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The activity of plant inner membrane anion channel (PIMAC) can be performed by a chloride channel (CLC) protein in mitochondria from seedlings of maize populations divergently selected for cold tolerance

Elisabetta Tampieri · Elena Baraldi · Francesco Carnevali · Elisabetta Frascaroli · Aurelio De Santis

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Abstract The proteins performing the activity of the inner membrane anion channel (IMAC) and its plant counterpart (PIMAC) are still unknown. Lurin et al. (Biochem J 348: 291–295, 2000) indicated that a chloride channel (CLC) protein corresponds to PIMAC activity in tobacco seedling mitochondria. In this study, we investigated: (i) the presence of a CLC protein in maize seedling mitochondria; (ii) the involvement of this protein in plant cold tolerance; and (iii) its possible role in PIMAC activity. We validated the presence of a CLC protein (ZmCLCc) in maize mitochondria by immunoassay using a polyclonal antibody against its C-terminus. The differential expression of the ZmCLCc protein in mitochondria was measured in seedlings of maize populations divergently selected for cold tolerance and grown at different temperatures. The ZmCLCc protein level was higher in cold stressed than in non-stressed growing conditions. Moreover, the ZmCLCc level showed a direct relationship with the cold sensitivity level of the populations under both growing conditions, suggesting that selection for cold tolerance induced a constitutive change of the ZmCLCc protein amount in mitochondria. The anti-ZmCLCc antibody inhibited (about 60%) the channel-mediated anion translocations by PIMAC, whereas the same antibody did not affect the free diffusion of potassium thiocyanide through the inner mitochondrial membrane. For this reason, we conclude that the mitochondrial ZmCLCc protein can perform the PIMAC activity in maize seedlings.

Keywords Chloride channel · Cold tolerance · Inner membrane anion channel · Maize · Plant mitochondria · *Zea mays*

Abbreviations

IMAC	mitochondrial inner membrane anion channel
VDAC	mitochondrial voltage dependent anion channel
PIMAC	plant mitochondrial inner membrane anion channel
PT-pore	mitochondrial permeability transition pore
CLC	chloride channel
CLC-Nt1	tobacco chloride channel
C0	source maize population
C4H	divergently selected maize population for high cold tolerance
C4L	divergently selected maize population for low cold tolerance
PVDF	polyvinylidene difluoride
V-ATPase	vacuolar-type H^+-ATPase
NS	25 °C for 7 days (non-stressed)
AC	25 °C for 3 days followed by an

E. Tampieri · F. Carnevali · A. De Santis (✉)
Laboratory of Plant Physiology, Dipartimento di Scienze del Mare, Università Politecnica delle Marche,
Via Brecce Bianche,
60123 Ancona, Italy
e-mail: aurelio.desantis@univpm.it

E. Baraldi
Dipartimento di Protezione e Valorizzazione Agro-Alimentare, Alma Mater Studiorum, Università di Studi di Bologna, Bologna, Italy

E. Frascaroli
Dipartimento di Scienze e Tecnologie Agroambientali, Alma Mater Studiorum, Università di Studi di Bologna, Bologna, Italy

	acclimation for 7 days at 14 °C (acclimated)
ACS	25 °C for 3 days followed by 2 days acclimation at 14 °C and then by a cold stress treatment for 7 days at 5 °C (acclimated and stressed)
S	25 °C for 3 days followed by a cold stress treatment for 7 days at 5 °C, without any acclimation period (stressed)
DEPC	diethyl pyrocarbonate
ZmCLC	maize chloride channel
PBS	phosphate buffered saline solution
PBS (T)	phosphate buffered saline solution containing Tween 20
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
EGTA	ethylene-glycol tetraacetic acid
J	anion fluxes
AtCLC	*Arabidopsis thaliana* chloride channel
OsCLC	rice chloride channel

Introduction

In mammalian cells, the inner membrane anion channel (IMAC) has been demonstrated to play crucial functions, such as the regulation of the mitochondrial volume and respiratory rates (Beavis 1992), and the control of the superoxide traffic among mitochondria (Brown and O'Rourke 2011). The IMAC activity has been widely studied in rat liver mitochondria (Beavis et al. 1985; Garlid and Beavis 1985) where it was proven that this channel activity is inhibited by Mg^{2+} ions (Beavis and Powers 1989), H^+ ions (Beavis and Powers 1989; Liu et al. 1996) and P_i content (Ng et al. 1993). The IMAC activity was also found to be markedly dependent on temperature (Liu et al. 1996; Beavis and Powers 2004). In cardiac muscle mitochondria, it was reported that the IMAC activity has a function both in the transport of superoxide anions from the mitochondrial matrix to the cytoplasm and in superoxide signalling between neighboring mitochondria in order to regulate the membrane potential oscillations during post-ischemic arrhythmias (see Brown and O'Rourke 2011, for a review). Moreover, it was proposed that the GSH/GSSG ratio regulates IMAC activity and that IMAC can operate in conjunction with the voltage dependent anion channel (VDAC) of the outer membrane (Aon et al. 2007; Brown et al. 2010).

In plant cells, the plant inner membrane anion channel (PIMAC), which is the IMAC counterpart, was shown to operate in mitochondria isolated from tubers of potato and of Jerusalem artichoke as well as from seedlings of durum wheat and of maize. PIMAC activity was reported to allow a high rate of electrophoretic fluxes of various anions such as iodide, nitrate, chloride, succinate, malate and oxaloacetate (Beavis and Vercesi 1992; Laus et al. 2008; De Santis et al. 2011). As compared with IMAC activity of mammalian mitochondria, PIMAC activity shows specific properties, i.e., it is inhibited by exogenous ATP or by free fatty acids (Laus et al. 2008; De Santis et al. 2011) while it is not inhibited by Mg^{2+}, mersalyl or N,N_0-dicyclohexylcarbodiimide (Beavis and Vercesi 1992). Moreover, in mitochondria from Jerusalem artichoke tubers, the levels of superoxide anion and of H_2O_2 do not regulate the PIMAC activity (Laus et al. 2008). These different properties suggest that PIMAC can perform additional specific physiological functions compared to those performed by mammalian IMAC. Consistently, PIMAC involvement in the malate/oxaloacetate shuttle (Beavis and Vercesi 1992) and PIMAC cooperation with dicarboxylate carrier activity (Laus et al. 2008) were reported in plants only. Furthermore, in maize populations divergently selected for different cold tolerance level, chloride fluxes (J_{Cl}-) through PIMAC show differential temperature dependences and thermodynamic parameters, suggesting a role of PIMAC in plant adaptation to cold (De Santis et al. 2011).

Despite the number of detailed studies reporting different regulatory mechanisms and functions of IMAC or PIMAC activities (Beavis et al. 1985; Garlid and Beavis 1985; Beavis and Powers 1989; Beavis and Vercesi 1992; Liu et al. 1996; Beavis and Powers 2004; Laus et al. 2008; De Santis et al. 2011), the protein performing such activities still remains to be identified. Analogously, as reported by Brown and O'Rourke (2011), it remains to be identified another largely studied channel of the mitochondrial membrane, i.e., the permeability transition pore (PT-pore) (De Marchi et al. 2008; Zoratti et al. 2009).

Borecky et al. (1997) showed that the 108 pS (Cl^-) mitochondrial anion channel of brown adipose tissue has substrate specificity and inhibitor sensitivity very similar to that of IMAC, suggesting that the 108 pS anion channel—which is a chloride channel (CLC)—can perform the IMAC activity. CLCs showed different physiological functions in mammals, ranging from salt homeostasis in the kidneys to acidification of cellular compartments where they reside (see for reviews Jentsch 2008; Accardi and Picollo 2010).

In plant cells, CLCs were reported to be involved in osmoregulation, cell signalling, metal tolerance, control of both plant nutrition and compartmentalization of metabolites (see for reviews De Angeli et al. 2009a; Barbier-Brygoo et al. 2011). A great number of different CLC proteins were identified in *A. thaliana,* rice and poplar, and their localization was proposed to be in vacuolar, chloro-

plastic or Golgi membranes (see Zifarelli and Pusch 2010 and Barbier-Brygoo et al. 2011, for reviews). Moreover, in maize seedlings, a *ZmCLCd* gene (GenBank Accession number for the mRNA sequence is GU344733) was recently reported to increase its expression during the adaptation of seedlings in response to chilling stress (Yang et al. 2011). Lurin et al. (2000) reported results addressing the molecular identification of PIMAC in tobacco cell membrane fractions. These authors showed that an antiCLC-Nt1 antibody co-localized with mitochondrial-specific membrane markers or antibodies (in particular with cytochrome c oxidase activity and with anti-NAD9 protein antibody), demonstrating for the first time a possible mitochondrial localization of the CLC-Nt1 protein in tobacco seedlings and suggesting that this protein may actually correspond to the PIMAC activity.

Based on these premises, we undertook this study on the possible involvement of a CLC protein in the PIMAC activity of purified maize mitochondria. This study was carried out on seedlings of maize populations divergently selected from the same source (C0), for high (C4H) and low (C4L) level of cold tolerance during germination (Landi et al. 1992). In these populations, chloride fluxes (J_{Cl^-}) through PIMAC proved to vary in dependence on their cold tolerance level and on the growing temperature of the seedling (De Santis et al. 2011). Because of their common origin, the two divergently selected populations are expected to share most of the genes, with the main exception of those controlling the selected trait, i.e., the cold tolerance level. These populations thus represent suitable materials to verify whether a CLC protein is differentially expressed in maize mitochondria purified from seedlings subjected to different growing conditions. Therefore, objectives of the present study were to investigate: (i) the presence of a CLC protein in maize seedling mitochondria; (ii) the dependence of the expression of this CLC protein on the different cold tolerance level of investigated maize populations and on the temperature of the seedling growth; and (iii) the possible role of this mitochondrial CLC protein in performing the PIMAC activity.

Materials and methods

Chemicals

TRIZOL reagent was purchased from Invitrogen, Carlsbad, CA, USA; Turbo DNA-free™ from Applied Biosystem - Ambion, Austin, TX, USA; ImProm-II™ Reverse Transcriptase and plasmid pGEM-T Easy from Promega, Milan, Italy; degenerate primers from Primm, Milan, Italy; GeneRacer™ kit and ProPure™ *Caulobacter* Expression System from Invitrogen s.r.l., Milan, Italy; polyvinylidene difluoride (PVDF) membrane Hybond-p and enhanced chemioluminescence kit from Western Blotting Detection Reagents, Amersham Biosciences Europe GmbH, Freiburg, Germany; V-ATPase antibody from Agrisera, Vännäs, Sweden. All other chemicals were of analytical grade and were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Plant materials

Three maize (*Zea mays* L.) populations, identified as C0, C4H and C4L were investigated. These populations were obtained (Landi et al. 1992) by four cycles of divergent recurrent selection for low (C4L) and high (C4H) germination at 9.5 °C using as source the C0 population (i.e., the F_2 generation of the cross B73 x IABO78). Hence, C4H and C4L show different levels of tolerance to low temperature (9.5 °C) at germination (Landi et al. 1992), as well as different behaviours during post-germinative growth at 14 °C (De Santis et al. 1999).

C0, C4H and C4L seedlings were grown, as reported by De Santis et al. (2011), in a growth chamber at 90% RH in the darkness, and were subjected to the following different temperature protocols: (i) 25 °C for 7 days (non-stressed or NS treatment); (ii) 25 °C for 3 days, followed by an acclimation for 7 days at 14 °C, (acclimated or AC treatment); (iii) 25 °C for 3 days, followed by 2 days acclimation at 14 °C and then by a cold stress treatment for 7 days at 5 °C (acclimated and stressed or ACS treatment); and (iv) 25 °C for 3 days, followed by a cold stress treatment for 7 days at 5 °C, without any acclimation period (stressed or S treatment):.

All experiments were replicated three times, and within each experiment, assays were also performed three times. Considering the genetic heterogeneity of the material, about 500 seedlings per population were grown in each experimental unit.

Cloning of full-length *ZmCLC* cDNA

Using the TRIZOL reagent, total RNA was extracted from 500 seedlings of each combination among populations (three) and growth temperature treatments (four). RNA pellets were resuspended in DEPC water and stored at −80 °C until use. To generate first strand cDNA, 5 μg of total RNA was treated with Turbo DNAse (Turbo DNA-free™) and used in RT-PCR with the ImProm-II™ Reverse Transcriptase. A 120 bp long *ZmCLC* cDNA partial sequence was initially isolated from first strand cDNA by PCR, using degenerate primers (forward: GGWAARGARGGNCCNATGGTN; reverse: RTCDATNACNGGRAANCCRTTRTG) annealing to conserved regions of homologous *CLC* cDNAs. PCR product was ligated into the plasmid pGEM-T Easy and sequenced.

To obtain the full-length sequence of *CLC* cDNA, the Rapid Amplification of the 5′ and 3′ cDNA Ends (RACE) method was used with the "GeneRacer™ kit". The nested PCR products (of 850 bp and 900 bp, for the 5′ and 3′ end respectively) were cloned into the plasmid vector pGEM-T Easy and sequenced (about 20 clones). Sequences were then combined with that of the partial *ZmCLC* cDNA to determine the full-length sequence of *ZmCLC* cDNA.

Assessment of the homologies of ZmCLCc amino acid sequence with sequences of CLC proteins from other plants

The ZmCLCc protein sequence was aligned with sequences of CLC proteins from *Arabidopsis*, rice and tobacco reported in literature by using Clustal X software (Larkin et al. 2007) Then, a phylogenetic tree was obtained and visualized by using Treeview software (http://www.treeview.net/). Similarity and identity percentage values among these sequences were calculated using the EMBOSS Needle Global Alignment software (Rice et al. 2000).

ZmCLC protein expression in *Caulobacter crescentus* and anti-ZmCLC antibody production

A fusion protein between the bacterial RsaA protein and a 169 aminoacid C-terminal polypeptide of *Zea mays* CLC (ZmCLC) was produced by using the ProPure™ *Caulobacter* Expression System. Briefly, a DNA fragment encoding ZmCLC was generated by PCR with specific primers (forward primer: 5′-CACAATGGTTTCCCTGTGCTTGAT-3′; reverse primer: 5′-GTGAGGATTGAGGTTTGGGAA CAG-3′). This fragment was cloned in pCX-TOPO vector, downstream and in frame of the RsaA protein. B5 BAC *Caulobacter crescentus* cells were transformed with the resulting pCX-TOPO-RsaA-CLC-plasmid. Bacteria were grown at 30 °C in M11 medium for 30 h. Aggregates of recombinant fusion protein ZmCLC-RsaA were then recovered from periplasmic fraction and purified as described in the kit instruction, to obtain a final yield of about 70 mg/L of fusion protein. The aggregates were solubilized in one volume of 8 M urea in 100 mM Tris–HCl (pH 8.5) and stored at 4 °C. The solubilized proteins were resolved by SDS-PAGE using 15% acrylamide Tricine-buffered gel. The obtained gel bands, containing 4 mg of recombinant protein, were used to produce anti-ZmCLC polyclonal antibody in rabbit by Primm (Milan, Italy).

Polyclonal antibodies were immunopurified by blotting the fusion protein onto PVDF membrane Hybond-p and isolating only antigenic bands upon Ponceau staining. These isolated bands were treated with phosphate-buffered saline (PBS) solution in the presence of 0.05% (*v*/*v*) Tween 20 (PBS-T) and 5% (*w*/*v*) fatty free milk powder. Treated bands were incubated overnight at 4 °C in the same buffer with the serum (1:2500 dilution). After two washes with PBS-T, the antibody was stripped with the elution buffer (0.2 M glycine pH 2.8, 1 mM EGTA) and neutralized by one volume of 1 M Tris base; 0.1 volume of PBS 10x was added and the purified antibody was stored at 4 °C.

Isolation and purification of mitochondria

Mitochondria were isolated and purified from shoots of seedlings of the three populations grown in the four different temperature treatments previously described (i.e., NS, AC, ACS and S). From 200 g of seedling shoots, an average yield of 12 mg protein (biuret base) of purified mitochondria was obtained. In these mitochondrial fractions, the level of contamination from non-mitochondrial cellular membranes was routinely tested according to Douce et al. (1987). In particular, we assayed the activity of a specific marker enzyme for each cell membrane compartment: (i) the antimycin A-insensitive Cyt c reductase for the ER; (ii) the isocitrate lyase for glyoxysomes; (iii) the glycolate oxidase for peroxysomes; (iv) the vanadate-sensitive K1-ATPase for plasma membranes; and (v) the nitrate-sensitive V-ATPase for vacuolar membranes. The putative contamination of purified mitochondria with vacuolar membranes was also immunoassayed in Western Blot with a V-ATPase antibody. Finally, etioplast contamination of purified mitochondria was assayed spectrophotometrically by testing the carotenoid level (Venturoli et al. 1986).

For each preparation of purified mitochondria, the intactness of the outer membrane was measured by assaying the cyanide-sensitive succinate-Cyt c oxidoreductase, as reported by Douce et al. (1973) and Chiandussi et al. (2002). Moreover, the intactness of the inner membrane of mitochondria was assayed by testing the activity level of fumarate hydratase, a marker enzyme of the internal mitochondrial matrix compartment (Douce et al. 1987).

Immunoblot analysis of the mitochondria

Mitochondria purified from shoots of seedlings of the three populations grown in the four different temperature treatments were lysed in 1% SDS at 100 °C. Total mitochondrial proteins were then resolved by SDS-PAGE (0.108 mg mitochondrial protein per lane) and blotted on to PVDF membranes. The membranes were blocked with PBS-T containing 5% (*w*/*v*) fatty free milk powder and incubated overnight at 4 °C in the same buffer with purified antibody (at a dilution of 1:5). After two washes in PBS-T, the secondary antibody (goat anti-rabbit IgGs coupled to horseradish peroxidase conjugate, 1:5000 dilution in PBS) was added and the membranes were incubated for 1 h at room temperature. After washing, the antigen/antibody

complex was detected with an enhanced chemioluminescence kit on a BioMax Light Film.

Anti-ZmCLC antibody inhibition of PIMAC activity

Purified mitochondria (0.4 mg protein) were suspended in 45 μL of a solution containing 300 mM sucrose and 5 mM TrisCl pH 8.0 (resuspension medium), and subjected to one of the following treatments: (i) addition of 45 μL of the resuspension medium (as a control test), (ii) addition of 45 μL of resuspension medium containing rabbit pre-immune serum (at a dilution of 1:100), (iii) addition of 45 μL of the same medium containing purified anti-CLC antibody (at a dilution of 1:1000). Treated mitochondria were then incubated for 15 min at 0 °C. Anion fluxes through the inner mitochondrial membrane were determined spectrophotometrically by monitoring A_{540} changes of mitochondrial suspension, as reported by Beavis and Vercesi (1992) and by De Santis et al. (2011). In particular, treated mitochondria were added to 1.9 mL solutions containing either 200 mosM KSCN, or 200 mosM KCl, or 200 mosM KI, in the presence of 2 mM KHEPES (pH 8.0) and 0.1 mM KEGTA. In order to block the oxidation of endogenous substrates by the respiratory chain, these experiments were conducted in the presence of both 50 μg mL^{-1} antimycin A and 2 mM salycylhydroxamic acid. By the subsequent addition of saturating concentrations of valinomycin, the complete permeability to potassium anions was allowed through the mitochondrial inner membrane and the rate of swelling was definitely made dependent only on the anions flux kinetics through PIMAC. Experiments were conducted at pH 8.0, i.e., the optimal pH for the PIMAC activity in plant mitochondria (Beavis and Vercesi 1992; Laus et al. 2008; De Santis et al. 2011). Using the technique established by Beavis et al. (1985) and by Garlid and Beavis (1985) we calculated anion fluxes (J) through PIMAC, as detailed in De Santis et al. (2011).

In order to test the significance of the effects of the growth treatments and of the cold tolerance of populations on anion fluxes through PIMAC, the ANOVA was performed, according to a randomized complete block design with three replicates, with GLM procedure (SAS Institute 1996).

Results

Purity of isolated mitochondria and intactness of the inner membranes

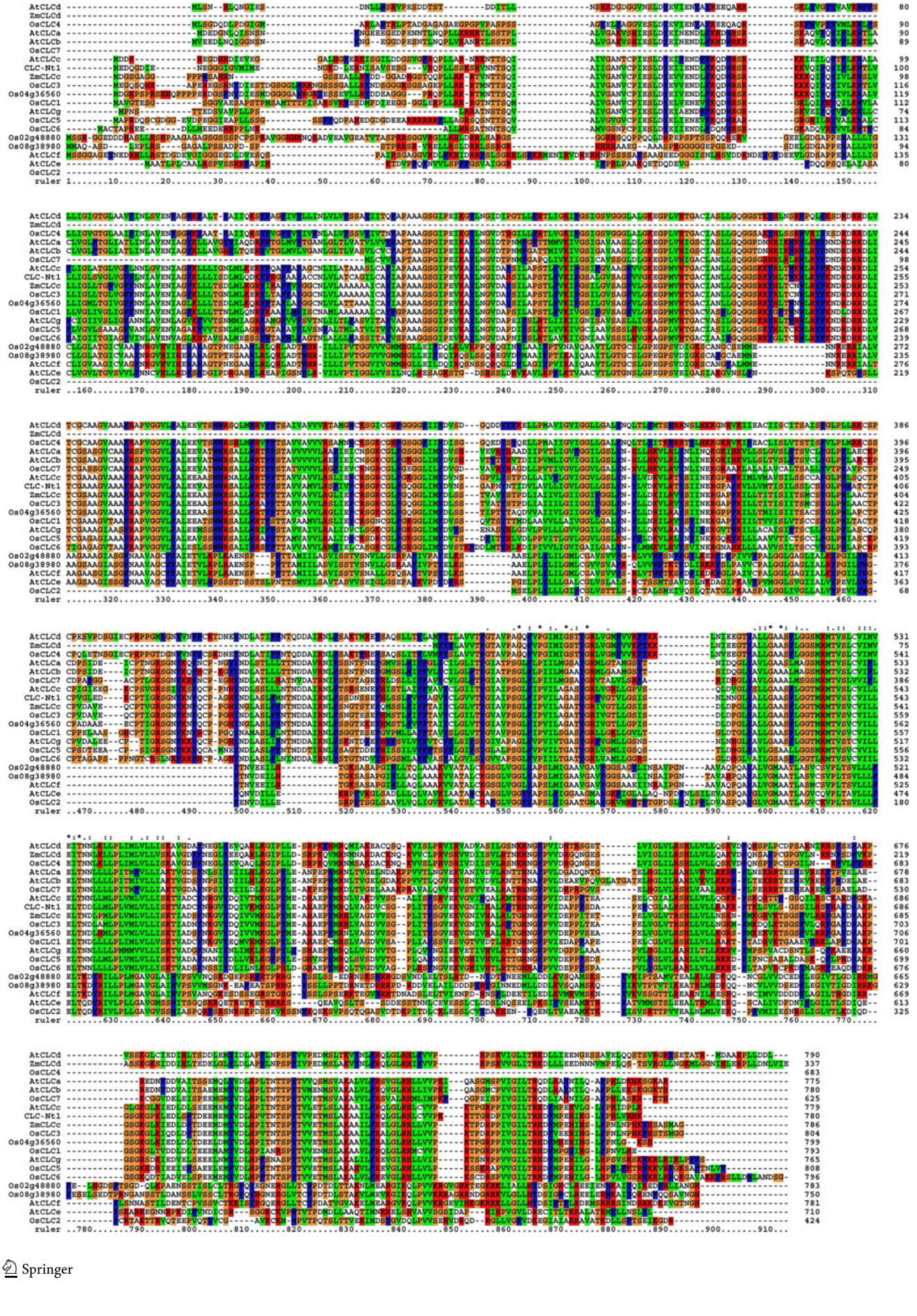
Purified mitochondria showed a minimal average contamination—less than 0.33%—from non-mitochondrial cellular membranes such as plasma membranes, ER membranes and etioplasts membranes. Moreover, the activity of the vacuolar ATPase was completely absent in purified mitochondria and no detection with a V-ATPase antibody was found when total protein extracts from purified mitochondria were analyzed in Western Blots. Consequently, purified mitochondria were completely free from vacuolar membrane contamination.

When purified mitochondria were suspended in either isotonic sucrose or salt mediums, fumarate hydratase activity was not more than 0.03% with respect to the same activity measured in frozen and thawed mitochondria. Based on all these findings, we can assume that this study was conducted using purified maize mitochondria with undamaged and osmotically active inner membranes.

Expression of a CLC protein in maize seedlings mitochondria

A 120 bp *ZmCLC* cDNA partial sequence was amplified from total first strand cDNA from seedling shoots of each population grown under all different temperature treatments, thus indicating that a *CLC* gene is expressed in maize seedlings in all these conditions. All the sequenced clones corresponded to a single *ZmCLC* cDNA sequence, 2361 bp long, encoding a polypeptide of 786 amino acids with a calculated molecular mass of 85 kDa. The complete mRNA sequence of the *ZmCLC* gene has been deposited in the GenBank database under GenBank Accession Number AY236970, whereas the encoded amino acid sequence of the corresponding protein can be accessed through NCBI Protein Database, under NCBI Accession Number AAP04392.2. The Entrez Gene LOC542114 (Chromosome 5, Locus pco116691, Maize GDB) displays the complete sequence of this *ZmCLC* gene.

On the basis of the alignment of the amino acid sequence encoded by *ZmCLC* gene with homolog CLC amino acid sequences from *Arabidopsis thaliana* (Fig. 1), the sequence of the protein encoded by *ZmCLC* gene showed the greatest similarity (82.5%) with the AtCLCc protein sequence (first part of Table 1). For this reason we named the maize protein here investigated as ZmCLCc. This choice was made following the same rational adopted by Yang et al. (2011), who named ZmCLCd the CLC protein they investigated, on the basis of the sequence similarity between ZmCLCd and AtCLCd. When compared with homolog CLC amino acid sequences from rice, and tobacco (Fig. 1 and second part of Table 1), the ZmCLCc sequence showed a similarity higher than that showed with AtCLCc, being 92.1% with OsCLC3, 88.5% with the OsCLC protein encoded by the locus Os04g36560, and 82.9% with CLC-Nt1. It is noteworthy that all these highly similar CLC protein sequences from *Arabidopsis thaliana*, rice, tobacco and maize cluster all together in a major clade of the



◀ **Fig. 1** Multiple alignment of amino acid sequences of ZmCLCc with known CLC protein of maize (ZmCLCd), *Arabidopsis* (AtCLCa, AtCLCb, AtCLCc, AtCLCd, AtCLCe, AtCLCf, AtCLCd), rice (OsCLC1, OsCLC2, OsCLC3, OsCLC4, OsCLC4, Os02g48880, Os04g36560, Os08g38980) and tobacco (CLC-Nt1). Conserved amino acid residues are represented by the same color. Identical residues in all sequences are represented by *, whereas dots (., :) indicate the degree of similarity. *Dashes* represent gaps in the sequence

phylogenetic tree (Fig. 2) and share several highly conserved domains (Fig. 1). In particular they contain (i) the complete GSGIPE motif (in ZmCLCc residues 224–229) reported to be crucial for the anion selectivity in EcCLC from *E. coli* (Dutzler et al. 2002), (ii) a conserved Tyr residue (445 in EcCLC and 564 in ZmCLCc), which directly interacts with Cl^- ions (Dutzler et al. 2002), and (iii) two conserved gating and proton transport glutamate residues (in ZmCLCc residues 269 and 328, respectively), which characterize proton/anion coupling in plant CLCs (De Angeli et al. 2009b; Zifarelli and Pusch 2010). On the other hand, these hallmark motifs are missing in the AtCLCe, AtCLCf and OsCLC2 sequences, which cluster together in a second major clade of the phylogenetic tree (Fig. 2). A third clade of the tree contains some CLC proteins having amino acid sequences showing low similarity with ZmCLCc (Tab. 1) as AtCLCd, OsCLC4 and the only CLC protein sequenced in maize, i.e. ZmCLCd.

In order to have an insight into the subcellular localization of the ZmCLCc protein, we generated a polyclonal antibody against the C-terminus of ZmCLCc. We tested the ZmCLCc antibody in Western Blot analysis against total proteins of mitochondria purified from seedling shoots of the selected populations. In this analysis, the ZmCLCc antibody specifically recognized a single band migrating at 65 kDa (Fig. 3), thus indicating that ZmCLCc is localized in mitochondria.

Effects of temperature treatments during seedlings growth and of the cold tolerance level of populations on the expression of ZmCLCc protein in seedling mitochondria

The effects of the different growth temperature treatments (NS, AC, ACS and S) on the expression level of ZmCLCc protein in mitochondria from seedling of the source C0 and in the two divergently selected populations C4H and C4L were immunoassayed (Fig. 4). Regardless of the maize populations, total ZmCLCc protein content increases in ACS and S as compared to NS and AC, indicating that the *ZmCLCc* gene becomes more induced when seedlings are subjected to cold treatments during growth. On the other hand, when comparing the three maize populations, the ZmCLCc expression level appeared to gradually decrease with the population cold tolerance level in both NS and ACS treatments. In fact, C4H showed the lowest and C4L the highest ZmCLCc content, whereas C0 was intermediate, thus revealing a symmetric trend in both ACS and NS. In S treatment, the ZmCLCc expression level changed independently from the cold tolerance level of the population (as C0 showed the lowest ZmCLCc protein content) while in AC treatment the ZmCLCc expression level

Table 1 Percentage of identity and similarity between ZmCLCc protein and *Arabidopsis*, rice and tobacco CLC protein

CLC protein	Chromosome	Locus	% Identity	% Similarity
AtCLCa	5	At5g40890	52.8	70.0
AtCLCb	3	At3g27170	52.9	69.0
AtCLCc	5	At5g49890	72.2	82.5
AtCLCd	5	At5g26240	44.4	58.1
AtCLCe	4	At4g35440	18.6	31.2
AtCLCf	1	At1g55620	20.3	32.9
AtCLCg	5	At5g33280	58.9	74.5
OsCLC1	1	Os01g65500	69.4	80.0
OsCLC2	1	Os01g50860	11.5	20.3
OsCLC3	2	Os02g35190	88.6	92.1
OsCLC4	3	Os03g48940	39.6	52.9
OsCLC5	4	Os04g55210	58.9	73.2
OsCLC6	8	Os08g20570	56.9	69.3
OsCLC7	12	Os12g25200	42.8	56.2
OsCLC (unnamed)	2	Os02g48880	18.1	30.1
OsCLC (unnamed)	4	Os04g36560	84.5	88.5
OsCLC (unnamed)	8	Os08g38980	18.2	31.5
CLC-Nt1	–	–	74.1	82.9
ZmCLCd	–	–	17.6	25.7

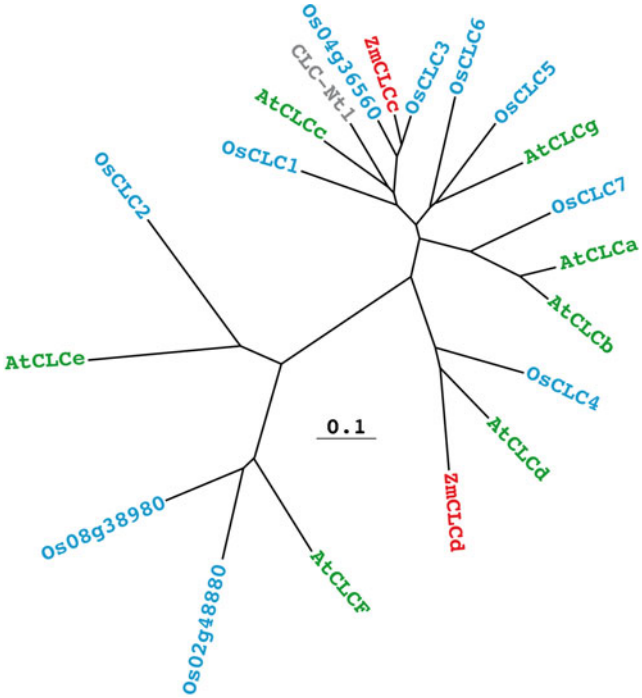


Fig. 2 Dendrogram of the deduced aminoacid sequences of the protein of the CLC family in *Arabidopsis*, rice, tobacco and maize

seemed to follow a trend opposite to the ones noted in NS and ACS treatments.

Inhibition of anion fluxes through PIMAC by anti-ZmCLC antibody

Since a previous study (Elthon et al. 1989) reported that specific antibodies against a protein can inhibit the activity of the same protein, we tested the effect of the anti-ZmCLC antibody on thiocyanide (SCN^-), chloride (Cl^-) and iodide (I^-) anion fluxes (J_{SCN^-}, J_{Cl^-} and J_{I^-}) in mitochondria isolated from seedlings of C0 population in NS growing condition (Table 2). No inhibition was detected upon addition of the rabbit pre-immune serum to the mitochondria. The addition of anti-ZmCLC antibody to the mitochondria did not show any effect on the free diffusion of SCN^- through the mitochondrial inner membrane, whereas it significantly inhibited J_{Cl^-} and J_{I^-} via PIMAC. From data reported in Table 2, we calculated an anti-ZmCLC antibody inhibitory effect of 61.5% for chloride and of 55.5% for iodide anion fluxes. The percentage of antibody inhibitory effect on PIMAC activity was similar for all populations and growth conditions (data not shown).

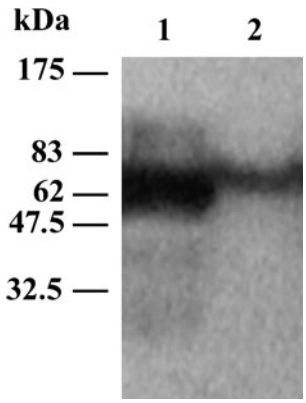


Fig. 3 Anti-ZmCLCc antibody affinity purification. Western blot analysis of the recombinant ZmCLC-RsaA protein (*lane 1*) and of the total protein extract from purified mitochondria from seedlings of C0 population grown in NS condition (*lane 2*), using the specific anti-ZmCLCc polyclonal antibody. Molecular weight marker bands were visualized on PVDF membrane by Ponceau staining before washing

Discussion

Since the proteins performing IMAC and/or PIMAC activity are still unknown, we investigated the hypothesis that PIMAC activity can be performed in maize seedling mitochondria by a *CLC* gene encoded protein. This hypothesis was previously suggested for tobacco seedlings by Lurin et al. (2000), who reported that the tobacco CLC-Nt1 antibody specifically co-localizes with the markers of the mitochondrial inner membrane, while it does not react with specific markers of the other cellular membranes. Mitochondrial localization of CLC-Nt1 was further supported by immunogold labelling experiments showing that CLC-Nt1 localized in mitochondrial membranes both in a suspension of tobacco whole cells and in a crude leaf fraction (Lurin et al. 2000).

In this work we sequenced the *ZmCLCc* cDNA and, as expected, we found that the encoded protein ZmCLCc showed the highest similarity with several CLC proteins of rice, which is a monocotyledoneous species as maize. A rather high similarity was found between sequences of ZmCLCc protein and sequences of CLC-Nt1 and AtCLCc proteins, i.e., with two proteins of dicotyledoneous species. All these three proteins, belonging to the same clade of the phylogenetic tree, contain sequence motifs important for anion selectivity and two glutamate residues specific for proton/anion exchange in plant CLCs.

Conversely, the ZmCLCc sequence exibited only a low similarity with the ZmCLCd sequence reported by Yang et al. (2011). However, this latter observation should be taken with care because the published ZmCLCd sequence is much shorter than other plant CLC reported sequences, and probably it is not complete. Moreover, the similarity of the ZmCLCc protein sequence with the other CLC protein sequences from animals and yeasts that are reported in literature was just moderate, ranging from 24% to 28% of the sequence.

Our experiments with purified polyclonal anti-ZmCLCc indicated that ZmCLCc is localized in maize seedling mitochondria. The discrepancy found between the apparent molecular weight (65 kDa) of the band recognized in mitochondria by the anti-ZmCLCc antibody and the molecular weight of the ZmCLCc protein calculated by the amino acid sequence (85 kDa) can be probably ascribed

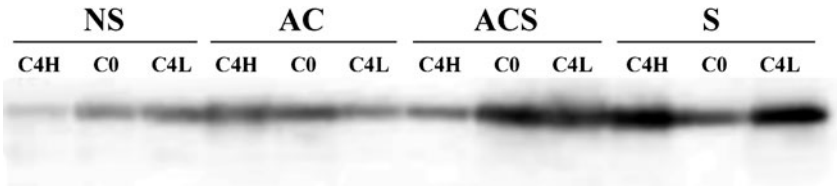


Fig. 4 ZmCLCc protein levels in C4H, C0 and C4L populations. Total mitochondrial protein extract from these populations grown in NS, AC, ACS and S were probed with affinity-purified antibody raised against ZmCLC-RsaA. Equal amounts of protein (calculated using the biuret method) were loaded into each lane

to a particular electrophoretic mobility of this membrane protein, as already shown for the renal CLC-K1 protein (Uchida et al. 1995).

Given the high similarity of ZmCLCc with AtCLCc, which has been localized in vacuolar membranes (Zifarelli and Pusch 2010; Barbier-Brygoo et al. 2011) and not in mitochondria, we have accurately addressed the control of the purity level of our mitochondria preparations used in immunoblot assays, to exclude any vacuolar contamination. The lack of V-ATPase activity and, above all, the failure of protein recognition by the antiV-ATPase antibody in the purified mitochondria indicate that our results are not biased by vacuolar membranes contamination. However, our findings can not exclude that other ZmCLC protein isoforms can be also localized in vacuolar or other cellular membranes in maize seedlings.

The ZmCLCc protein level in mitochondria varied with the cold tolerance level of the maize populations and with temperature conditions of seedlings growth, indicating that the ZmCLCc protein level in mitochondria is genetically and environmentally regulated. In particular, ZmCLCc protein level was higher in cold stress conditions, with (ACS) and without (S) acclimation, than in non-stressed conditions (NS and AC). Interestingly, a different maize CLC protein (ZmCLCd) was also reported to increase its expression in maize seedlings during chilling stress at 6 °C (Yang et al. 2011). The relationship between ZmCLCc protein level and the cold sensitivity level of the populations suggests that the divergent selection affected the intrinsic regulation of ZmCLCc protein level in maize seedlings mitochondria. In particular, since ZmCLCc protein level decreased along with cold tolerance level of the populations not only in ACS but also in NS treatment, we hypothesize that the selection determined a constitutive change of ZmCLCc protein level. Consistently with this conclusion, De Santis et al. (2011) reported that, in mitochondria isolated from seedlings of the same maize populations, and subjected to the same temperature treatments during growth, the PIMAC activity, too, was greatly dependent on both the level of cold tolerance of the populations and the temperature treatments. All together, these findings thus support a similar genetic and environmental control of both ZmCLCc protein expression level and PIMAC activity in maize seedlings mitochondria.

Our results on inhibitory effect of anti-ZmCLCc on PIMAC activity indicate that the *ZmCLCc* gene encodes for a mitochondrial protein involved in PIMAC activity. This conclusion is supported by the significant inhibition of the PIMAC activity by the anti-ZmCLCc antibody. Conversely, the treatment of mitochondria with the anti-ZmCLCc antibody did not lead to any inhibition of free diffusion of thiocyanide anion through the inner membrane. The specific anti-ZmCLCc inhibitory effect on PIMAC activity indicates that this antibody was able to pass through the outer membrane of purified mitochondria and to reach the external side of the inner membrane to react with PIMAC. De Santis et al. (2011) indicated that, when mitochondria are suspended in KCl medium plus valinomycin, the outer membranes become broken and partially permeable to cytochrome c, while the inner membranes remain intact and osmotically active. This finding can explain the accessibility of the anti-ZmCLCc antibody to the inner membrane spanning proteins as PIMAC in whole mitochondria. The observed lack of complete inhibition of chloride and iodide anion fluxes through PIMAC by the anti-ZmCLCc antibody may be due to: (i) a slow

Table 2 Effect of purified anti-ZmCLCc antibody on PIMAC activity of mitochondria isolated from maize seedlings of C0 population grown in the NS condition. The reaction medium contained 200 mosM KSCN (or 200 mosM KCl, or 200 mosM KI), 2 mM KHEPES (pH 8.0) and 0.1 mM KEGTA. Mitochondria were preincubated with the rabbit pre-immune serum or with the anti-ZmCLCc antibody and anion fluxes (J_{SCN^-}, J_{Cl^-} and J_{I^-}) (± SE) were assayed

Treatment	J_{SCN^-} (nmol min^{-1} mg^{-1} protein)	J_{Cl^-} (nmol min^{-1} mg^{-1} protein)	J_{I^-} (nmol min^{-1} mg^{-1} protein)
Untreated	5056±43	5276±11	6947±27
Rabbit pre-immune serum	5077±13	5286±27	6976±27
Anti-ZmCLCc antibody	5062±25	2032±28	3094±12

permeation rate of the anti-ZmCLCc antibody through the partially broken outer membranes; (ii) a particular structural property of the antibody-ZmCLCc protein complex; and/or (iii) the occurrence in mitochondria of some other proteins which may perform PIMAC activity.

Several studies have shown that, when higher plants are exposed to low temperatures, the calcium concentration increases in the cytoplasm and, at a higher level, in the mitochondrial matrix (Ruelland et al. 2009). Consistently, the increase in ZmCLCc protein level at low temperatures of growth, i.e., in ACS and S conditions, observed in this study for all the three populations, could be linked to changes in calcium concentration in the mitochondrial matrix, as previously indicated for other cold-responsive genes (Ruelland et al. 2009). Therefore, accordingly to what is reported for the cold-induced activation of anion channels in the plasma membrane of various plant species (Ruelland et al. 2009), the *ZmCLCc* encoded PIMAC activity in maize seedlings grown at low temperatures could play a role in the control of a cold-dependent increase of mitochondrial calcium level.

In conclusion, the results reported herein for maize are in agreement with the suggestion made by Lurin et al. (2000) for tobacco that PIMAC activity is performed by a mitochondrial CLC protein. In particular, our results (i) indicate that the ZmCLCc protein is present in maize mitochondria, (ii) show that the expression level of the *ZmCLCc* gene is genetically and environmentally regulated as it is the PIMAC activity, and finally (iii) support the role of ZmCLCc protein in determining PIMAC activity.

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