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Recombinant plant gamma carbonic anhydrase homotrimers bind inorganic carbon

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ABSTRACT

Gamma carbonic anhydrases (γCA) are widespread in Prokaryotes. In Eukaryotes, homologous genes were found only in plant genomes. In Arabidopsis and maize, the corresponding gene products are subunits of mitochondrial Complex I.
At present, only γCA homotrimers of *Methanosarcina thermophila* (CAM) show reversible carbon dioxide (CO_2) hydration activity. In the present work, it is shown that recombinant plant γCA2 could form homotrimers and bind $H^{14}CO_3^-$. However, they are unable to catalyse the reversible hydration of CO_2.
These results suggest that plant γCAs do not act as carbonic anhydrases but with a related activity possibly contributing to recycle CO_2 in the context of photorespiration.

Structured summary:
MINT-7266044: *gamma CA2* (uniprotkb:Q9C6B3) and *gamma CA2* (uniprotkb:Q9C6B3) *physically interact* (MI:0914) by *dynamic light scattering* (MI:0038)
MINT-7266036: *gamma CA2* (uniprotkb:Q9C6B3) and *gamma CA2* (uniprotkb:Q9C6B3) *physically interact* (MI:0914) by *molecular sieving* (MI:0071)

1. Introduction

Plant mitochondrial NADH ubiquinone oxidoreductase (complex I) contains a spherical extra domain in the membrane arm which is specific of eukaryotic photosynthetic organisms [1]. This domain is absent in complex I particles of all other investigated organisms, except for the alga Polytomella, which is closely related to Chlamydomonas. Biochemical data strongly suggest that this extra domain is composed of trimers of gamma carbonic anhydrases (γCAs) facing the mitochondrial matrix. Thus, presence of γCA subunits within complex I correlates with the occurrence of this extra, so-called CA domain.

Carbonic anhydrases (CA-EC 4.2.1.1) are Zn-containing enzymes catalyzing the reversible hydration of carbon dioxide according to the reaction:

$$HCO_3^- + H^+ \rightarrow CO_2 + H_2O$$

Currently, five types of CAs (α, β, γ, δ and ζ) showing a convergent evolution have been described [2–5]. Gamma type CAs are present in the Bacteria and Archaea domains. In eukaryotes, however, they are found only in photosynthetic organisms [6]. The Arabidopsis γCA protein family is represented by five members. Three of them contain nearly all functionally important amino acids: γCA1 (At1g19580), γCA2 (At1g47260), γCA3 (At5g66510). The two other members are more divergent proteins: γCAL1 (CAL: carbonic-anhydrase-like; At5g63510) and γCAL2 (At3g48680). All five Arabidopsis γCA/γCAL subunits were found to be associated with mitochondrial complex I [7,8]. All photosynthetic eukaryotes examined so far contain at least one γCA and one γCAL [9]. Structural modelling of these proteins revealed a left-handed-β-parallel helix (LβH) conformation [6]. Sequence comparisons showed highest conservation of these proteins to the only biochemical and structurally investigated γCA (CAM) of *Methanosarcina termophila*. The functionally important amino acids His 81, His 117, His 122 (zinc coordination), as well as Arg 59, Asp 61, Gln 75, Asp 76 and Asn 202 are conserved between CAM and most of the complex I subunits of plants. Two other functionally important residues (Glu 62 and Glu 84 of CAM) are

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missing, but alternative amino acids which could substitute their roles were suggested [6].

The Arabidopsis γCA2 subunit is exclusively located in the membrane fraction of plant mitochondrial complex I and it is thought to interact with other family members forming trimers. Carbonate treatment of isolated mitochondrial membranes did not allow extraction of the protein, indicating a direct anchoring of γCA2 within the inner mitochondrial membrane most likely by their C-terminal extensions [7]. Furthermore, protease protection experiments using mitoplasts (mitochondria lacking the outer membrane) suggest that a small part of this protein (~2 kDa) is exposed to the mitochondrial intermembrane space [7,10].

Proteomic and physiological analyses of knock out mutants for each of the *CA* Arabidopsis genes indicate that only γCA2 appears to be important for complex I assembly since *γca2* null mutant exhibits only 10% of normal complex I levels [11]. On the other hand, ectopic over-expression of γCA2, causes a failure in anther dehiscence and consequently a male sterile phenotype. Impairment of complex I function was detected in these plants [12].

Until present, there is no direct physiological evidence of carbonic anhydrase (CA) activity associated to complex I in plants. Several efforts to determine this activity have been performed using different biochemical fractions, including purified Arabidopsis complex I [11]. Nevertheless, CA activity of this group of plant-specific complex I subunits is strongly supported by computer modelling. As summarized above, most of the residues important for catalysis are conserved between the prototype γCA of *Methanosarcina thermophila* (CAM) and plant γCAs [6,10]. Recently, a related protein from cyanobacteria (*Synechocystis* CcmM), which is a component of carboxysome (structure involved in CO_2 concentration) was shown to bind inorganic carbon. However, carbonate dehydration activity for CcmM has not been detected [13]. CcmM also contains most of the catalytic residues except for Asp 76 and Asn 202 residues (CAM annotation), which are replaced by His and Arg in CcmM, respectively. These residues in CAM are postulated as essential for proton transfer in the CO_2 hydration reaction [4,14]. Thus, it currently cannot be ruled out that the γCA subunits of complex I do not show CA activity and only bind and perhaps transport CO_2 and/or bicarbonate.

In this work, a subunit of complex I, γCA2 was expressed in *Escherichia coli* in a soluble homotrimeric form, purified to homogeneity and subjected to $H^{14}CO_3$ binding and CA activity assays.

2. Materials and methods

2.1. Protein determinations

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed as described previously [15]. In silico analysis of molecular weight and extinction coefficients (ε) were determined with the Protparam tool (www.expasy.ch/tools/protparam.html). The apparent native molecular mass was monitored by Dynamic light scattering (DLS) using a DynaPro™ Titan from Wyatt Technology and by using a molecular exchange column (Superose 12/300 GL, GE) equilibrated in buffer A (50 mM NaH_2PO_4, 150 mM NaCl, 1 μM $ZnSO_4$, pH 7.4) Protein concentration was determined by using the A_{280} with a ε: 21 555 M^{-1} cm^{-1}.

2.2. Bioinformatics

The mitochondrial signal peptide of γCA2 was predicted using: MitoProt: mips.helmholtz-muenchen.de/cgi-bin/proj/medgen/mitofilter, TargetP: www.cbs.dtu.dk/services/TargetP and SignalP: www.cbs.dtu.dk/services/SignalP/. Multiple Sequence Alignment (MSA) was performed with T-coffee: http://tcoffee.vital-it.ch [16]. Sequences used for MSA were 2EG0 (Hypothetical Protein from *G. kaustophilus*), 1V3W (Ferripyochelin Binding Protein from *P. horikoshii*), 1XHD (Putative Acetyltransferase from *B. cereus*), 1QRL (carbonic anhydrase from *M. thermophila*). For secondary structure prediction, Jpred 3 (http://www.compbio.dundee.ac.uk/www-jpred/) [17] was used.

2.3. Expression and purification of recombinant γCA2 protein

RNA from *Arabidopsis thaliana* was extracted and used for RT-PCR with the forward primer: 5′GGGGACAAGTTTGTACAAAAAAGCAGGCTCGGAAAACCTGTATTTTCAGGGCGATAAATCACCATTGGTGGATAAAGATG, and reverse primer: GGGGACCACTTTGTACAAGAAAGCTGGGTTTAATTCTCTGAGGCGTGAATCTGTGC, to generate a recombinant soluble 6XHis-tagged form of γCA2 (aa 50–218). This PCR fragment was cloned using in vitro recombination (GATEWAY, Invitrogen), in the expression plasmid pDEST17. Competent *E. coli* Rosetta (DE3)pLysS (Novagen) cells were transformed with pDEST17-50-218 (21 kDa). *E. coli* cells were grown at 30 °C in LB containing 100 μg/ml of ampicillin and 25 μg/ml of chloramphenicol, and induced at an A_{600} of 0.6–1 with 1 mM isopropylthiogalactopyranoside (IPTG). After additional growth for 18 h at 20 °C, cells were sonicated and resuspended in His binding buffer (20 mM NaH_2PO_4 y 500 mM NaCl, 30 mM Imidazole, pH 7.4). The soluble protein fraction was collected after centrifugation (10 000×*g*, 30 min, 4 °C). Soluble protein extract was applied to His trap HP column (GE Healthcare), then washed with five column volumes with His binding buffer. The His-tagged protein was eluted from the column with elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 500 mM Imidazole, pH 8). The purified recombinant 21 kDa γCA2 was dialyzed ON against 20 mM NaH_2PO_4, 100 mM NaCl and 1 μM $ZnSO_4$. Western blotting was performed to monitor purification steps using a commercial anti-HIS antibody (GE). Furthermore, the identity of the eluted protein was assigned by automated EDMAN degradation.

2.4. $H^{14}CO_3^-$ binding assays

Soluble protein extract containing His_6-tagged γCA2 (21 kDa) was incubated at 4 °C with a Ni affinity resin (GE Healthcare) for 1 h. Resin-Bound γCA2 was incubated with $NaH^{14}CO_3$ as previously described [13]. The radioactivity of ^{14}C was measured with a scintillation counter Beckman LS 7000.

2.5. Fluorometric CA activity assays

The dehydration of bicarbonate reaction was followed by measuring the pH change using a fluorescent pH-indicator, 8-hydroxypyrene-1,3,6-trisulfonate (pyranine) [18]. Fluorescence measurements were collected with an AMINCO–Bowman Series 2 Luminescence Spectrofluorometer equipped with a Bio SX-18 MV stopped flow (Applied photophysics). The reaction was started by mixing a solution containing $KHCO_3$ in 0.5 mM bicine–KOH at pH 8 with an equal volume of 100 nM pyranine with or without enzyme in 0.5 mM HEPES–KOH at pH 6.0. The fluorescence intensity change was followed by measuring fluorescent emission at 512 nm after excitation at 466 nm.

2.6. Esterase activity

Activity for p-nitrophenylacetate hydrolysis was determined at 25 °C in a reaction mixture (1.35 ml) containing 0.5 ml of freshly prepared 3 mM p-nitrophenylacetate in aqueous 3% (vol/vol) acetone and 0.85 ml of water. The uncatalyzed rate of the reaction was determined by adding 0.15 ml of 100 mM potassium phosphate (pH 7.0) containing 1 mM zinc sulfate and recording the

change in A_{348} per min ($\Delta\varepsilon$ 5000 M^{-1} cm^{-1}). After 2 min, 15 μl of enzyme solution was added, and the catalyzed reaction was monitored for additional 3 min [19].

3. Results

To provide insight into the function of γCAs in plants, the cDNA encoding one complex I subunit, γCA2, was cloned in an expression vector containing a removable 6XHis tag at the N-terminus. Mitochondrial signal peptide predictions and alignments with crystallized protein homologues [2EG0, 1V3W, 1XHD and 1QRL (CAM)] were used with in order to find structural restriction to perform γCA2 deletions (Fig. 1).

Since the full-length cDNA produced a 30 kDa recombinant protein in inclusion bodies, several deletions were assayed to obtain soluble proteins. According to the alignment analysis, a series of three constructs were performed: (2–218, 18–218 and 50–218) which were termed according to the first and last amino acid positions. Only the construct 50–218 (coding for a 21 kDa protein) was expressed as a soluble protein (Fig. 2A and B). This recombinant protein contains all the predicted amino acids important for catalysis and Ci binding (Fig 1). However, it does lack the predicted signal peptide and the non-conserved C-terminus. Accordingly, it was chosen for further analyses.

3.1. Purification of soluble γCA2

The *E. coli* strain containing the 50–218 construct induced with IPTG as indicated in Section 2 as grown ON at 20 °C and subjected to lysis. Soluble proteins were applied to a Ni-column in order to retain the recombinant His-tagged protein. A great proportion (∼98%) was eluted with Imidazole. A 21 kDa protein is recognized in a Western blot using the commercial anti-His-tag antibody (Fig. 2C). The protein was stable at 4 °C for more than 10 days without any sign of proteolysis or precipitation. A relatively low yield of about 1 mg/4 l of culture was routinely obtained.

3.2. Recombinant plant γCA2 forms stable homotrimers

The best biochemical and structurally characterized γCA is CAM from the methanogenic archeon *M. thermophila* (for a review see [4]). This protein was crystallized and shown to form homotrimers

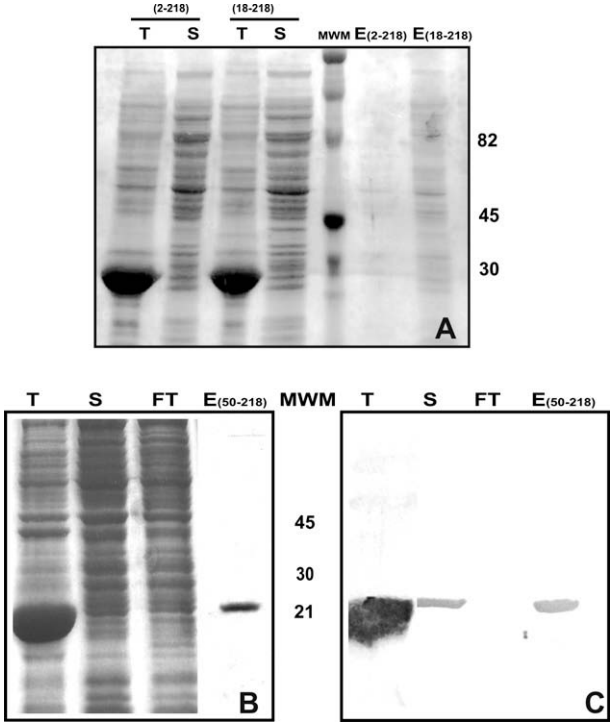

Fig. 2. Expression and purification of γCA2. (A) Coomasie blue stain of insoluble recombinantly expressed γCA2 (2–218 and 18–218). T: whole bacterial cell lysate. S: whole bacterial soluble cell lysate. E: Eluted fraction. (B and C) Coomasie stain of soluble recombinantly expressed γCA2 (50–218) and Western blot revealed with Anti -His Antibody. T: whole bacterial cell lysate. S: whole bacterial soluble cell lysate. FT: Flow through of Ni-column. E: Eluted fraction. MWM: Molecular weight marker. In all case the SDS–PAGE was performed at 10%.

that are essential for activity. In order to know the oligomerization state of recombinant γCA2, Dynamic light scattering was used. A spherical particle of 3.4 nm corresponding to ∼58 kDa was mainly seen (96.3% in mass) (Fig. 3A). Furthermore, purified recombinant γCA2 was applied to a Superose 12 column. A specie of ∼57 kDa was clearly detected (Fig. 3B). Accordingly, it was concluded that recombinant γCA2 folded in the form of homotrimers as previously predicted [6].

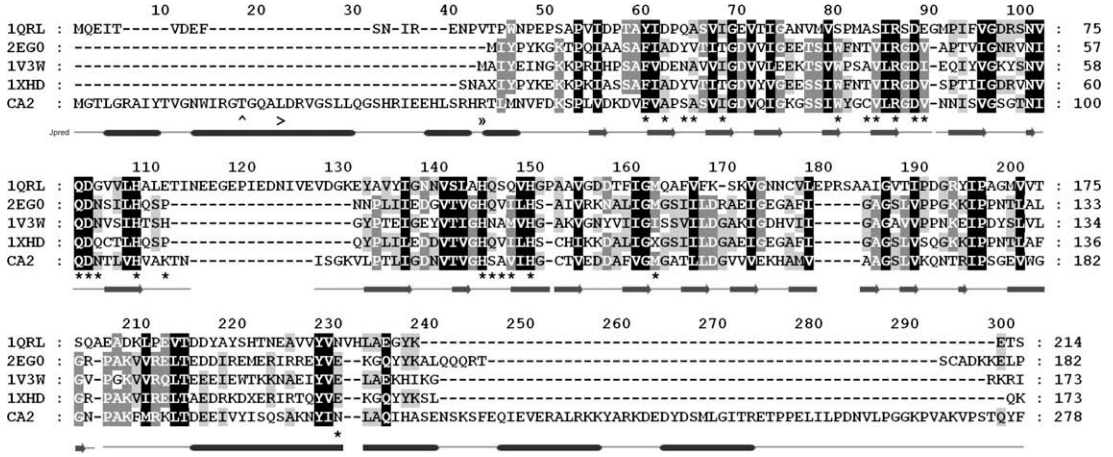

Fig. 1. Sequence comparison of γCA2 with crystallized γCAs. MSA of γCA2, CAM (1QRL) and other crystallized protein homologues as described in Section 2. Asterisks (∗) indicate important amino acids for trimer formation, catalysis and metal coordination, according to CAM structure. Signal peptide cleavage sites predicted for γCA2 are marked: MitoProt, ^; SignalP, >; TargetP, ». Secondary structure predicted for γCA2 by Jpred: cylinders, α-helix; arrows, β-sheet.

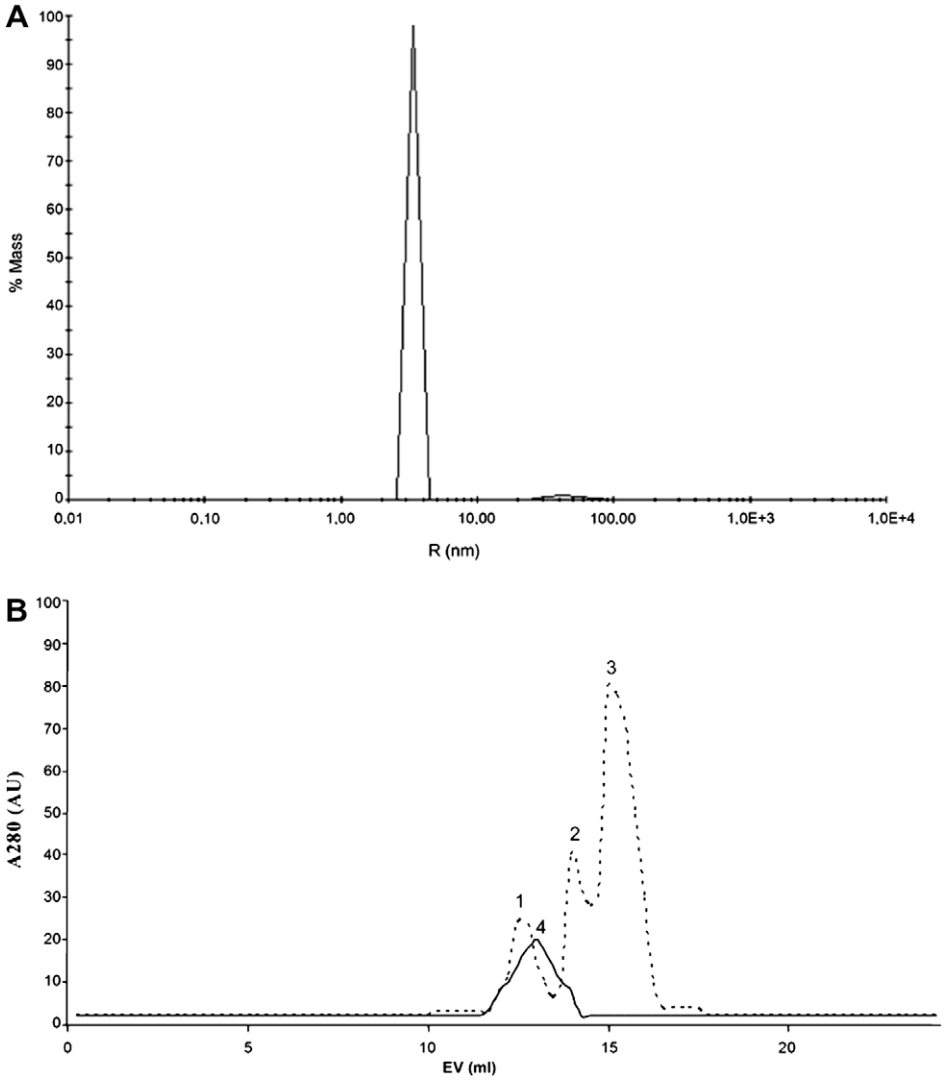

Fig. 3. Recombinant γCA2 fold as homotrimers. (A) Dynamic light scattering of recombinantly expressed γCA2 (50–218) R, ratio in nm. Main peak: 3.4 nm = 58 kDa. (B) Purified γCA2 (50–218) was applied to a molecular gel filtration Superose 12 column (GE) in buffer A. Elution profiles were monitored by UV absorbance at 280 nm. EV: Elution Volume in ml. Molecular mass standards: Peak 1: Bovine seroalbumin (66 kDa), Peak 2: Bovine Carbonic Anhydrase II (20 kDa) and Peak 3: Cytochrome c from Equine heart (12 kDa).

3.3. γCA2 homotrimers do not exhibit detectable bicarbonate dehydration

The dehydration reaction of bicarbonate was assayed in the absence of enzyme (spontaneous reaction, SR), and in the presence of either γCA2 or Bovine Carbonic Anhydrase (BCAII). As shown in Fig. 4, the presence of γCA2 in the reaction mixture does not produce a significant change in the initial velocity of dehydration reaction of bicarbonate respect to the spontaneous reaction (0.0682 S.D. 0.0005 au s^{-1} and 0.0581 S.D. 0.0003 au s^{-1}, respectively, $n = 3$). The same observations were achieved at several concentrations of γCA2 (1–10 μM) and $NaHCO_3$ (5 and 10 mM) (data not shown). Notice that sevenfold less of BCA in the reaction mixture produces a noticeable increase of the initial velocity (0.319 S.D. 0.005 au s^{-1}). It was thus concluded that recombinant plant γCA2 homotrimers do not exhibit carbonic anhydrase activity, at least, in the conditions tested.

Some carbonic anhydrases from mammalian sources catalyse the reversible hydrolysis of esters [20]. With p-nitrophenylacetate as a substrate, commercially available Bovine Carbonic Anhydrase showed an esterase activity of 34.7 mol of p-nitrophenylacetate per min per mol of enzyme, in contrast no activity was detected for the plant enzyme (0.02 mol of p-nitrophenylacetate per min per mol). Thus, γCA2 is one of several carbonic anhydrases [14] for which esterase activity appears to be absent.

3.4. γCA2 is able to bind inorganic carbon

Structural and amino acid sequence comparisons indicated that all of the CAM residues involved in Zn coordination, binding of HCO_3^- and the formation homotrimer are spatially conserved in γCA2 [6]. Thus, bio-informatic analyses support the view that γCA2 homotrimers may retain HCO_3^-/CO_2 binding capability, although the experimental evidence indicates that recombinant γCA2 (50–218) is apparently unable to catalyse the reversible CO_2 hydration (Fig. 4). When incubated with $H^{14}CO_3^-$, resin-retained, recombinant γCA2 binds 2.4×10^{-5} nanomol of Ci per ng of γCA2 protein (S.D. 1.71×10^{-6}, $n = 5$), corresponding to 17.95 ± 2.74 picomol in total. This level of binding was threefold greater than that observed for the resin alone or resin-retained *E. coli* background proteins, on an equal volume basis (Fig 5). Due to the spontaneous HCO_3^- dehydration to form CO_2, the exact nature of the bonded molecule to γCA2 remains elusive.

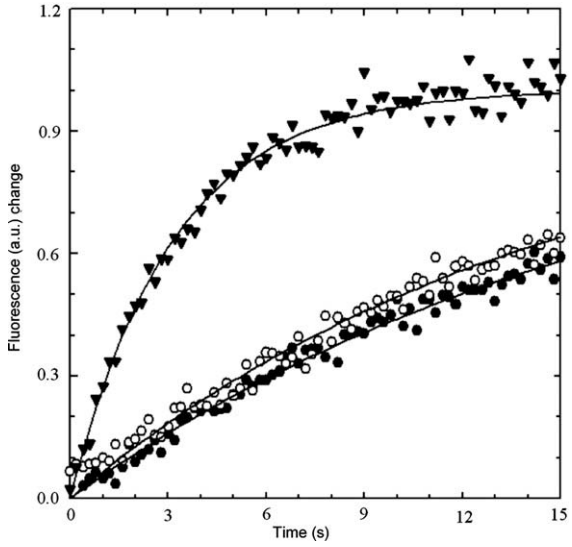

Fig. 4. Fluorometric carbonic anhydrase activity assay. Changes in pyranine fluorescence intensity as a function of time were obtained in media containing 20 mM $KHCO_3$ and either (▼) 3 μg/ml of Bovine Carbonic Anhydrase II BCA II, (○) 20 μg/ml of γCA2 or (●) without protein (SR). The continuous lines are plots of equation, signal = $A\ (1 - e^{-k\ \mathrm{time}}) + C$ for the best fitting values of the parameters A and k. Each fluorescence trace was normalized to the maximum increase in fluorescence (A).Values of initial velocity (vi) were calculated as: $vi = A \times k$.

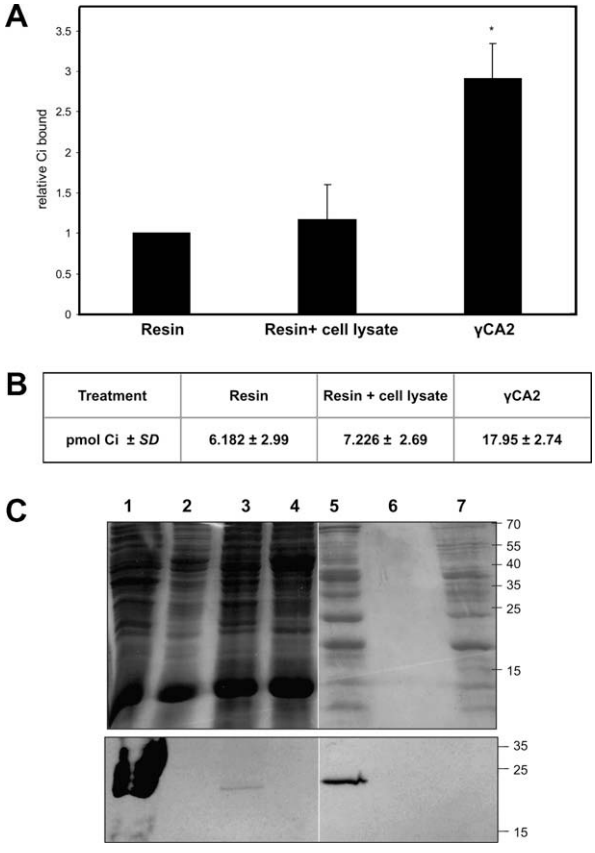

Fig. 5. ^{14}C carbonate binding assay. (A) Relative (^{14}C) carbon inorganic (Ci) bound respect to the control. (B) Quantitative amount of Ci expressed in picomol. (C) Coomasie stain (top) and Western blot analysis with Anti-His (bottom). 1: overexpressed γCA2 bacterial cell lysate, 2: bacterial cell lysate, 3: overexpressed γCA2 bacterial cell lysate after incubation with Ni-resin, 4: bacterial cell lysate after incubation with Ni-resin, 5: γCA2 bound to the resin, 6: Resin alone, 7: resin + bacterial cell lysate.

4. Discussion

In this work, a functional characterization of gamma carbonic anhydrase subunit of plant mitochondrial complex I, γCA2 was performed. Recombinant homotrimers of γCA2 are able to bind carbon dioxide/bicarbonate, however, they appear to be unable to catalyse the reversible bicarbonate dehydration in the conditions tested. These results could suggest that the so-called eukaryotic γCAs, in plants, do not function as *bona fide* carbonic anhydrases, however, with a related activity.

In Chlamydomonas as well as in Arabidopsis βCA and γCA families were described [6,7,21–23]. In Chlamydomonas there are two mitochondrial βCAs encoded by nuclear genes which are expressed in the light at low external CO_2 concentrations [24,25]. In Arabidopsis only one of six active genes, *AtβCA6*, encode a product which is imported into mitochondria [23]. Different hypothesis were suggested concerning the function of mitochondrial βCAs: (i) buffering matrix H^+ concentration upon initiation of photorespiration [21] or depending on NH_4^+ concentration [26]; (ii) HCO_3^- formation for anaplerotic reactions [26]; (iii) limiting loss of CO_2 produced by photorespiration [27]. The latter hypotheses require the presence of a bicarbonate transporter within the mitochondrial membranes, which so far has not been described. Bicarbonate could thus be transported actively from mitochondria to the cytoplasm and afterwards into plastids, allowing recycling of excess of mitochondrial CO_2 for carbon-fixation by RubisCO.

4.1. Possible function of γCA proteins

Phylogenetic studies revealed the conservation of relevant active site residues of the large family of γCAs [6,8]. All members contain the three His residues (or conservative replacements) important to bind a metal ion. Cyanobacterial CcmM shows a C-terminal region which may serve to interact with RubisCO and an N-terminal region similar to γCAs [28,29]. It does contain all the catalytically important residues described previously, except those involved in proton transfer [30–32]. Conversely, mitochondrial γCAs are integral proteins of complex I. All five γCA/γCALs contain an N-terminal extension which may function as mitochondrial signal peptide [6,10] and a C-terminal extension which is most likely required to anchor the proteins to the membrane arm of complex I [7]. In addition, putative γCAs from α and γ proteobacteria (considered as mitochondrial ancestors) and from plant and green algae mitochondria lack the Glu residues essential for proton transfer. However, the Tyr 159 of γCA2, postulated as proton transfer residue [6] is conserved in all γCA homologues with the intriguing exception of CAM, which is the only example showing CA activity.

Could these modifications in proton shuttle residues mean that all other homologous sequences encode for proteins with another but related activity? Experimental evidences from both, cyanobacteria [13] and plants (this work) suggest that this could be the case. Neither the CcmM protein nor γCA2 seem to exhibit reversible carbon dioxide hydration but both of them are able to bind carbonate and/or carbon dioxide at similar levels suggesting a related activity. Furthermore, their transcription was shown to be dependent on CO_2 concentration [11,29,33]. The CcmM protein is proposed to transport bicarbonate from the cytoplasm to the centre of the carboxysome where a conserved βCA, the CcaA protein, efficiently converts bicarbonate into carbon dioxide and thus, RubisCO could fixate through the Calvin Cycle [13].

In eukaryotes, RubisCO, especially at low CO_2, is able to use molecular O_2 and initiates a glycolate cycle or photorespiration which involves the glyoxysome and finishes in the mitochondria. Within this organelle, decarboxylation of two molecules of glycine occurs to give serine, ammonium and carbon dioxide which is most

likely rapidly converted in bicarbonate by a mitochondrial βCA which has been suggested to be AtβCA6 [23]. The CO_2 could be recycled to increase the efficiency of photosynthetic carbon-fixation. However, this hypothesis requires the presence of a bicarbonate transporter across the inner mitochondrial membrane which, as CcmM, transports the bicarbonate from one side to another. Mitochondria represent an important source of CO_2 for photosynthesis [34]. Accordingly, it is proposed that plant complex I integrated γCAs may function together with mitochondrial βCAs in CO_2 recycling.

The CA domain seems to be a general feature of plant complex I, since it now was described for the C_3 plant Arabidopsis, the C_4 plant maize and the green alga *Polytomella* [1,7,35]. If the CA domain is important in photorespiration, a difference should be observed between mitochondrial complex I from C_3 and C_4 plants. The latter show anatomical and biochemical specializations to avoid photorespiration, pre-fixation in four carbon compounds in mesophyll cells and CO_2 fixation by RubisCO in bundle sheath cells. However, Peters et al. [35] suggest that γCA function in these two kinds of plants might be different, in C_3 plants in the context of photorespiration and in C_4 plants in the CO_2 liberation step in the bundle sheath cells [36]. Furthermore, it would be interesting to know the tissue specific expression pattern of maize γCAs which may differ between bundle sheath and mesophyll cells.

Thus, it is proposed that complex I integrated γCAs could eventually represent this elusive bicarbonate transporter with the following characteristics: (1) attached to the membrane and/or complex I membrane arm by a C-terminal extension only present in plant γCAs [7], (2) binding of bicarbonate at comparable level to CcmM and (3) do not show bicarbonate dehydration activity being thus most likely able to transport this molecule without further changes.

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