLC-a is 87% identical to that of AtCLC-b but only 53% identical with AtCLC-c and 48% identical with the AtCLC-d protein. All four cDNAs show significant homology to other CLC proteins, with CLC-6 and CLC-7 (35) being the most closely related mammalian CLC genes (~30% identity) (Fig. 1). AtCLC-c is 75% identical to CLC-Nt1, a putative chloride channel recently cloned from tobacco (14), and thus both genes may represent species homologues. Homology is observed in particular throughout the membrane spanning region of the proteins. Hydrophy analysis supports the current topology model of CLC proteins with up to 12 transmembrane spans (48) which predicts that all potential N-glycosylation sites present in the AtCLC clones (four sites in AtCLC-a and -b, and one site in AtCLC-c and -d, respectively) are intracellularly. Similar to CLC-7, AtCLC proteins lack an N-linked glycosylation site between D8 and D9, which is present in all other eukaryotic CLC proteins, including the plant homologue CLC-Nt1.

Organ-specific Expression of AtCLC Genes—RT-PCR analysis was performed on total RNA isolated from seedling; root; stem; sink-, source-, and cauline-leaf; flower; and siliqua. Oligonucleotide primers for the different genes were chosen to specifically amplify fragments containing diagnostic restriction sites. Specificity of each set of AtCLC primers was tested in parallel PCR reactions with DNA of the other three cDNA clones as template (not shown). α2-Tubulin primers were included to control for integrity of the RNA, for the presence of equal amounts of cDNA, and as a control for contamination with genomic DNA. As shown in Fig. 2, PCR fragments of the correct sizes were amplified from all tissue samples with primers specific for the respective AtCLC gene. Demonstration of RT-PCR products was evident after 30 cycles for AtCLC-a and -c, 31 cycles for AtCLC-d, but 38 cycles were required to detect AtCLC-b products, suggesting that AtCLC-b transcripts may be expressed at lower levels. However, a quantitative estimation of expression levels by RT-PCR, even when compared with the tubulin signal, is difficult, since efficiency of PCR detection is dependent on several parameters, such as primers and annealing temperature, and could be an alternative explanation for the variance seen among the four genes.

Despite their ubiquitous expression, differences in tissue specificity seem to exist among the four genes. Thus the level of AtCLC-a mRNA seems to be higher in source leaf, that of AtCLC-b in root, whereas AtCLC-d mRNA levels are highest in root and source leaf.

To further characterize the tissue distribution, we performed a Western analysis of different Arabidopsis tissues using an antisera developed against the C-terminal part of the AtCLC-d protein. To demonstrate that the antibody is specific for AtCLC-d and does not recognize the related AtCLC-a, -b, and -c proteins, yeast cells transformed with expression plasmids encoding the different AtCLC proteins, were analyzed by a Western blot. Fig. 3A shows that the antibody detects a protein of the predicted molecular mass of ~85 kDa only in cells expressing AtCLC-d. No protein is detected in cells transformed with the related AtCLC-a, -b, and -c cDNAs, as well as in cells transformed with the expression vector alone, demonstrating specificity of the antisera for the AtCLC-d protein.

A protein of the same size is present in all investigated Arabidopsis tissues (Fig. 3B), confirming that the AtCLC-d protein is widely expressed, as indicated from the distribution of its mRNA. However, and in contrast to its mRNA, the AtCLC-d protein is predominantly expressed in the siliqua.

Expression of AtCLCs in Xenopus Oocytes—In contrast to CLC-0, -1, -2, and -5, but similar to other CLC proteins (27, 35), we could not detect novel currents in oocytes injected with any of the four AtCLC cRNAs. Various experimental conditions, such as different voltage programs, increasing intracellular cAMP concentration, or coexpression of different AtCLC cRNAs in several combinations did not result in currents differing from control oocytes.

To exclude that lack of chloride currents is due to a failure of oocytes to express the corresponding proteins, crude membranes from oocytes injected with cRNAs encoding AtCLC proteins tagged C-terminally with the green fluorescent protein (46) were subjected to Western blot analysis using an anti-GFP antibody (these membranes are a mixture of plasma membrane and intracellular membranes). As is evident from Fig. 4 oocytes were capable of synthesizing all four AtCLC proteins with similar efficiencies.

Complementation of gef1 Mutant Strain of Saccharomyces cerevisiae—Green et al. (1993) described a yeast mutant with iron-limited growth and could demonstrate that its phenotype is due to a defective gene they named GEF1. This gene encodes
a protein highly homologous to mammalian voltage-gated chloride channel proteins of the CLC family. Their work suggests that GEF1 (which we termed ScCLC to conform with the nomenclature of the CLC family2) is an intracellular chloride channel that has some unknown, probably indirect role in iron metabolism.

By disrupting the ScCLC gene in a different yeast strain, we generated a mutant with a phenotype similar to the original gef1 mutant(17). All four AtCLC genes from Arabidopsis were tested for their ability to functionally complement the growth defects.
defect. The gene-disrupted yeast strain (K700a ΔScCLC) was transformed with the different plant CLC cDNAs, cloned into both a low and high copy yeast expression vector (p416Met25 and pDR46, respectively). 50% of the transformants were plated on 2URA/HIS synthetic complete medium to assess transformation efficiency, whereas the remaining 50% was screened for functional complementation on iron-limited medium LIM50 (41). Only one of the four AtCLC homologues was able to functionally complement the growth defect of the yeast strain disrupted for the ScCLC gene under conditions of iron-limited growth (Fig. 5A). AtCLC-d permitted wild-type rate of growth of the gene-disrupted yeast strain, both at low and high expression levels. We mutated several positions within the AtCLC-d protein that are of functional importance for other CLC chloride channel proteins. The proline located at position 471 is strictly conserved in all CLC proteins and, if mutated to a leucine (P480L) in the muscle chloride channel CLC-1, leads to dominant myotonia (49). Introduction of this mutation into the AtCLC-d protein completely abolished its capacity for complementation, even when expressed at high levels (Fig. 5B).

In CLC-5 introduction of a positive charge at the end of the third transmembrane domain (L200R) destroys chloride channel activity and results in hypercalciuric kidney stones (34). Although this residue is not exactly conserved in the plant protein, mutating an adjacent isoleucine (I180R) rendered the AtCLC-d protein nonfunctional. Similarly, a highly conserved leucine at the end of D3, when mutated for an arginine (L225R), functionally inactivates the yeast ScCLC protein (3). The corresponding mutant of AtCLC-d (L187R) was not able to

\[ \text{FIG. 5. Functional complementation of gef1}^- \text{ yeast mutant by AtCLC-d. Yeast cells disrupted in the gene coding for ScCLC (GEF1) were transformed either with wild-type AtCLC-d (A) or mutated AtCLC-d cDNAs (B) and cloned into either low or high expression vector, or with the respective vector alone. Cells were then grown on synthetic complete medium without uracil and histidine (SC-URA/HIS), allowing growth of all transformed cells, or on iron-limiting medium (LIM50) to select for complementation. Only cells transformed with wild-type AtCLC-d cDNA grow in the presence of low iron concentration. Introduction of mutations I180R, L187R, or P471L abolished the ability of AtCLC-d to complement the growth defect. Serial dilutions of transformed cells were plated. Only plates with single colonies are shown. With non-complementing cDNA clones no colonies were observed at any dilution.}\]
In this study we describe the molecular cloning of putative chloride channels from *Arabidopsis* and functional expression of one of them, AtCLC-d, in *S. cerevisiae*. They belong to the CLC family of voltage-gated chloride channels, and, together with the most recently identified CLC-Nt1 (14) from tobacco, represent the first putative chloride channels identified in plants at the molecular level.

The four *Arabidopsis* genes are more closely related to mammalian CLC-6 and CLC-7 (35) than to the yeast homologue ScCLC (17). CLC-Nt1 is most closely related to AtCLC-c (75% identity), suggesting that both proteins may represent species homologues (we therefore suggest to call it NtCLC-c, instead of CLC-Nt1, as originally dubbed by these authors (14)). Similar to CLC-Nt1 (14), and to several mammalian CLC genes, such as CLC-2 (22), CLC-6, and -7 (35), *Arabidopsis* AtCLC genes display a broad tissue expression. All four transcripts are ubiquitously expressed, but show some tissue preferences. Thus, strongest expression of AtCLC-a, -c, and -d transcripts seems to occur in source leaf, whereas AtCLC-b is most strongly expressed in root. We have developed an antisera against the AtCLC-d protein, and could demonstrate its specificity for this protein. Interestingly, we found that in the plant the AtCLC-d protein is mainly expressed in the fruit and not in leaf and root, as suggested from its mRNA distribution. This intriguing result might be due to a greater metabolic stability of the protein in the siliques as compared to the other tissues.

We were not able to functionally express any of the AtCLC proteins as a chloride channel in *Xenopus* oocytes. This is in contrast to Lurin *et al.* (14), who recently reported functional expression of CLC-Nt1, which may represent the tobacco homologue of AtCLC-c. Currents induced by CLC-Nt1 have properties similar to that reported for chloride currents endogenous to oocytes (50, 51). Similar currents can also be induced in oocytes by overexpressing different integral membrane proteins (51, 52), and in our hands by CLC-6, and -7 (35), and even by a nonfunctional mutant of CLC-1 (49). Thus it cannot be excluded that the currents elicited by CLC-Nt1 are due to endogenous oocyte chloride channels. Several possible reasons may explain a lack of functional expression of AtCLC proteins. We cannot exclude that we have not yet identified the proper physiological stimulus for channel activation, e.g. a second messenger pathway. It is possible that oocytes lack additional subunits necessary for functional expression, either belonging to the same or to a different gene family. CLC chloride channels are oligomers (49, 53) and functional heterooligomeric channels can be formed by CLC-1 and CLC-2 subunits (25). Since AtCLC proteins seem to have overlapping expression patterns, we tested the possibility that different AtCLC proteins combine to heterooligomeric channels. However, coexpression of AtCLC proteins in various combinations again failed to produce functional chloride channels. Heterologous expression of ion channel proteins and their detection by electrophysiological recording is mainly limited to proteins localized to the plasma membrane. Proteins of intracellular membranes usually are not targeted to the oocyte plasma membrane. Thus AtCLC proteins may play an intracellular role. The ability of the AtCLC-d to functionally complement the growth defect of the gef1 yeast mutant points in this direction.

Transport-deficient yeast strains have served as valuable tools for cloning of heterologous plant transport proteins by functional complementation (reviewed in Ref. 54). Thus, strains deficient in potassium uptake have permitted the identification of plant K+ channels (55, 56). As an alternative expression system, we constructed a yeast mutant lacking the CLC homologue ScCLC. As is the case with the ScCLC-knockout on a different genetic background (17), our mutant strain needs high iron levels for normal growth. Since uptake of extracellular iron into the original mutant cells appeared to be unaffected (17), the ScCLC protein probably indirectly plays a role in intracellular iron metabolism. Intracellular steps in iron metabolism are presently not well understood, but presumably involve transport of iron in vesicles to the vacuole, storage in the vacuole, and release from the vacuole prior to utilization, e.g. as a component of mitochondrial electron transport chain proteins. In principal, defects at any given step in the intracellular route of iron transport could result in an increased iron requirement for normal growth. This is exemplified by the finding that a mutated subunit of the vacuolar H+-ATPase...
produces a very similar iron-responsive phenotype as described for the ScCLC knockout strain (57). The vacuolar H^+ -ATPase is responsible for acidifying the vacuole, and the resulting proton gradient provides the driving force for accumulation of some metal ions in the vacuole (58). Acidification can be more efficient if chloride channels in the vacuolar membrane allow for chloride influx into the vacuole, thereby compensating for the accumulation of positive charges (59). Nevertheless, the importance of an acidic vacuole for iron storage seems controversial (60, 61).

The ability of AtCLC-d to functionally substitute for the yeast ScCLC protein implies similar physiological functions. Balancing the charge of transported protons may well be an intracellular function of both proteins. Chloride channels thought to be involved in the development and regulation of H^+ gradients have been described in the vacuolar membrane (13, 62), in photosynthetic (thylakoid) membranes of chloroplasts (63, 64), and in the inner mitochondrial membrane (65).

The similar function of both proteins is also reflected by their subcellular localization. Both proteins appear in the same intracellular compartment.

Elucidation of the precise cellular localization of AtCLC-d, as well as that of the other three AtCLC proteins, is an important goal for future studies. In addition, the successful expression of AtCLC-d in yeast offers the opportunity to use this genetic system for structure-function analysis. The isolation of four Arabidopsis CLC Chloride Channels

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