CLC-Nt1, a putative chloride channel protein of tobacco, co-localizes with mitochondrial membrane markers

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The voltage-dependent chloride channel (CLC) family of membrane proteins has cognates in animals, yeast, bacteria and plants, and chloride-channel activity has been assigned to most of the animal homologues. Lack of evidence of CLC functions in plants prompted us to characterize the cellular localization of the tobacco CLC-Nt1 protein. Specific polyclonal antibodies were raised against an N-terminal polypeptide of CLC-Nt1. These antibodies were used to probe membrane proteins prepared by various cell-fractionation methods. These included aqueous two-phase partitioning (for plasma membranes), free-flow electrophoresis (for vacuolar and plasma membranes), intact vacuole isolation, Percoll-gradient centrifugation (for plastids and mitochondria) and stepped, linear, sucrose-density-gradient

centrifugation (for mitochondria). Each purified membrane fraction was characterized with specific marker enzyme activities or antibodies. Our studies ruled out the possibility that the major cell localization of CLC-Nt1 was the vacuolar or plasma membranes, the endoplasmic reticulum, the Golgi apparatus or the plastids. In contrast, we showed that the tobacco CLC-Nt1 specifically co-localized with the markers of the mitochondrial inner membrane, cytochrome *c* oxidase and NAD9 protein. CLC-Nt1 may correspond to the inner membrane anion channel ('IMAC') described previously in animal and plant mitochondria.

Key words: anion transport, membranes, mitochondria, plant cell, voltage-dependent chloride channels.

INTRODUCTION

The voltage-dependent chloride channel (CLC) family, with members in bacteria, yeast, animals and plants, defines a large class of structurally related membrane proteins with putative chloride channel activities [1]. Eukaryotic CLCs can be distributed into three homology subfamilies, with mammalian CLC-0, CLC-1, CLC-2, CLC-K1 and CLC-K2 defining the first branch. All five of these proteins have been shown to function as plasma membrane chloride channels and participate in distinct cellular functions, such as membrane excitability, regulation of cell volume and epithelial transport [1]. Animal CLC-3, CLC-4 and CLC-5, together with yeast Gef1p, define the second branch of the CLC family. Whereas CLC-3 encodes a plasma membrane volume-regulated chloride channel [2], CLC-5 and Gef1p have been recently localized in intracellular membranes [3–5]. Gef1p may provide an electrical shunt for H⁺-ATPase in acidic vesicles of the late Golgi apparatus and thus participate in anion and cation homeostasis in this compartment [3,6]. CLC-5 is predominantly localized in endocytotic membranes, where it might play a role similar to that of Gef1p [4]. The two putative chloride channels, CLC-6 and CLC-7, from mammals, together with all the plant CLCs define the last branch of the family. The function of these putative chloride channels remains unknown, but CLC-6 was recently localized in the endoplasmic reticulum [7].

Whereas, in animals, several types of molecular chloride channels different from CLCs have been recognized, the CLC

genes from tobacco (*Nicotiana tabacum*) [8] and *Arabidopsis thaliana* [9] represent the only known putative anion channel genes of plants. Heterologous expression of tobacco CLC-Nt1 in *Xenopus* oocytes elicited inward chloride currents upon membrane hyperpolarization [8]. However, evidence for chloride-channel activity of plant CLCs in the native membrane is still lacking. These proteins may encode some of the various voltage-dependent anion channel activities which have been established in plant cell membranes [10,11]. Because of its critical physiological role and because it is easily amenable to patch-clamp analysis, the plant plasma membrane has been the object of most studies. However, anion channels have also been reported in internal membranes, such as the tonoplast, which delimits the large central vacuole, and in mitochondrial and plastid membranes [10,11].

Lack of evidence of CLC functions in plants prompted us to characterize the cellular localization of the tobacco CLC-Nt1 protein. Specific antibodies were raised against the protein and used to probe membrane fractions purified using various cell-fractionation methods. These studies showed that CLC-Nt1 co-localized with markers of the mitochondrial membrane.

EXPERIMENTAL

Plant materials

Tobacco (*Nicotiana tabacum* cv. Xanthi; wild-type clone XHFD8) plants were grown in a greenhouse at 22 °C with a

Abbreviations used: CLC, voltage-dependent chloride channel; DHFR, dihydrofolate reductase; IMAC, inner membrane anion channel; N1, 53-amino-acid N-terminal polypeptide of CLC-Nt1.

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photoperiod of 9 h of light/day for vegetative development and of 16 h for flowering induction. Tobacco (*N. tabacum* cv. Xanthi) suspension-cultured cells were maintained in B5 Gamborg medium containing $1\ \mu\text{M}$ 2,4-dichlorophenoxyacetic acid and 60 nM kinetin in the light at 26 °C, and were transferred to fresh medium every 7 days. Exponentially growing cells were used 4–5 days after transfer.

Protein expression in *Escherichia coli* and antibody production

A fusion protein between the rat dihydrofolate reductase (DHFR) and a 53-amino-acid N-terminal polypeptide of CLC-Nt1 (Met¹–Gln⁵³) (N1) was produced using a Qiagen expression system (Les Ulis, France). Briefly, a DNA fragment encoding N1 was generated by PCR with appropriate oligonucleotides as primers and CLC-Nt1 cDNA as template. This fragment was cloned in a pQE30 plasmid (Qiagen), downstream and in-frame of the His₆ motif. A 561-bp cDNA fragment encoding rat DHFR was then inserted in-frame between the His₆ motif and the CLC fragment of the plasmid. M15 (pREP4) bacteria were transformed with the resulting pQE30-DHFR-N1 plasmid. For production of the fusion protein, bacteria were grown to a D_{600} of 0.8 and induced by 2 mM isopropyl β -D-thiogalactopyranoside for 5 h. Proteins were then extracted and purified by affinity chromatography on a metal-chelation resin charged with Ni²⁺ under denaturing conditions, as described in the Qiagen instruction manual. Approx. 20 mg of fusion protein per litre of culture was usually obtained.

Two rabbits were immunized with 1 mg of fusion protein in complete Freund's adjuvant, injected subcutaneously. Three booster injections were administered at 4-week intervals with the same amount of protein but in incomplete Freund's adjuvant. Blood samples were collected 10 days after each boost, kept at room temperature for 8 h and then centrifuged at 1000 *g* for 15 min. The serum was collected and stored at –80 °C. For immunopurification of antibodies, the antigenic fusion protein was coupled in anhydrous conditions to Affi-Gel 10 beads (Bio-Rad, Hercules, CA, U.S.A.). Briefly, 500 μg of DHFR fusion protein solubilized in DMSO was added to 0.5 ml of activated beads and reaction was carried out for 2 h at room temperature. Unreacted groups were blocked with 50 μl of 1 M ethanolamine, pH 8, and the beads were washed and equilibrated with 10 mM Tris/HCl, pH 7.5. Antiserum (1 ml) was heated at 56 °C for 30 min and cleared by centrifugation at 10000 *g* for 5 min. The serum was then diluted 5-fold in 10 mM Tris/HCl, pH 7.5, and added to the pre-equilibrated affinity beads. The beads were maintained for 1 h at room temperature to allow binding of the antibodies. The beads were then transferred to a chromatography column and washed successively with 10 mM Tris/HCl, pH 7.5, and with 500 mM NaCl/10 mM Tris/HCl, pH 7.5. The specific antibodies were eluted first with 100 mM glycine buffer, pH 2.5, followed by 100 mM triethylamine buffer, pH 11.5. The eluted antibody fractions were combined and dialysed against PBS.

Membrane purification

A microsomal membrane fraction was prepared from suspension cells or leaves, as described by Mathieu et al. [12]. Membranes from suspension cells were further separated by free-flow electrophoresis as described previously [13]. A plasma-membrane-enriched fraction was purified from leaf microsomes by aqueous two-phase partitioning as described by Ephritikhine et al. [14]. Vacuoles were purified according to Höfte et al. [15] from tobacco mesophyll protoplasts [16]. Plastids were isolated from tobacco leaves by a three-step Percoll gradient according to the method of Douce and Joyard [17]. Mitochondria were isolated

from tobacco leaves on a self-generating gradient of Percoll (28%) in combination with a linear gradient of polyvinylpyrrolidone (0–10%) and a linear gradient of raffinose (0–300 mM) according to Douce et al. [18]. For organelle membrane purification, vacuoles, plastids and mitochondria were lysed by a hypo-osmotic treatment, collected by centrifugation at 100000 *g* for 30 min and resuspended in 300 mM sucrose, 9 mM KCl, 5 mM dithiothreitol, 10 mM Mes/Tris, pH 6. Marker enzyme assays were performed as described previously [19].

A fractionation procedure for tobacco leaf membranes by linear sucrose gradient centrifugation was adapted from that of Chaumont et al. [20], and is briefly described. Leaves (30 g) were homogenized in a blender in 150 ml of 330 mM sucrose, 50 mM Tris/HCl, 0.2% (w/v) BSA, 0.04% (v/v) 2-mercaptoethanol, pH 8.0, filtered through sieves (pore sizes 500 μm and 56 μm) and the homogenate was centrifuged at 3000 *g* for 5 min. The supernatant was then centrifuged for 25 min at 25000 *g* and the pellet was resuspended in 1 ml of 400 mM mannitol, 10 mM potassium phosphate buffer, pH 7.2. The suspension was layered on to a linear sucrose gradient [25–50% (w/w) sucrose in 50 mM KCl, 10 mM Tris/HCl, pH 7.5] and centrifuged for 16 h at 30000 *g*. Membrane fractions of 500 μl were removed, washed in 50 mM KCl, 10 mM Tris/HCl, pH 7.5, and centrifuged at 100000 *g* for 30 min.

Immunoblot analysis

Proteins were resolved by SDS/PAGE (10% gel) and blotted on to PVDF membranes (Millipore, Bedford, MA, U.S.A.). The membranes, blocked with TBST [250 mM NaCl, 0.1% (v/v) Tween 20, 50 mM Tris/HCl, pH 7.6] containing 7.5% (w/v) fat-free milk powder, were incubated overnight at room temperature in TBST, with the appropriate dilutions of serum or purified antibodies. After two washes with TBST, secondary antibodies (goat anti-rabbit IgGs coupled to alkaline phosphatase conjugate; BioRad) (1:2000 dilution in TBST) were added and the membrane was incubated for 1 h at room temperature. After washing, the antigen–antibody complex was detected by colour development with 5-bromo-4-chloro-3-indolyl phosphate (2.5%, w/v) and Nitro Blue Tetrazolium (5%, w/v). Rabbit antisera anti-NAD9 (mitochondrial marker) and anti-E37 (plastid marker) were kindly provided by Dr J.-M. Grienenberger (CNRS, Strasbourg, France) and Dr J. Joyard (CEA, Grenoble, France) respectively. The polyclonal antibodies directed against a tobacco plasma membrane H⁺-ATPase and the E subunit of a barley vacuolar H⁺-ATPase were a gift from Professor Marc Boutry (University of Louvain-la-Neuve, Belgium) and Dr K.-J. Dietz (University of Würzburg, Germany) respectively.

RESULTS

Production and characterization of the antiserum

Antibodies were raised against a fusion protein between rat DHFR and N1. To reduce the background signal observed with both preimmune and immune sera (results not shown), antibodies were affinity-purified using the DHFR–N1 fusion. As shown in Figure 1(A), the purified antibodies recognized a single band at 70 kDa, whereas the preimmune purified serum did not react with any microsomal protein. This indicates that the 70-kDa signal was specifically related to the DHFR–N1 antigens.

The antibodies, however, may recognize a plant protein immunologically related to rat DHFR. To check for this possibility, the antibodies were pretreated with an excess of recombinant DHFR protein. As shown in Figure 1(B), DHFR pre-adsorption completely abolished the reactivity of the DHFR–N1

Figure 1 Specificity of polyclonal antibodies raised against CLC-Nt1 polypeptide

(A) Immunoblot analysis of microsomal membrane proteins (10 µg) from suspension cells using an affinity-purified preimmune (1) or immune (2) serum raised against CLC-Nt1. (B) Immunoblot analysis of microsomal membrane proteins (m) (20 µg), DHFR-N1 (10 ng) or DHFR (10 ng) using affinity-purified antibodies raised against CLC-Nt1. Where indicated, antibodies preabsorbed with 10 µg of DHFR were used as primary antibodies (DHFR Pretreated).

Figure 2 Immunoblot analysis of free-flow electrophoresis membrane fractions

(A) Microsomal proteins (m) and pooled membrane fractions (A–F) purified from tobacco cells by free-flow electrophoresis (10 µg of protein per lane) were probed with affinity-purified antibodies raised against CLC-Nt1. (B) Corresponding free-flow electrophoresis profile, as determined by absorbance at 280 nm. The letters A–F indicate the positions of the pooled fractions.

antibodies to DHFR protein, whereas the reactivity to the DHFR-N1 fusion protein and to the 70-kDa microsomal protein was unchanged. This result indicates that antibodies to DHFR-N1 detected a membrane protein immunologically related to the N1 fragment of CLC-Nt1, and that the 70-kDa protein revealed in immunoblot experiments is very likely to be the tobacco CLC-Nt1 protein. A signal at 70 kDa did not exactly match the calculated molecular mass of CLC-Nt1 (84.7 kDa), but abnormal electrophoretic mobility of many membrane proteins, for example renal CLC-K1 [21] has been reported.

Figure 3 Immunoblot analysis of fractions enriched in tonoplast, mitochondrial, plastidial and plasma membranes

Microsomal proteins (m), plasma membrane (PM), tonoplast (T), mitochondrial membrane (M) and plastid membrane (P) fractions were probed with affinity-purified antibodies raised against CLC-Nt1. Equal amounts of proteins (10 µg) were loaded on to each lane. The proteins were also probed with antibodies raised against a *N. plumbaginifolia* plasma membrane H⁺-ATPase (PM-ATPase), the subunit E of barley vacuolar H⁺-ATPase (TpP31), the wheat mitochondrial protein Nad9 (Nad9) and the spinach chloroplast protein E37 (E37). The large signal with a weak intensity, observed in the tonoplast, is not specific and corresponds to the major protein of this extract, as observed after Coomassie Blue staining (results not shown).

Biochemical subcellular localization of CLC-Nt1 proteins

In plants, the activity of voltage-dependent anion channels has been generally characterized in the vacuolar (tonoplast) and plasma membranes. Because CLCs are obvious candidates for these activities, we first searched for a putative localization of CLC-Nt1 in these two membranes. Membrane vesicles from tobacco suspension cells were fractionated by free-flow electrophoresis and characterized using the antibodies raised against CLC-Nt1. Figure 2 shows that the signals specific for the protein were reduced in the least and most electronegative membrane fractions. These fractions have been shown to be being specifically enriched in plasma membrane and tonoplast respectively [13]. In contrast, strong signals were detected in the central fractions, which contained most of the internal cellular membranes. Plasma membrane and tonoplast were also prepared from tobacco leaves by two independent procedures, aqueous two-phase partitioning and intact vacuole purification. As shown in Figure 3, a faint signal was obtained with CLC-Nt1 antibodies in a crude microsomal fraction, but no specific signal could be detected in the corresponding plasma membrane and tonoplast fractions. Yet, the enhancement of signals obtained in the plasma membrane and the tonoplast fractions with antibodies raised to a tobacco plasma membrane H⁺-ATPase [22] or the subunit E of a barley vacuolar H⁺-ATPase [23] demonstrates the efficiency of our purification procedures. Altogether, these data indicate that CLC-Nt1 proteins are most likely not localized in the plasma membrane or in the tonoplast of tobacco cells.

In preliminary experiments, we observed that the immunodetection of the CLC-Nt1 signal was very pronounced in a 1000–10000 g fraction (results not shown). This fraction is typically enriched in mitochondria and plastids and, indeed, anion channels have been reported in these two organelles [10]. To investigate these findings, mitochondria and plastids were prepared from tobacco leaves by centrifugation on Percoll gradients. To evaluate the efficiency of Percoll-gradient separation, the mitochondria- and plastid-enriched fractions were

Table 1 Purity of mitochondria purified from tobacco leaves by Percoll centrifugation

A 1000–10000 *g* fraction was prepared from a tobacco leaf homogenate, as described in the Experimental section, and was used to isolate mitochondria by Percoll centrifugation. Enzyme marker activities (means $\pm$ S.E.M.) specific for mitochondria (cytochrome *c* oxidase; μ mol/min per mg of protein), endoplasmic reticulum (NADH cytochrome *c* reductase; μ mol/min per mg of protein) and Golgi apparatus (latent UDPase and latent IDPase; μ mol/h per mg of protein) were measured at each step of the purification procedure. Numbers of replicates are shown in parentheses. ND, not detected. The enrichment in enzyme marker activities obtained after Percoll centrifugation (purified mitochondria/1000–10000 *g* fraction) is indicated in the last column.

Enzyme marker	Cell fraction			Fold increase
	Homogenate	1000–10000 <i>g</i>	Purified mitochondria	
Cytochrome <i>c</i> oxidase	0.27 $\pm$ 0.08 (3)	3.71 $\pm$ 0.33 (6)	27.75 $\pm$ 0.59 (3)	7.5
NADH cytochrome <i>c</i> reductase	0.89 $\pm$ 0.15 (2)	0.25 $\pm$ 0.01 (3)	0.15 $\pm$ 0.01 (3)	0.6
Latent UDPase	0.97 $\pm$ 2.92 (5)	3.91 $\pm$ 1.60 (5)	ND	–
Latent IDPase	1.27 $\pm$ 0.96 (5)	3.63 $\pm$ 1.03 (5)	ND	–

Figure 4 Immunoblot analysis of membranes fractionated by sucrose-gradient centrifugation

(A) Sucrose-gradient membrane fractions (20 μ g of protein/lane) were probed with affinity-purified antibodies raised against CLC-Nt1 or with antibodies raised against the wheat mitochondrial Nad9. Fractions were numbered from the bottom of the gradient. Asterisks alongside fractions 11–15 indicate a faint CLC-Nt1 signal. (B) Percentage sucrose concentration (w/w) of the corresponding fractions. (C) Protein concentration was estimated by the Bradford technique [34]. Cytochrome *c* oxidase activity (% of maximum value) was measured using 2 μ g of each membrane fraction.

characterized by marker antibodies, which included antibodies raised against spinach chloroplast protein E37 [24] and wheat mitochondrial protein Nad9 [25]. The purity of the mitochondrial fraction was also estimated using enzyme markers specific for the inner mitochondrial membrane (cytochrome *c* oxidase), the endoplasmic reticulum (cytochrome *c* reductase) and the Golgi apparatus (latent UDPase and IDPase) (Table 1). As shown in Figure 3, no signal was observed after immunoblot analysis of the plastid fraction with the CLC-Nt1 antibodies. In contrast, the mitochondrial fraction was strongly enriched in proteins immunologically related to CLC-Nt1. Consistent with our purification procedure, we found that the mitochondrial fraction was strongly enriched in Nad9 marker (Figure 3) and cytochrome *c*

oxidase activity (Table 1). A 6- to 7-fold increase in the latter activity during mitochondrial purification was consistently paralleled by a 8- to 11-fold increase in the CLC-Nt1 immunoblot signal. The contamination of the mitochondrial fraction by plasma membrane, tonoplast (Figure 3) or Golgi membranes (Table 1) was very low and was barely detectable. In contrast, a significant level of cytochrome *c* reductase was measured in the mitochondrial fraction. However, this endoplasmic reticulum marker was depleted nearly 2-fold after Percoll centrifugation, whereas the immunodetection of CLC-Nt1 was enhanced. The mitochondrial fraction was also labelled by the chloroplast marker antibodies. However, the CLC-Nt1 antibodies gave a strong signal in the mitochondrial fraction but not in the plastid fraction, indicating that the channel protein was not localized in this fraction. Altogether, these results support the idea that, in tobacco leaves, CLC-Nt1 is not localized in plastids or in any compartment of the secretory pathway, but rather in mitochondria.

To ensure that the signal for CLC-Nt1 in purified mitochondria was not due to a contamination by yet another cellular membrane, we used equilibrium linear sucrose-gradient centrifugation (25–50%, w/w) as a distinct method for fractionation of tobacco leaf membranes. The antibodies raised against CLC-Nt1 showed a typical signal at 70 kDa, with a major peak in fractions 1–5 (50–42% sucrose) and a minor peak in less dense fractions 11–15 (36–32% sucrose) (Figure 4). Cytochrome *c* oxidase activity and Nad9 protein, two markers of the inner mitochondrial membrane, also exhibited a consistent bimodal separation profile. These two peaks are characteristic of mitochondrial membranes [20]. Precise comparison of CLC-Nt1, cytochrome *c* oxidase and Nad9 showed slight differences between the three profiles, in particular in the position and intensity of the second peak. This result might indicate small differences in the populations of vesicles harbouring the three proteins.

The localization of CLC-Nt1 in tobacco cells was also investigated by immunogold electron microscopy. The number of gold particles remained very low, because of the low abundance of the CLC-Nt1 proteins and/or a loss of antigenicity after chemical fixation. Nevertheless, CLC-Nt1 antibodies specifically labelled mitochondria in suspension cells and in a crude 1000–10000 *g* leaf fraction (results not shown). This result confirms that the signal obtained in the immunoblot analyses of mitochondria-enriched fractions was indeed associated with mitochondrial membranes.

DISCUSSION

In contrast with their animal counterparts, little is known about the functions of plant CLCs. The anion currents observed after expression of CLC-Nt1 in *Xenopus* oocytes did not provide any obvious parallel to the various anion channels described in the plasma membrane and internal membranes of plant cells [8]. Also, it has been suggested that these currents may reflect activation of endogenous currents [8,26]. Yeast has a unique CLC homologue (Gef1p), and functional complementation of a Gef1p null strain (*gef1*) has been used to evaluate the function of *Arabidopsis* CLCs (AtCLCs) [9]. However, the *gef1* phenotype is multifactorial and could be rescued to various extents by distinct AtCLCs. For example, AtCLC-d complemented both the iron-dependent respiratory and pH-sensitive growth phenotypes of *gef1*, whereas AtCLC-c rescued only the latter deficiency [3]. CLC-0, a *Torpedo* plasma membrane channel involved in the electric organ excitability, also complemented Gef1p function in the Golgi apparatus [3]. These results show that the yeast

complementation assay does not allow a definitive conclusion on the cellular localization and function of animal and plant CLCs.

To determine the cellular localization of CLC-Nt1, and thus get some idea of its function, we raised antibodies against an N-terminal polypeptide of the protein. The antibodies were used to probe various plant membrane fractions and consistently identified a 70-kDa protein immunologically related to CLC-Nt1 antigens. The use of independent membrane purification procedures allowed us to establish that CLC-Nt1 was not localized in the plasma membrane or in the tonoplast. These data are consistent with the report by Hechenberger et al. [9], who showed that the staining patterns of AtCLC-GFP (green fluorescent protein) chimeric constructs in yeast were intracellular and did not match the position of the vacuole. In the present work, we show more specifically that the CLC-Nt1 protein co-localizes with two mitochondrial membrane markers, the Nad9 protein and cytochrome *c* oxidase activity. This conclusion is reliant on the preparation of highly purified mitochondria by Percoll centrifugation. The extensive characterization of this preparation showed that the enhanced CLC-Nt1 signal observed in this fraction cannot be explained by contamination with plasma, vacuolar, plastid, endoplasmic reticulum or Golgi membranes. Similarly to the two mitochondrial markers, CLC-Nt1 also exhibited a typical bimodal separation profile after sucrose-gradient centrifugation. The localization of CLC-Nt1 was confirmed in suspension cells and in a crude leaf fraction by faint but specific immunogold labelling of mitochondria with the CLC-Nt1 antibodies.

The localization and/or function of CLCs in the intracellular membranes of yeast or animal cells has been unambiguously established by several groups [3–5,7]. Yet, the localization of tobacco CLC-Nt1 in the mitochondrial fraction remains unique, because this compartment does not directly derive from the cell secretory pathway. Similar cases have been reported for Shaker-like K⁺ channels and porin-like voltage-dependent anion channels, where members of the same molecular family show very distinct cellular distributions with some members in the secretory pathway and others in mitochondria [27,28]. The unexpected localization of CLC-Nt1 in mitochondria now raises specific questions concerning the post-translational import of the protein into this compartment. The N-terminal region of CLC-Nt1 does not show any transit-peptide signature, but several inner membrane proteins, including the recently identified plant uncoupling proteins [29], do not contain any cleavable peptide extension.

The outer mitochondrial membrane harbours large conductance voltage-dependent anion channels (VDAC) encoded by porin-like channel proteins [30]. Anion transport in mitochondria is thus primarily limited by a specific pathway in the inner membrane, referred to as the inner membrane anion channel (IMAC) [31]. This pathway has been detected in the mitochondria of animals [31] and plant species, such as potato [32] and tobacco (C. Lurin and C. Maurel, unpublished work), by means of a classical mitochondrial-swelling assay. Patch-clamp experiments also revealed a voltage-gated 108-pS chloride channel in the inner mitochondrial membrane of several animal cell types, and this activity has been tentatively associated with IMAC permeability [33]. The molecular identity and the cellular functions of these channels remains unknown and the results of the present study lead to the idea that CLC-Nt1 maybe a candidate for these activities in tobacco. The *CLC-Nt1* gene may thus provide a unique molecular tool to study the putative role of mitochondrial

anion transport in the regulation of mitochondrial volume and in the control of membrane electrical potential.

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