THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 52, Issue of December 27, pp. 33632–33638, 1996

Printed in U.S.A.

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

(Received for publication, May 25, 1996, and in revised form, October 17, 1996)

Mirko Hechenberger‡, Blanche Schwappach, Wolf N. Fischer§, Wolf B. Frommer§, Thomas J. Jentsch, and Klaus Steinmeyer¶

From the Center for Molecular Neurobiology (ZMNH), Hamburg University, Martinistr. 52, D-20246 Hamburg, Germany and §Institut für Botanik, Universität Tübingen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany

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-Nt1*; 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 silique. 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 elicited 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 Ca^{2+} 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[1] 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 branch 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-

* This work was supported in part by the Deutsche Forschungsgemeinschaft by a Fellowship (to K. S.) and Grants (to T. J. J. and K. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ This work constitutes part of a Ph.D. thesis.

¶ To whom correspondence should be addressed: Tel.: 49-40-4717-6614; Fax: 49-40-4717-4839.

[1] The abbreviations used are: CLC, chloride channel of the *CLC* gene family; CLC-*X*, member *X* of the *CLC* gene family; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); EST, expressed sequence tag; GFP, green fluorescent protein.

ney stone disorders (34). The single CLC homologue in yeast, *ScCLC* (originally termed *GEF1*), 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*. Whereas transcripts of AtCLC-a, -b, -c, and -d show a nearly ubiquitous tissue distribution, the AtCLC-d protein is highly expressed in the silique. None of the newly cloned proteins could be expressed functionally as a chloride channel in *Xenopus laevis* oocytes. Among the four cDNA clones only AtCLC-d rescued the iron-sensitive phenotype of a mutant yeast strain lacking the gene for ScCLC. The ability to functionally complement the yeast ScCLC protein suggests an intracellular function for AtCLC-d.

EXPERIMENTAL PROCEDURES

Isolation of cDNAs—AtCLC-a and AtCLC-d were cloned from an *Arabidopsis* cDNA library (λgt10, Clontech) using three non-overlapping 42-bp oligonucleotides complementary to each of the ESTs with accession nos. T44914 (which overlaps with T76902), T44555, and T44764. They were end-labeled with ^{32}P and hybridized to the library at 42 °C in 1 × SSC, 5 × Denhardt's solution, and 0.1% SDS. 13 independent cDNA clones hybridizing to either two or all three oligonucleotide probes based on the individual EST sequences were isolated and analyzed. To complete the sequences, two additional *Arabidopsis* cDNA libraries (λMax, Clontech; λZap, Stratagene) were rescreened with cDNA probes derived from these original cDNA clones. During this screen, a different cDNA sequence, AtCLC-b, was isolated, closely related to AtCLC-a. An initial 300-bp fragment of AtCLC-c was amplified by RT-PCR from total *Arabidopsis* RNA, using two primers complementary to the EST with the accession no. T04412, and then used to isolate a full-length cDNA clone from a cDNA library (λZap, Stratagene). In cDNA clones AtCLC-a, -c, and -d typical polyadenylation signal sequences were found (13–18, 24–29, and 10–15 nucleotides, respectively) upstream of the poly(A)$^+$ tail, indicating that these are the correct 3′ ends of the transcripts.

The sequence of all four cDNA clones was fully confirmed on both strands by using an automated DNA sequencer (ABI 373). For AtCLC-a, -b, and -d, partial sequences were obtained from independent clones that change the amino acid at the following positions in the respective proteins: in AtCLC-a, at position 330 from H to D, and at position 709 from T to I; in AtCLC-b, at position 43 from L to P, and at position 612 from D to V; in AtCLC-d, at position 532 from E to D. In case of AtCLC-d, six independent cDNA clones were sequenced to confirm the 3′ end. Three of these clones have a 5-bp deletion (at position 2305–2309 relative to the A in the initiator codon), leading to a frameshift and a premature stop codon. The corresponding truncated protein sequence (772 *versus* 792 amino acids) continues at position 768 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 GenBank™/EMBL data base, accession nos. Z71445, Z71446, Z71447, 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, Life Technologies, Inc.) in 50 μl at 42 °C for 60 min, and 2 μl of cDNA were amplified in a 25-μl total volume, containing 60 mM KCl, 20 mM Tris-HCl, pH 9.2, 200 μM dNTP, 2 mM $MgCl_2$, and 2.5 units of *Taq* plus polymerase (Stratagene). The following primers were used at 1 μM concentration: AtCLC-a, 5′-AGGATCAGACCATGGAGC-3′; 5′-GGCATGGTTTCTCTCTGG-3′; AtCLC-b, 5′-ACCGTTGGTGAGCTTGGT-3′; 5′-CCCACTTGCCGGAAAAGT-3′; AtCLC-c, 5′-ACCAGTGGCAGCATTAGG-3′; 5′-CTCCCATGGCTTTCACAG-3′; AtCLC-d, 5′-TGGCGTCGGAACTCTTTG-3′; 5′-ACAGTCATCCGCATCGAG-3′; α_2-tubulin, 5′-GAAATGCTTGCTGGGAGC-3′; 5′-CCCACGGGCGAAATTGTT-3′.

The tubulin primers used were designed to amplify a 269-bp DNA fragment from cDNA and, in case of a contamination with genomic DNA, to amplify a DNA fragment of 731 bp. All AtCLC primers have been tested for specificity for the respective cDNA by PCR on the different plasmid DNAs. Amplification reactions were subjected to 27–38 cycles of the following thermal program: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s. With these individual cycle numbers, amplification is in the linear range and allows rough quantitation of amplified products. For each set of primers at least three RT-PCR reactions, using two different batches of RNA, were performed, yielding similar results. Amplification products were run on a 1.4% agarose gel and stained with ethidium bromide. The identity of the amplified products was controlled by digestion with diagnostic endonucleases, or by direct sequencing.

Xenopus Oocyte Expression—Using PCR-based mutagenesis, an *Nco*I site was introduced at the initiator ATG codon in the respective cDNA clones (in AtCLC-d this changes the leucine at position 2 to a valine). The cDNAs were then cloned into the *Nco*I site of the expression vector pTLN (25), which places the start site for translation immediately downstream of the *Xenopus* β-globin 5′-untranslated region. Using Sp6 RNA polymerase, capped cRNA was prepared from this construct after linearization. 10–25 ng of cRNA were injected into *Xenopus* oocytes prepared and handled as described elsewhere (15). Oocytes were kept in modified Barth's solution (88 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 10 mM Hepes, pH 7.6). After 2–4 days at 18 °C, they were investigated by two-electrode voltage clamping using a Dagan amplifier and pCLAMP software (Axon Instruments). Recordings were performed at room temperature in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 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 isoosmolar concentrations of malate and nitrate. To increase intracellular cAMP oocytes were perfused with a mixture containing 200 μM chlorophenylthio-cAMP, 12 μM forskolin, and 500 μM 3-isobutyl-1-methylxanthine. Similar concentrations lead to a robust increase in cystic fibrosis transmembrane conductance regulator currents (not shown).

Production of Antiserum—For generation of antisera the prokaryotic pRSET expression system (Invitrogen) was used. A *Xho*I/*Eco*RI fragment of cDNA clone AtCLC-d, coding for the last 192 C-terminal amino acid residues was cloned into the vector pRSET-C, and XL-1 Blue bacteria were transformed. For induction of fusion protein cells at 0.3 A_{600} were treated with isopropyl-β-D-thiogalactopyranoside and infected with M13 helper virus, carrying the T7 RNA-polymerase gene. After 4 h, cells were harvested and lysates were analyzed on a preparative 11% SDS-polyacrylamide gel. The gel was stained in 250 mM KCl (37) and the protein band corresponding to the fusion protein (molecular weight ~25,000) was excised and homogenized in phosphate-buffered saline. Rabbits were immunized with 1 ml of the antigen homogenate mixed 1:1 with Freund's adjuvant. Rabbits were boosted three times at 6-week intervals.

Yeast Strains—K700α ΔScCLC, *Matα; ade2-1; trp1-1; can 1-100; leu2-3, 112; his3-11, 15; ura3; ssd1; gef1::HIS3;* derived from K700α (38). GPY385, *MAT***a***; ura3-52; leu2-3, 112; trp1-289; sst1-3; his4* and/or *his6;* Δ*pep4 :: LEU2* (39).

Functional Complementation of gef1⁻ Yeast Mutant—Yeast cells were transformed using the lithium acetate method as described previously (40). All media were used as described previously (40), except for LIM50, an iron-limiting medium prepared as described elsewhere (41). The low copy vector used was p416 MET25 (42). A yeast expression vector derived from the yeast expression vector pSEY8, pDR46, carries the URA3 marker gene and the MET3 promoter fragment (43). Methionine was generally omitted from the medium to allow maximal expression from pDR46.

Transformation efficiencies were 5 × 10^4–10^5 colonies/μg of DNA. Each set of experiments was performed three times, always with the same result.

Western Blot Analyses—Xenopus oocytes injected with cRNAs encoding AtCLC-green fluorescent protein (GFP) fusion proteins were homogenized in 20 μl/oocyte phosphate-buffered saline buffer containing 1 mM phenylmethylsulfonyl fluoride. Homogenates were cleared four times by centrifugation at 4,000 rpm, for 1 min at 4 °C in an Eppendorf centrifuge. Crude membranes were pelleted at 65,000 rpm in a Beckman tabletop ultracentrifuge for 30 min at 4 °C and dissolved in 2 μl of Lämmli SDS-buffer/oocyte. 16-μl aliquots were analyzed on a 10% SDS-gel.

AtCLC proteins expressed in yeast were analyzed as described previously (44). Briefly, 15 A_{600} 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_{600} 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*-aminobenzamidine, 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-rGFP 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 L187R 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 AtCLCs and the GFP (46) were generated by ligating a cDNA encoding the GFP in frame to the C terminus of the AtCLC cDNAs. For expression in oocytes, fusion protein constructs were cloned into the pTLN 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). Spheroblasts were prepared as described (47) and mounted onto poly-L-lysine-coated glass slides. GFP fluorescence was analyzed in UV light using a Zeiss Axiophot microscope, equipped with a fluorescein isothiocyanate filter set.

RESULTS

Cloning and Sequence Analysis of Arabidopsis Chloride Channels—Search of the GenBank™/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. Hydropathy 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 located 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 silique. 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). α_2-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 antiserum 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. 3*A* 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 antiserum for the AtCLC-d protein.

A protein of the same size is present in all investigated *Arabidopsis* tissues (Fig. 3*B*), 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 silique.

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—Greene *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

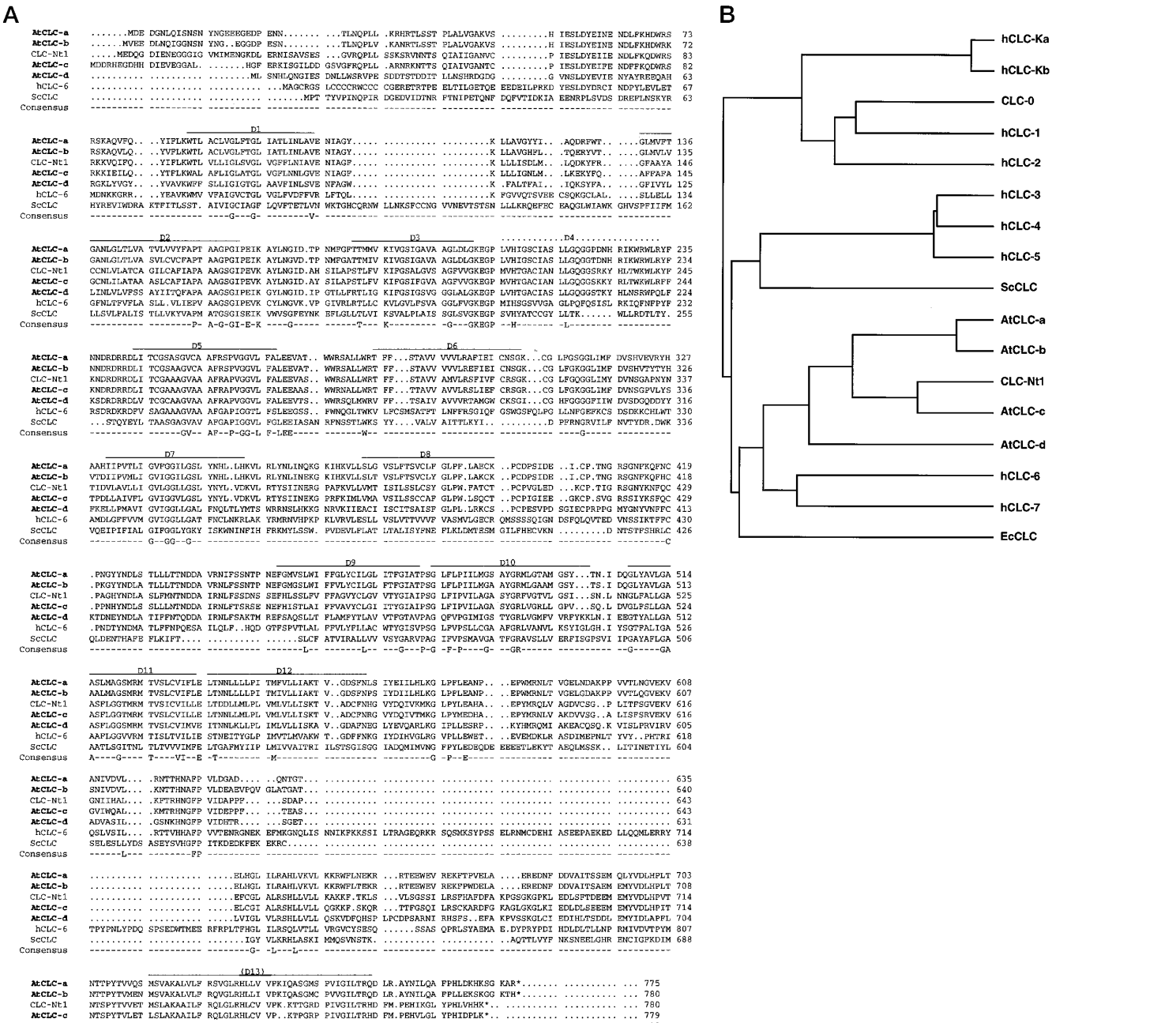

FIG. 1. *A*, amino acid sequence of AtCLC proteins and alignment of these with recently isolated tobacco CLC-Nt1, mammalian CLC-6, and yeast ScCLC. The consensus sequence below gives identical amino acid residues for all seven proteins. Conserved domains of CLC channels are indicated above the sequences (*D1–D13*). Except for D4 and D13, these domains are proposed to be transmembrane domains (48). Potential protein kinase A consensus sites predicted to be intracellular are present in AtCLC-a (position 662), AtCLC-b (positions 74 and 667), in AtCLC-c (positions 27, 49, and 664), and in AtCLC-d (positions 351 and 385). *N*-Linked glycosylation sites are present in AtCLC-a (positions 25, 560, 586, and 617), AtCLC-b (positions 35, 585, 616, and 718), AtCLC-c (position 659), and in AtCLC-d (position 95). *B*, dendrogram showing the degree of similarity between the known members of the CLC superfamily. The CLC family can be divided into three branches, the plant CLC proteins belonging to the branch comprising mammalian CLC-6, CLC-7, and EcCLC, a bacterial *CLC* gene identified by an *E. coli* genome project (16). The plot has been generated with the Pileup program of the GCG software package. *h* refers to human sequences.

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 family[2]) 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

[2] The yeast genome project reveals that there is only one *CLC* gene in the *S. cerevisiae* genome. For a standardized nomenclature of CLC chloride channels, we suggest the addition of the species name as prefix to CLC and the use of numbers only for vertebrate channels and lower case letters for other channels. Thus we suggest to name the yeast *CLC* gene *ScCLC*, instead of *GEF1*, and, if it turns out that *CLC-Nt1* is the species homologue of *AtCLC-c*, to rename it to *NtCLC-c*.

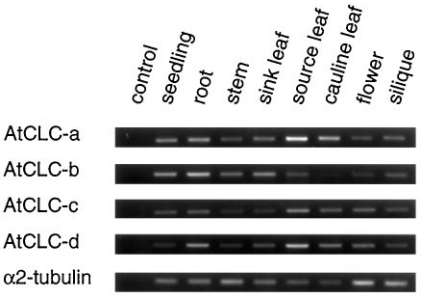

FIG. 2. **Tissue distribution of AtCLC messages.** RNA was isolated from various plant tissues and subjected to RT-PCR. RNA samples were amplified (30 cycles for AtCLC-a and -c, 31 cycles for AtCLC-d, 38 cycles for AtCLC-b, and 27 cycles for α_2-tubulin) using oligonucleotide primer pairs specific for individual AtCLC cDNAs and α_2-tubulin. No amplified products were detected when cDNA was omitted as a control.

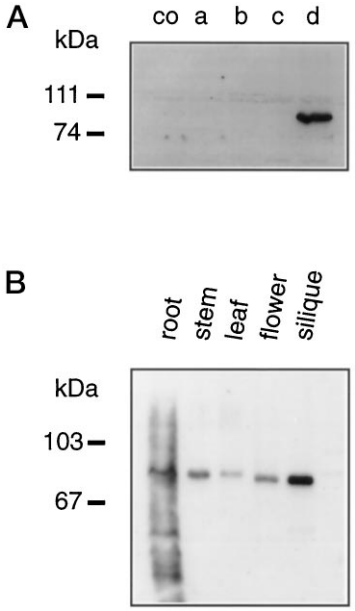

FIG. 3. **Expression pattern of the AtCLC-d protein in *Arabidopsis*.** *A*, specificity of the anti-AtCLC-d antiserum. Homogenates were prepared from PEP4-deficient (GPY385) yeast cells, transformed with AtCLC-a, -b, -c, and -d (*lanes a–d*) or with vector without cDNA insert (*lane co*) and analyzed by Western blotting. The antibody specifically detects the AtCLC-d protein (*lane d*). The same protein was detected in K700α ΔScCLC cells transformed with AtCLC-d (not shown). The corresponding preimmune serum did not recognize any protein (not shown). *B*, crude membrane preparations from different *Arabidopsis* tissues were analyzed by Western blotting with an antiserum raised against the C-terminal part of the AtCLC-d protein. Leaf homogenates contain both source and sink leaves. All lanes were loaded with 20 μg of solubilized membrane extract.

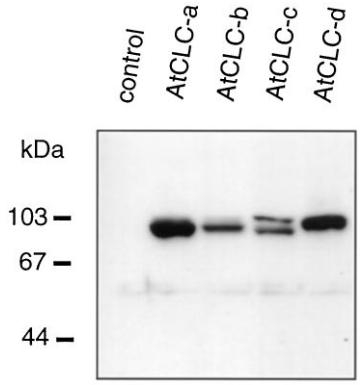

FIG. 4. **Expression of AtCLC proteins in *Xenopus* oocytes.** Homogenates of oocytes expressing the different AtCLC proteins, C-terminally tagged with a GFP, were analyzed by Western blotting using an anti-rGFP antibody. All four AtCLC proteins were synthesized with similar efficiency. No protein was detected in noninjected (*control*) oocytes.

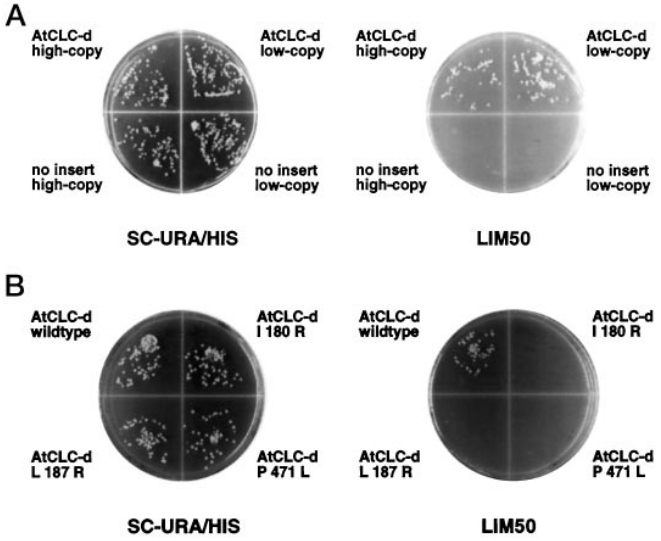

FIG. 5. **Functional complementation of *gef1*$^-$ yeast mutant by AtCLC-d.** Yeast cells disrupted in the gene coding for ScCLC (GEF1) were transformed either with wild-type AtCLC-d (*A*) or mutated AtCLC-d cDNAs (*B*) and cloned into either low or high expression vector, or with the respective vector 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 noncomplementing cDNA clones no colonies were observed at any dilution.

defect. The gene-disrupted yeast strain (K700α 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 −URA/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. 5*A*). 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. 5*B*). 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

[3] B. Schwappach, and T. J. Jentsch, unpublished observation.

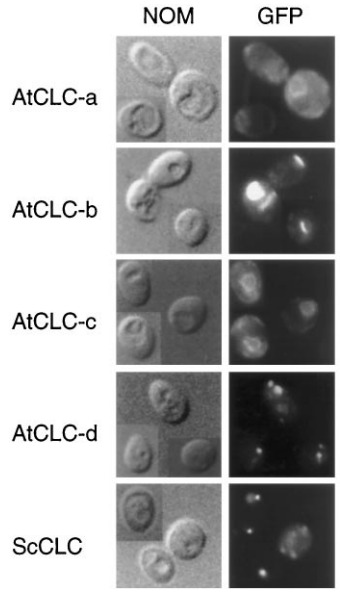

FIG. 6. **Staining pattern of AtCLC-GFP fusion proteins in yeast cells.** ScCLC-disrupted yeast cells were transformed with plasmids encoding the different AtCLC-GFP fusion proteins. Nomarski optics images (*left panels*) and green fluorescence images (*right panels*) were analyzed with a Zeiss Axiophot fluorescence microscope equipped with a fluorescein isothiocyanate filter set. For none of the GFP-tagged proteins the observed fluorescence matched the Nomarksi-visible vacuole. The staining pattern was rather dispersed for AtCLC-a, -b, and -c, but more localized for AtCLC-d and ScCLC.

rescue the growth defect of the *gef1*$^-$ yeast cells (Fig. 5*B*). This is compatible with, but does not prove, a function of AtCLC-d as a chloride channel.

Expression of GFP-tagged AtCLC Proteins in Yeast Cells—To investigate whether the ability of AtCLC-d to functionally substitute for the ScCLC protein is also reflected by a similar subcellular localization of both proteins in yeast, ScCLC knockout yeast cells were transformed with AtCLC-GFP chimeric constructs and analyzed for GFP fluorescence. The addition of the C-terminal GFP did not influence the capacity of ScCLC and of AtCLC-d proteins to complement the mutant yeast phenotype, indicating that it did not disrupt sorting of these proteins (not shown). Fig. 6 shows that all four GFP-tagged AtCLC proteins were synthesized in mutant yeast cells. The complementing AtCLC-d protein exhibits a staining pattern, similar to that of the ScCLC-GFP fusion protein. In contrast, staining observed with AtCLC-a, -b, and -c proteins is clearly different, suggesting different intracellular localizations, which in turn would easily explain their inability to substitute for the yeast ScCLC protein. In addition, staining patterns of the different AtCLC proteins do not match the position of the vacuole, as visualized by Nomarski optics, suggesting that they reside in a different compartment.

DISCUSSION

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 antiserum 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 silique 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-sensitive 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 location in the yeast, whereas the other three proteins are clearly differently localized, and therefore most likely cannot fulfill the function of the endogenous yeast ScCLC protein. Greene *et al.* (17) discussed the possibility of a vacuolar localization for the yeast CLC protein. Our studies with the GFP-tagged ScCLC protein did not support this and rather suggest that the yeast ScCLC protein plays its role in a different 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 novel *CLC* genes should facilitate the identification of other members of this chloride channel family in plants and provides an important step to further increase our understanding of the roles chloride channels play in plant physiology.

Acknowledgments—We thank Patricia Hausmann for excellent technical assistance, and S. Michaelis, E. Pratje, and S. Jentsch for the gift of yeast strains and vectors.

REFERENCES

1. Tyerman, S. D. (1992) *Plant Mol. Biol.* **43,** 351–373
2. Hedrich, R., and Jeromin, A. (1992) *Phil. Trans. R. Soc. Lond. B* **338,** 31–38
3. Schroeder, J. I. (1996) *Plant Mol. Biol.* **28,** 353–361
4. Hedrich, R., and Becker, D. (1994) *Plant Mol. Biol.* **26,** 1637–1650
5. Keller, B. U., Hedrich, R., and Raschke, K. (1989) *Nature* **341,** 450–453
6. Linder, B., and Raschke, K. (1992) *FEBS Lett.* **313,** 27–30
7. Schroeder, J. I., and Keller, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5025–5029
8. Hedrich, R., Busch, H., and Raschke, K. (1990) *EMBO J.* **9,** 3889–3892
9. Marten, I., Lohse, G., and Hedrich, R. (1991) *Nature* **353,** 758–762
10. Hedrich, R., and Marten, I. (1993) *EMBO J.* **12,** 897–901
11. Dangl, J. L., Preuss, D., and Schroeder, J. I. (1995) *Cell* **83,** 1071–1077
12. Assmann, S. M. (1993) *Annu. Rev. Cell Biol.* **9,** 345–375
13. Plant, P. J., Gelli, A., and Blumwald, E. (1994) *J. Membr. Biol.* **140,** 1–12
14. Lurin, C., Geelen, D., Barbier-Brygoo, H., Guern, J., and Maurel, C. (1996) *Plant Cell* **8,** 701–711
15. Jentsch, T. J., Steinmeyer, K., and Schwarz, G. (1990) *Nature* **348,** 510–514
16. Fujita, N., Mori, H., Yura, T., and Ishihama, A. (1994) *Nucleic Acids Res.* **22,** 1637–1639
17. Greene, J. R., Brown, N. H., DiDomenico, B. J., Kaplan, J., and Eide, D. J. (1993) *Mol. Gen. Genet.* **241,** 542–553
18. Jentsch, T. J. (1996) *Curr. Opin. Neurobiol.* **6,** 303–310
19. Steinmeyer, K., Ortland, C., and Jentsch, T. J. (1991) *Nature* **354,** 301–304
20. Koch, M. C., Steinmeyer, K., Lorenz, C., Ricker, K., Wolf, F., Otto, M., Zoll, B., Lehmann-Horn, F., Grzeschik, K.-H., and Jentsch, T. J. (1992) *Science* **257,** 797–800
21. Steinmeyer, K., Klocke, R., Ortland C., Gronemeier, M., Jockusch, H., Gründer, S., and Jentsch, T. J. (1991) *Nature* **354,** 304–308
22. Thiemann, A., Gründer, S., Pusch, M., and Jentsch, T. J. (1992) *Nature* **356,** 57–60
23. Gründer, S., Thiemann, A., Pusch, M., and Jentsch, T. J. (1992) *Nature* **360,** 759–762
24. Malinowska, D. H., Kupert, E. Y., Bahinski, A., Sherry, A. M., and Cuppoletti, J. (1995) *Am. J. Physiol.* **268,** C191–C200
25. Lorenz, C., Pusch, M., and Jentsch, T. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13362–13367
26. Uchida, S., Sasaki, S., Furukawa, T., Hiraoka, M., Imai, T., Hirata, Y., and Marumo, F. (1993) *J. Biol. Chem.* **268,** 3821–3824
27. Kieferle, S., Fong, P., Bens, M., Vandewalle, A., and Jentsch, T. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6943–6947
28. Adachi, S., Uchida, S., Ito, H., Hata, M., Hiroe, M., Marumo, F., and Sasaki, S. (1994) *J. Biol. Chem.* **269,** 17677–17683
29. Kawasaki, M., Uchida, S., Monkawa, T., Miyawaki, A., Mikoshiba, K., Marumo, F., and Sasaki, S. (1994) *Neuron* **12,** 597–604
30. Van Slegtenhorst, M. A., Bassi, M. T., Borsani, G., Wapenaar, M. C., Ferrero, G. B., De Conciliis, L., Rugarli, E. I., Grillo, A., Franco, B., Zoghbi, H. Y., and Ballabio, A. (1994) *Hum. Mol. Genet.* **3,** 547–552
31. Fisher, S. E., Black, G. C. M., Lloyd, S. E. (1995) *Hum. Mol. Genet.* **3,** 2053–2059
32. Steinmeyer, K., Schwappach, B., Bens, M., Vandewalle, A., and Jentsch, T. J. (1995) *J. Biol. Chem.* **270,** 31172–31177
33. Sakamoto, H., Kawasaki, M., Uchida, S., Sasaki, S., and Marumo, F. (1996) *J. Biol. Chem.* **271,** 10210–10216
34. Lloyd, S. E., Pearce, S. H. S., Fisher, S. E., Steinmeyer, K., Schwappach, B., Hatchwell, E., Wrong, O., Thakker, R. V., and Craig, I. W. (1996) *Nature* **379,** 445–449
35. Brandt, S., and Jentsch, T. J. (1995) *FEBS Lett.* **377,** 15–20
36. Ninnemann, O., Jauniaux, J.-C., and Frommer, W. B. (1994) *EMBO J.* **13,** 3464–3471
37. Hager, D. A., and Burgess, R. R. (1980) *Anal. Biochem.* **109,** 76
38. Schwob, E., and Nasmyth, K. (1993) *Genes Dev.* **7,** 1160–1175
39. Berkower, C., Loayza, D., and Michaelis, S. (1994) *Mol. Biol. Cell* **5,** 1185–1198
40. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1993) *Current Protocols in Molecular Biology*, pp. 13.0.1–13.13.9, J. Wiley & Sons, New York
41. Eide, D., and Guarente, L. (1992) *J. Gen. Microbiol.* **138,** 347–354
42. Mumberg, D., Mueller, R., and Funk, M. (1994) *Nucleic Acids Res.* **22,** 5767–5768
43. Mountain, H. A., and Korch, C. (1991) *Yeast* **7,** 873–880
44. Loison, G. (1994) in *Molecular Genetics of Yeast. A Practical Approach* (J. R. Johnston, ed), pp. 161–180, Oxford University Press, New York
45. Stolz, J., Stadler, R., Opekarova, M., and Sauer, N. (1994) *Plant J.* **6,** 225–233
46. Heim, R., Cubitt, A. B., and Tsien, R. Y. (1995) *Nature* **373,** 663–664
47. Roberts, C. J., Raymond, C. K., Yamashiro, C. T., and Stevens, T. H. (1991) *Methods Enzymol.* **194,** 644–661
48. Jentsch, T. J., Günther, W., Pusch, M., and Schwappach, B. (1995) *J. Physiol.* **482P,** 19S–25S
49. Steinmeyer, K., Lorenz, C., Pusch, M., Koch, M. C., and Jentsch, T. J. (1994) *EMBO J.* **13,** 737–743
50. Kowdley, G. C., Ackerman, S. J., John, E. III, Jones, L. R., and Moorman, J. R. (1994) *J. Gen. Physiol.* **103,** 217–230
51. Shimbo, K., Brassard, D. L., Lamb, R. A., and Pinto, L. H. (1995) *Biophys. J.* **69,** 1819–1829
52. Tzounopoulos, T., Maylie, J., and Adelman, J. P. (1995) *Biophys. J.* **69,** 904–908
53. Middleton, R. E., Pheasant, D. J., and Miller, C. (1994) *Biochemistry* **33,** 13189–13198
54. Frommer, W. B., and Ninnemann, O. (1995) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 419–444
55. Anderson, J. A., Huprikar, S. S., Kochian, L. V., Lucas, W. J., and Gaber, R. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3736–3740
56. Sentenac, H., Bonneaud, N., Minet, M., Lacroute, F., Salmon, J.-M., Gaymard, F., and Grignon, C. (1992) *Science* **256,** 663–665
57. Eide, D. J., Bridgham, J. T., Zhao, Z., and Mattoon, J. R. (1993) *Mol. Gen. Genet.* **241,** 447–456
58. Klionsky, D. J., Herman, P. K., and Emr, S. D. (1990) *Microbiol. Rev.* **54,** 266–292
59. Wada, Y., Ohsumi, Y., and Anraku, Y. (1992) *Biochim. Biophys. Acta* **1101,** 296–302
60. Raguzzi, F., Lesuisse, E., and Crichton, R. R. (1988) *FEBS Lett.* **231,** 253–258
61. Bode, H. P., Dumschat, M., Garotti, S., and Fuhrmann, G. F. (1995) *Eur. J. Biochem.* **228,** 337–342
62. Hedrich, R., and Kurkdjian, A. (1988) *EMBO J.* **7,** 3661–3666
63. Schönknecht, G., Hedrich, R., Junge, W., and Raschke, K. (1988) *Nature* **336,** 589–592
64. Pottosin, I. I., and Schönknecht, G. (1995) *J. Membr. Biol.* **148,** 143–156
65. Beavis, A. D., and Vercesi, A. E. (1992) *J. Biol. Chem.* **267,** 3079–3087