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Effect of CO₂ Content in Air on the Activity of Carbonic Anhydrases in Cytoplasm, Chloroplasts, and Mitochondria and the Expression Level of Carbonic Anhydride Genes of the α- and β-Families in Arabidopsis thaliana Leaves

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Abstract: The carbonic anhydrase (CA) activities of the preparations of cytoplasm, mitochondria, chloroplast stroma, and chloroplast thylakoids, as well as the expression levels of genes encoding αCA1, αCA2, αCA4, βCA1, βCA2, βCA3, βCA4, βCA5, and βCA6, were measured in the leaves of Arabidopsis thaliana plants, acclimated to different CO₂ content in the air: low (150 ppm, lCO₂), normal (450 ppm, nCO₂), and high (1200 ppm, hCO₂). To evaluate the photosynthetic apparatus operation, the carbon assimilation and chlorophyll a fluorescence were measured under the same conditions. It was found that the CA activities of the preparations of cytoplasm, chloroplast stroma, and chloroplast thylakoids measured after two weeks of acclimation were higher, the lower CO₂ concentration in the air. That was preceded by an increase in the expression levels of genes encoding the cytoplasmic form of βCA1, and other cytoplasmic CAs, βCA2, βCA3, and βCA4, as well as of the chloroplast CAs, βCA5, and the stromal forms of βCA1 in a short-term range 1–2 days after the beginning of the acclimation. The dependence on the CO₂ content in the air was most noticeable for the CA activity of the preparations of the stroma; it was two orders higher in lCO₂ plants than in hCO₂ plants. The CA activity of thylakoid membranes from lCO₂ plants was higher than that in nCO₂ and hCO₂ plants; however, in these plants, a significant increase in the expression levels of the genes encoding αCA2 and αCA4 located in thylakoid membranes was not observed. The CA activity of mitochondria and the expression level of the mitochondrial βCA6 gene did not depend on the content of carbon dioxide. Taken together, the data implied that in the higher plants, the supply of inorganic carbon to carboxylation sites is carried out with the cooperative functioning of CAs located in the cytoplasm and CAs located in the chloroplasts.

Keywords: carbonic anhydrase; CA activity; photosynthesis; carbon assimilation; Arabidopsis; gene expression; cytoplasm; chloroplasts; thylakoids

1. Introduction

In all living organisms, cells have several buffer systems, including those based on forms of Ci, CO₂, and HCO₃⁻. This is, apparently, one of the main reasons why the enzyme carbonic anhydrase (CA), which accelerates the interconversion of CO₂ and bicarbonate with the release and consumption of a proton, emerged at least eight times in the course of evolution in different groups of living organisms.

In autotrophs, which include inorganic carbon (Ci) into organic compounds, the interconversion of Ci forms is of particular importance. On the way from the medium to the carboxylation centers, where the fixation of inorganic carbon into organic compounds takes place, this interconversion must repeatedly occur to overcome the membranes and aqueous...
phases of cells. The presence of CAs is required to accelerate the feeding of the necessary Ci form and to ensure a high rate of transport. In unicellular photoautotrophs, bacteria and green microalgae, CAs function in the system of Ci concentration in the cells as compared with that of the environment. In all plants, CAs are needed to maintain the required local concentration of a particular form of Ci near the carboxylation sites. The substrate for ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) in C3 plants and in bundle sheath cells of C4 plants is CO$_2$, and in the mesophyll cells of C4 plants, a substrate for phosphoenolpyruvate carboxylase (PEP carboxylase) is bicarbonate. In higher C3 plants, despite the attempts to study the mechanisms of CO$_2$ entry into cells to the sites of their fixation in metabolic pathways and, first of all, to Rubisco, the proven statements on how CAs participate in the conversion of Ci forms to achieve the physiologically necessary result are almost absent.

In cells of angiosperms, as well as of most algae, there are representatives of three families, $\alpha$, $\beta$, and $\gamma$, which are soluble or membrane-bound, and have a wide range of molecular masses and pH optima. In the genomes of higher C3 plants, the number of genes encoding CAs ranges from four in *Spirodela polyrhiza* to seventy-nine in *Triticum aestivum*.

CAs are present in all cell structures. In plasma, the membrane of Arabidopsis leaves $\beta$CA4 was found [1]. The other form of this enzyme was found in cytoplasm [2], with three other CAs, $\beta$CA2, and $\beta$CA3 [1], and $\beta$CA1.4, which has been recently determined as the cytoplasmic form of $\beta$CA1 [3]. In mitochondria, $\beta$CA6 was found in the mitochondrial matrix [1] and all five $\gamma$CAs were identified as a part of the CA-domain attached to the inner surface of the mitochondrial membrane [4].

In chloroplasts, there are several CAs of $\beta$- and $\alpha$-families. Two forms of $\beta$CA1, $\beta$CA1.1 and $\beta$CA1.2 [3], are situated in chloroplast stroma as well as $\alpha$CA1 [5]. The presence of $\beta$CA1.3 was detected in the chloroplast envelope [3]. $\beta$CA5 is one more chloroplast CAs [1] with an unknown precise position in this organ. The thylakoid lumen of pea and Arabidopsis chloroplasts possess CA activity [6,7]. Based on a number of properties, this CA was identified as $\beta$CA, but not $\beta$CA1, and it was assumed that this CA was $\beta$CA5 [8,9]. Proteomic analysis of proteins in thylakoid membranes from Arabidopsis revealed the product of the $\text{At4g20990}$ gene encoding $\alpha$CA4 [10]. Ignatova et al. [11] showed that this CA is situated in the thylakoid membrane close to PSII complexes. In [12], the evidence was presented that the position of $\alpha$CA2 is also in the thylakoid membranes. The presence of $\alpha$CA5 in the stromal parts of thylakoid membranes of Arabidopsis was revealed using mass-spectrometry analysis [13].

The data of the literature indicate that CAs function mutually with other CAs. Wang et al. [14] have shown the involvement of the plasma membrane form of $\beta$CA4 in complex with aquaporin PIP2:1 in the enhancement of CO$_2$ transport in Arabidopsis cells. Hu et al. [15], using $\Delta \beta$-ca1ca4 double mutants, showed that $\beta$CA4 regulates the stomatal conductance of leaves in the presence of carbon dioxide together with chloroplast stromal $\beta$CA1. Under low CO$_2$ conditions, the growth of the mutants with knocked-out genes of two cytoplasmic CAs, $\beta$CA2, and $\beta$CA4, was significantly suppressed [2], which means that at least $\beta$CA1, $\beta$CA2, and $\beta$CA4 participate in the process of carbon dioxide entry into higher plants cells.

The main hypotheses on the physiological role of chloroplast stromal CAs, taking into account the changing levels of substrates and products catalyzed by the CA reaction, were formulated by Coleman [16]: (1) facilitating Ci fluxes in the stroma to the site of Rubisco-mediated carboxylation; (2) the catalysis of bicarbonate dehydration to CO$_2$ in the alkaline stroma to supply the latter for Rubisco; and (3) the acceleration of pH changes in biological buffering systems. Despite copious evidence of a spatial and functional connection of stromal $\beta$CA ($\beta$CA1) and Rubisco [17–21], no definite effects from the inhibition of this CA synthesis on photosynthesis have been shown. Price et al. [22], in transgenic plants of *Nicotiana tabacum* with 10% or less activity of soluble $\beta$CA, have not found significant differences from in a number of parameters characterizing photosynthesis compared to WT plants. However, the carbon isotopic composition of the leaf dry matter was changed...
in these mutants. One of the promising ideas in solving the problem of the conversion of bicarbonate to CO$_2$ and the supply of the latter to Rubisco is the assumption that thylakoid CAs are involved in this process, additionally to the stromal CAs, $\alpha$CA1, and/or $\beta$CA1 [23]. Most likely, this function can be carried out by $\alpha$CA5, since this CA is situated on the stromal side of stromal thylakoid membranes, where the part of Rubisco associated with the thylakoid membrane is located [24,25].

The presence of Ci forms is important not only for the dark phase of photosynthesis but also for the optimal functioning of the electron transport chain. Bicarbonate is required for the electron transport on the acceptor side of PSII [26]. HCO$_3^-$ interaction with non-heme iron ensures rapid electron transfer from Q$_{A}$ to Q$_{B}$ [27]. Bicarbonate is also required on the donor side of PSII, and it was suggested that CA located there could be involved in HCO$_3^-$ delivery [27,28]. Studies on the functioning of $\alpha$CA4 in Arabidopsis thylakoid implied that this CA is located close to light-harvesting PSII antennae [10,11,29]. The assumption about the role of $\alpha$CA4 is that it directs the protons that arise from CO$_2$ hydration to the proteins (PsbS and/or violaxanthin de-epoxidase), which initiate the non-photochemical quenching of leaf chlorophyll $a$ fluorescence (NPQ) [30–32]. These processes protect PSII from photoinhibition. Because of the CO$_2$ hydration bicarbonate molecules released in this reaction, they can bind with protons, which produce during water oxidation in the water-oxidizing PSII complex [33].

In recent years, more data on the involvement of CAs in higher plant cells for protection against stresses, in particular in transmitting a response signal to a negative environmental impact, have begun to appear [34–37]. Dabrowska-Bronk et al. [38] have shown that Arabidopsis plants with single mutations in all six $\beta$CAs genes had a weakened stress tolerance under high light conditions. At the same time, these authors emphasize that the regulation of $\beta$CAs’ gene expression and the enzymatic activities of CAs are important for optimal plant growth and photosynthesis.

An understanding of the physiological roles of CAs in higher plants is still something elusive. Studies using mutants with the suppressed synthesis of CA genes usually turn out to be of little information, and we assume that the reason for this may be that CAs in plants cell function together, and some can replace the weakened function of one of them. The main aim of the present work was to determine the changes in the activity of CAs in different compartments of plant cells in parallel with the expression level of the genes encoding these CAs by exposing Arabidopsis plants to altered carbon dioxide content in the air, which is the main nutrition source for plants and the main substrate of CA reaction.

2. Results

2.1. Photosynthetic and Stress State Characteristics of Arabidopsis Plants during Acclimation to Changed CO$_2$ Conditions

The acclimation of Arabidopsis plants grown under normal CO$_2$ levels in the air (450 ppm, nCO$_2$) to changed CO$_2$ levels in the air, low (150 ppm, lCO$_2$) and high (1200 ppm, hCO$_2$), was measured as changes of the induction of the chlorophyll $a$ fluorescence before and 3, 9, and 16 days after exposure to changed CO$_2$ content. Plants grown at nCO$_2$ were used as a control for lCO$_2$ and hCO$_2$ plants of the same age.

In lCO$_2$- and hCO$_2$-acclimated plants versus nCO$_2$ plants, all parameters of OJIP kinetics commonly used for stress evaluation were changed. The photosynthetic performance index of PSII (PI$_{abs}$), which is estimated to absorption energy per the photochemically active reaction center of PSII, as well as the maximum quantum yield of PSII (Fv/Fm), decreased in the lCO$_2$ plants (Figure 1A,B) after three days, whereas in hCO$_2$ plants, a decrease in these parameters was observed only after 9 and 16 days, respectively. These changes were in parallel with an increase in the dissipation of light energy per the active reaction center ($\text{D}_{\text{lo/RC}}$), i.e., a parameter which characterizes the activation of the defense mechanisms of the photosynthetic apparatus in response to stress, also three days after exposure to lCO$_2$ and 16 days after the exposure to hCO$_2$ (Figure 1C). The effect of the carbon dioxide level on the performance index of the total photosynthetic electron chain...
(PI\textsubscript{total}) was weaker than Pl\textsubscript{abs}, Fv/Fm, and Dlo/RC (Figure 1D). A decrease in the PI\textsubscript{total} in hCO\textsubscript{2} and lCO\textsubscript{2} plants compared to nCO\textsubscript{2} plants has appeared only by the ninth day.

Figure 1. Effect of CO\textsubscript{2} content in the air on photosynthetic parameters of Arabidopsis plants before (0), 3, 9, and 16 days after introduction to low (150 ppm), normal (450 ppm), and high (1200 ppm) CO\textsubscript{2} levels in the air. Red boxes are control plants; green boxes are plants under low CO\textsubscript{2} conditions; blue boxes are plants under high CO\textsubscript{2} conditions. (A) Pl\textsubscript{abs}, Photosynthetic performance index, which is estimated to absorption energy per photochemically active reaction center of PSII; (B) Fv/Fm, maximum quantum yield of PSII; (C) Dlo/RC, dissipation of light energy per active reaction center; (D) PI\textsubscript{total}, performance index. Values are presented from three independent populations. The parameters of 30 leaves were measured in each population.

After 16 days of acclimation to low, normal, and high CO\textsubscript{2} content in the air, we have measured the expression level (expression intensity) of the genes, which are the markers of the induction of stress transcriptional cascades activated by the molecules of phytohormones: abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) (Figure 2). ABA and JA are synergists and both of them are antagonists of SA [39]. At1g29395 and At1g52890 are both ABA-induced genes. At1g29395 encodes the integral membrane protein Cold Regulated 414 Thylakoid Membrane 1 (COR414-TM1), located in the inner envelope of chloroplasts, which provides tolerance to cold and water deprivation. At1g52890 encodes NAC Domain Containing Protein19, one of the main transcription factors in plant abiotic stress responses. At1g17420 (lox3) and At5g42650 (aos) are induced by JA. At1g74710 encodes a lipoxygenase, which catalyzes the oxygenation of fatty acids. At5g42650 encodes the allene oxide synthase that catalyzes the dehydration of the hydroperoxide to oxide in the JA biosynthetic pathway. At1g74710 (csci), At3g52430 (pad), and At1g64280 (npr1) are SA-induced genes. At1g74710 encodes a protein with isochorismate synthase activity, which is important for SA accumulation. At3g52430 encodes a lipase-like gene that is important for salicylic acid signaling. The nonexpressor of Pathogenesis-Related (NPR1) is a key regulator of the SA-mediated systemic acquired resistance (SAR) pathway.
At1g64280 and At1g17420 (Figure 3A). This difference became noticeable after about two weeks after the exposure to CO2 concentrations varying from 0 to 1200 ppm has been used. By the ninth day of acclimation, the difference in CO2 assimilation rate between lCO2, nCO2, and hCO2 plants was imperceptible (Figure 2A–C). The other analyzed stress-marker genes had a tendency to be 10–50% higher in hCO2 plants than in nCO2 plants (Figure 2A–C).

2.2. CO2 Assimilation and Carbon Levels in Leaves of Arabidopsis Plants Acclimated to Changed CO2 Content in the Air

The CO2 assimilation rate was measured in Arabidopsis plant leaves after 9 (Figure 3A) and 14–16 (Figure 3B) days of exposure to lCO2 and hCO2 with nCO2 plants of the same age as a control. For this purpose, the measurement chamber of the LI-6800 Portable Photosynthesis System using the dynamic assimilation technique at CO2 concentrations varying from 0 to 1200 ppm has been used. By the ninth day of acclimation, the difference in CO2 assimilation rate between lCO2, nCO2, and hCO2 plants was imperceptible (Figure 3A). This difference became noticeable after about two weeks after the exposure to the changed CO2 level (Figure 3B). In lCO2 plants, the CO2 assimilation rate was lower than in nCO2 plants. This difference was the highest in atmospheric CO2 content in the measurement chamber.

Figure 2. The content of transcripts of the genes inducible by plant immune signals in Arabidopsis plant leaves after 16 days of acclimation to low (150 ppm), normal (450 ppm), and high (1200 ppm) CO2 levels in the air. (A) At1g29395 and At1g52890 induced by abscisic acid; (B) At1g17420 (lox3) and At5g42650 (aos) induced by jasmonic acid; (C) At1g74710 (icsi), At3g52430 (pad) and At1g64280 (npr1) induced by salicylic acid. Data were normalized for actin gene expression. Values are means ± S.E. of three independent experiments each formed by three repetitions.

In lCO2 plants, the contents of the transcripts of ABA- and JA-inducible genes At1g29395, At1g17420 (lox3), and At5g42650 (aos) were 16, 10, and 3 times higher than in nCO2 plants, respectively (Figure 2A,B). The expression levels of SA-induced genes At3g52430 (pad) and At1g64280 (npr1) were 7 and 15 times lower in lCO2 plants than in nCO2 plants (Figure 2C). In hCO2 plants, the response of stress marker genes was less expressed. Only a four-to five-fold increase in the content of the transcripts of the SA-induced At1g74710 (icsi) gene versus nCO2 plants was observed (Figure 2C). The other analyzed stress-marker genes had a tendency to be 10–50% higher in hCO2 plants than in nCO2 plants (Figure 2A–C).
This difference became noticeable after about two weeks after the exposure to the changed CO₂ level (Figure 3B). In CO₂ plants, the CO₂ assimilation rate was lower than in nCO₂ plants. This difference was the highest in atmospheric CO₂ content in the measurement chamber.

Figure 3. CO₂ response curves measured using the dynamic assimilation technique at CO₂ concentrations in the measurement chamber varying from 0 to 1200 ppm in leaves of Arabidopsis plants after 9 (A) and 14–16 days (B) of exposure to low (150 ppm) and high (1200 ppm) CO₂ level. Assimilation rate in plants of the same age grown in the air with normal (450 ppm) CO₂ level is the control. Curves show the data of a typical experiment.
The lowest CO₂ assimilation rate has been detected in the hCO₂ plants (Figure 3B), which was the most expressed at 1200 ppm in the measurement chamber. Photosynthetic down-regulation in the hCO₂ plants (Figure 1B), which has also been observed by Zheng et al. [40] at elevated CO₂ levels, could be the reason for the relatively low CO₂ assimilation rate in the hCO₂ plants against the nCO₂ and even the ICO₂ ones.

The carbon fixation products, starch and soluble carbohydrates, were determined in the Arabidopsis plants’ leaves after 16 days of acclimation to the changed CO₂ level in the air. It was the lowest in the ICO₂ plants’ leaves (Table 1). The starch content in these plants was 70–80% lower, and the content of soluble carbohydrates was 20–30% lower than in the nCO₂ plants. In the hCO₂ plants, the content of starch was also lower than in the nCO₂ plants by about 30–40%, which correlates with the lower CO₂ assimilation rate in these plants (Figure 3B). However, the content of soluble carbohydrates was slightly higher in the hCO₂ plants than in the nCO₂ ones.

Table 1. The content of starch and soluble carbohydrates in Arabidopsis plant leaves after 16 days of acclimation to low (150 ppm), normal (450 ppm), and high (1200 ppm) CO₂ levels in the air. The table shows the data of a typical experiment. In the parentheses are given the data in % of those from control values, i.e., from data for plants grown at 450 ppm CO₂.

| CO₂ Level in Air | Starch Content, mg/g of Fresh Weight | Soluble Carbohydrates Content, mg/g of Fresh Weight |
|------------------|--------------------------------------|--------------------------------------------------|
| 150 ppm          | 0.82 ± 0.04 (23%)                    | 2.92 ± 0.35 (74%)                                |
| 450 ppm          | 3.60 ± 0.34 (100%)                   | 3.95 ± 0.31 (100%)                               |
| 1200 ppm         | 2.19 ± 0.22 (61%)                    | 4.25 ± 0.36 (108%)                               |

Thus, the presented results demonstrate that Arabidopsis plants grown at short day photoperiods and low photosynthetically active radiation (see Materials and Methods) require at least two weeks of acclimation to the changed CO₂ content in the air, both low and high.

2.3. CA Activity in Preparations of Cytoplasm, Mitochondria, and Chloroplasts Isolated from Leaves of Plants Acclimated to Low, Normal, and High CO₂ Level in the Air

We have found that CA activity in preparations of cytoplasm, where βCA4, βCA2, βCA1.4, and βCA3 are located, was about 30% higher in ICO₂ plants than in nCO₂ and hCO₂ ones (Figure 4A). The CA activity of mitochondria preparations incubated with 1% Triton X-100 was independent of the CO₂ level in the air (Figure 4B).

The CA activities of preparations isolated from chloroplasts were the most sensitive to the CO₂ levels in the air (Figure 4C–E). We measured the CA activity of the stroma and thylakoids separately. The CA activity of the preparations of chloroplast stroma, where βCA1.1, βCA1.2, and αCA1 are located, increased three-fold in the ICO₂ plants compared to the nCO₂ plants. In the hCO₂ plants, the CA activity in these preparations was reduced to 3% of that in the nCO₂ plants. Thus, the activity of the CAs in the stroma of the ICO₂ plants was two orders higher than of those in the stroma of the hCO₂ plants (Figure 4C).
Figure 4. Carbonic anhydrase activities of preparations isolated as described in Materials and Methods from two-month-old Arabidopsis plants after 16 days of acclimation to low (150 ppm), and high (1200 ppm) CO₂ levels in the air. The 100% value is the CA activity of preparations isolated from the leaves of Control plants of the same age grown at 450 ppm of CO₂. Data are shown as mean ± the SE. (A)—preparations of cytoplasm. Briefly, 100% is 941.30 μmol H⁺ (min⁻¹) (mg Chl⁻¹). (B)—preparations of mitochondria, incubated with 1% Triton X-100. In total, 100% is 7093.50 (min⁻¹) (mg Pr⁻¹). (C)—preparations of chloroplast stroma. In addition, 100% is 9896.50 μmol H⁺ (min⁻¹) (mg Pr⁻¹); (D)—preparations of thylakoids incubated with Triton X-100 at Triton/Chl ratio of 0.3. In total, 100% is 148.5 μmol H⁺ (min⁻¹) (mg Chl⁻¹). (E)—Preparations of thylakoids incubated with Triton X-100 at a Triton/Chl ratio of 1.0. In brief, 100% is 201.00 μmol H⁺ (min⁻¹) (mg Chl⁻¹).

The thylakoids during the isolation process were thoroughly washed from extrathylakoid CAs according to [7,41,42]. The CA activity of the thylakoids was determined after incubation with the detergent Triton X-100. The addition of Triton X-100 at a Triton/Chl ratio of 0.3 (Thyl.0.3) exhibits the maximum activity of the CA located in the stromal parts of the thylakoid membranes, i.e., close to PSI [6,42–44]. We have recently identified it as αCA5 [13]. The CA activity of Thyl.0.3 increased by about six times after acclimation to 150 ppm of CO₂ and by 2.5 times after acclimation to 1200 ppm of CO₂, respectively (Figure 4D), if compared with nCO₂ plants.

The CA activity of CAs located in the granal thylakoid membranes, i.e., close to PSII, shows its maximum after the incubation of the thylakoids with Triton X-100 at a Triton/Chl ratio of 1.0 (Thyl.1.0) [6,42–44]. This CA activity is apparently determined due to the presence of αCA4 [10,11,30–32] and αCA2 [11]. The CA activity of Thyl.1.0 was twice higher in the hCO₂ plants than in the nCO₂ plants (Figure 4E). In the hCO₂ plants, the CA activity of Thyl.1.0 was significantly, about 97%, lower than in the nCO₂ plants, i.e., the activity of the CAs from the granal thylakoid membranes of the plants acclimated to the high CO₂ level in the air was 30 times higher than of those from plants acclimated to the high CO₂ level.
2.4. The Effect of Acclimation of Adult nCO$_2$ Plants to Changed CO$_2$ Content in the Air on the Expression Level of the Genes Encoding CAs of $\alpha$- and $\beta$-Families

We have measured the expression level, i.e., the content of transcripts, of the genes encoding $\alpha$CA1, $\alpha$CA2, $\alpha$CA4, $\beta$CA1, $\beta$CA2, $\beta$CA3, $\beta$CA4, $\beta$CA5, and $\beta$CA6 in Arabidopsis leaves of different ages grown at nCO$_2$ (Table 2). The content of the transcripts of the gene encoding thylakoid $\alpha$CA5, located in stromal thylakoid membranes, i.e., close to PSI, has not been measured, since it is too low to be detected by the Real-Time RT PCR [13].

There are four alternative splicing forms of the $\beta$CA1 gene, with two pairs with the same sequences on 3’ ends: $\beta$ca1.1 + $\beta$ca1.2 and $\beta$ca1.3 + $\beta$ca1.4. We have determined the correspondent pairs together and denoted them as $\beta$ca1.1+1.2 and $\beta$ca1.3+1.4. $\beta$CA1.1 and $\beta$CA1.2 are situated in the chloroplasts [3], and the $\beta$ca1.1+1.2 transcripts were related to the group of chloroplast CAs. The other form of $\beta$CA1, $\beta$CA1.3, was determined in the chloroplast envelope, whereas $\beta$CA1.4 was detected in the cytoplasm with a much stronger GFP fluorescence signal than for $\beta$CA1.3 [3]. Thereafter, we related the $\beta$ca1.3+1.4 transcripts to the group of extrachloroplast CAs. Alternative splicing forms of the $\beta$CA4 gene, which encodes $\beta$CA4.2 located in the cytosol [2] and $\beta$CA4.1 located in the plasma membrane [2], also possess the same sequences on 3’ ends of exons, and the correspondent transcripts could not be defined separately.

Table 2. Levels of expression of genes encoding CAs in Arabidopsis plants leaves of different ages grown at the CO$_2$ content of 450 ppm, temperature 19 $^\circ$C, short day photoperiod 8 h day/16 h night, photosynthetically active radiation of 50–70 $\mu$mol quanta m$^{-2}$ s$^{-1}$. Data were normalized for actin gene expression. Data are shown as mean ± the SE of three independent experiments each formed by four repetitions.

| Genes | 26$^{th}$ Day of Age | 44$^{th}$ Day of Age | 50$^{th}$ Day of Age | 59–60$^{th}$ Days of Age |
|-------|---------------------|---------------------|---------------------|------------------------|
| $\beta$ca4 | 1037.9 ± 12.46 | 60.6 ± 2.7 | 55.89 ± 3.97 | 7.22 ± 0.52 |
| $\beta$ca2 | 1416.38 ± 12.35 | 195.67 ± 9.41 | 190.88 ± 15.41 | 37.90 ± 6.92 |
| $\beta$ca1.3+1.4 | 1290.03 ± 7.46 | 153.08 ± 16.1 | 140.74 ± 12.30 | 76.54 ± 5.43 |
| $\beta$ca3 | 1.52 ± 0.20 | 0.98 ± 0.04 | 0.80 ± 0.02 | 0.25 ± 0.07 |
| $\beta$ca6 | 10.94 ± 0.09 | 7.99 ± 0.93 | 6.45 ± 0.32 | 2.94 ± 0.06 |
| $\beta$ca1.1+1.2 | 24.34 ± 4.67 | 19.96 ± 0.96 | 17.91 ± 0.55 | 7.91 ± 0.46 |
| $\alpha$ca1 | 69.48 ± 5.71 | 16.43 ± 0.78 | 14.04 ± 1.44 | 3.31 ± 0.27 |
| $\alpha$ca2 | 3.8 ± 0.28 | 0.93 ± 0.05 | 0.71 ± 0.09 | 0.16 ± 0.05 |
| $\alpha$ca4 | 0.78 ± 0.03 | 0.28 ± 0.01 | 0.26 ± 0.04 | 0.14 ± 0.02 |
| $\beta$ca5 | 14.8 ± 2.3 | 10.59 ± 0.13 | 10.3 ± 0.20 | 6.98 ± 0.25 |

The intensity of the expression of the CA genes in control plants under the constant CO$_2$ level at the normal growth conditions, i.e., at atmospheric CO$_2$ content, was the highest in the leaves of young, 26 days-old plants for most CA genes (Table 2) and gradually decreased with age. In the nCO$_2$ plants, this expression level was rather stable from 44 to 52 days of age (Figures 5 and 6, white columns) and decreased for all analyzed genes by the age of about 59–60 days. In addition, in 59–60 days (two months) old plants, the effects of CO$_2$ on the intensity of the CAs genes expression, are absent (Figures 5 and 6).
Figure 5. Levels of expression of genes encoding extrachloroplast carbonic anhydrases in Arabidopsis plant leaves after introduction to low (150 ppm), and high (1200 ppm) CO$_2$ levels in the air. Numbers on the X axis show the age of the plants and (in parentheses) the number of days after moving introduction of the plants of 43 days of age grown at the atmospheric CO$_2$ level (450 ppm) to conditions of changed CO$_2$ level in the air. The 100% value (dashed horizontal line) is the expression level of the corresponding genes encoding CAs in the plants of 44 days of age grown at 450 ppm (Table 2). Data are shown as mean ± the SE. The experiments were performed three times with similar results. (A)—βca4; (B)—βca2; (C)—βca1.3+1.4; (D)—βca3; (E)—βca6. Asterisks denote statistically significant differences between values for different CO$_2$ levels on the same experimental day, $p < 0.01$. 
Figure 6. Levels of expression of genes encoding chloroplast carbonic anhydrases in Arabidopsis plant leaves after introduction to low (150 ppm), and high (1200 ppm) CO$_2$ levels in the air. Numbers on the X-axis show the number of days after moving the plants of 43 days of age grown at 450 ppm at the atmospheric CO$_2$ level to conditions of changed CO$_2$ level in the air. The 100% value (dashed horizontal line) is the expression level of the corresponding genes encoding CAs in the plants of 44 days of age grown at 450 ppm (Table 2). Data are shown as mean ± the SE. The experiments were performed three times with similar results. (A)—βca1.1+1.2; (B)—αca1; (C)—αca2; (D)—αca4; (E)—βca5. Asterisks denote statistically significant differences between values for different CO$_2$ levels on the same experimental day, p < 0.01.

Figure 5 shows the content of the same transcripts (Table 2) in the leaves of adult plants during the acclimation to different CO$_2$ content in the air. In two-month-old plants, the intensity of the expression of βCA4 did not show a significant change under lCO$_2$ (Figure 5A). However, after 2 days of introduction to ICO$_2$, it was about 30% higher than in the nCO$_2$ plants of the same age. Its expression was not significantly changed in the ICO$_2$ plants during CO$_2$ acclimation.
Until recently, βCA2 was considered as the main CA in the cytoplasm of higher plants [45,46] due to its abundance [1,47] and high expression level [48,49]. The data in Table 2 also show that the expression level of βca2 was one of the highest. However, the contents of the βca4 and βca1.3+1.4 transcripts are also high. The expression level of the genes encoding cytoplasmic Cas βca2, βca1.3+1.4, and βca3 increased by two times on the second day under lCO₂ (Figure 5B,C) versus nCO₂. During further acclimation to lCO₂, the expression level of all four genes of the cytoplasmic Cas decreased, and by the ninth day, became lower than in nCO₂ (Figure 5A–D).

The significant effect of hCO₂ has been observed only for the expression of βca2. On the first day of exposure to hCO₂, it decreased to 12% from that in nCO₂ and increased to 212% by the third day of acclimation (Figure 5B).

The expression level of the mitochondrial βCA6 was independent from CO₂ concentration in the air during all the acclimation (Figure 5E). These data correspond with the constant CA activity in the preparations of mitochondria at any CO₂ content in the air (Figure 4B).

The intensity of the expression of the genes encoding chloroplast CAs was fluctuating during acclimation (Figure 6). However, in lCO₂ conditions versus nCO₂, the reliable increase in the content of the βca1.1+1.2 transcripts (Figure 6A) and the βca5 transcripts (Figure 6E), eight and three times, respectively, have been observed. During further acclimation to lCO₂, the expression levels of βca1.1+1.2 and βca5 decreased and became lower by the ninth day than in control plants, i.e., changed in the same way as the expression intensity of the genes of cytoplasmic CAs (Figure 5A–D). The expression level of the genes, encoding stromal αCA1 and thylakoid CAs αCA2 and αCA4 were not significantly dependent on CO₂ content in the air (Figure 6B–D).

Thus, the observed high-CA activity of cytoplasmic and chloroplast preparations in two-month-old lCO₂ plants if compared to nCO₂ plants and, to an even greater extent, to hCO₂ (Figure 4), was preceded by an increase in the expression levels of the genes encoding chloroplast βCA1 and βCA5 and cytoplasmic βCA2, βCA3, and βCA4 (Figures 5 and 6) in a short-term range 1–2 days after the beginning of the acclimation.

2.5. The effect of Acclimation of Young Plants to low CO₂ Content in the Air on the Expression Level of the Genes Encoding CAs of α- and β-Families

A low concentration of carbon dioxide in the air is a significant stress factor for plants (Figures 1 and 2), leading to a considerable increase in the CA activity of most of the studied fractions (Figure 4), with a much less pronounced increase in gene expression intensity (Figures 5 and 6). Therefore, the additional studies on changes in the levels of CA gene expression in Arabidopsis plants younger than two months of age after acclimation to lCO₂ are noted. In 26 days-age plants, a decrease in the CO₂ content for two weeks led to an increase in the expression levels of most genes of the cytoplasmic and chloroplast CAs (Figure 7A). This expression level was 1.5–2 times higher for αca2 and βca5, 3–4 times higher for βca2 and βca3 genes, and 7–9 times higher for βca1.1+1.2 and βca1.3+1.4. The expression level of only αca4 was about 60% lower in lCO₂ plants than in nCO₂ plants.

In 50 days-age plants, 16 days of acclimation to lCO₂ led to an increase in the intensity of the expression of other CA genes to a much lesser extent than in 26 days-age plants. The expression level of βca4 and αca1 increased by two times; the αca4 and βca5 gene expressions were about three times higher (Figure 7B). The expression levels of cytoplasmic βca2, βca3 and βca1.3+1.4 were about two to three times lower in lCO₂ plants than in nCO₂ plants.
Figure 7. Levels of expression of genes encoding CAs in leaves of Arabidopsis plants of 26 (A) and 50 (B) days of age after 16 days introduction to low (150 ppm) CO$_2$ level in the air. The 100% value (dashed horizontal line) is the expression level of the corresponding genes encoding CAs in the Control plants, according to Table 2, for 26 (A) and for 50 (B) days of age grown at 450 ppm. Data are shown as mean ± the SE. Asterisks denote statistically significant differences between values for different CO$_2$ levels on the same experimental day, $p < 0.01$.

3. Discussion

In the present study, we have determined the changes in CA activities not in the total leave extract from C3 higher plant *A. thaliana* in response to CO$_2$ content in the air, as in previous studies [50,51], but in different cell fractions isolated from leaves of C3 higher plant *A. thaliana* acclimated to the low and high CO$_2$ content in the air. The changes in the contents of transcripts of the correspondent CA genes during acclimation were analyzed in order to evaluate the response of their expression during such acclimations.

We have studied the above-listed changes when the negative consequences of acclimations had already taken place, manifesting primarily in a decrease in the maximum quantum yield of PSII (Fv/Fm) and performance indexes parameters ($P_{\text{total}}$ and $P_{\text{abs}}$). In the plants exposed to lCO$_2$, the changes in these photosynthetic parameters started
in three days and were expectedly lower than in nCO₂ plants (Figure 1). However, the decline in the carbon dioxide assimilation rate in these plants, as well as in the content of starch and soluble carbohydrates (Table 1) and the ICO₂ plant size (not shown) has been observed not earlier than about two weeks after exposure to ICO₂ (Figure 3B). These data are easily explained in terms of the shortage of the CO₂ content, i.e., of the basic material for photosynthesis. At the same time, the parameters of photosynthesis in plants exposed to hCO₂ also decreased versus those in nCO₂ ones, although later than in ICO₂ plants, only on the 9th day for PI_{total} and PI_{abs} and on the 16th day for Fv/Fm (Figure 1A,B,D). Despite the larger size of the hCO₂ plants, the CO₂ assimilation rate in their leaves and the content of starch (mg/g of fresh weight) were lower than in the nCO₂ plants (Figure 3B, Table 1). Photosynthetic down-regulation in the hCO₂ plants (Figure 1B), which has also been observed by Zheng et al. [40] at elevated CO₂ levels, could be the reason for the relatively low CO₂ assimilation rate in hCO₂ plants against nCO₂ and even ICO₂ ones. This can be explained by the known effects of the changes in leaf configuration, i.e., the reduction in stomatal apertures and mesophyll tissue size at an elevated level of atmospheric CO₂ [40,52]. Another reason for the decrease in the assimilation rate may be the acidification of the stroma as a result of indirect proton transfer because of the absorption of excessive CO₂ [53]. Probably, in the conditions of the changed carbon dioxide level, some compensatory mechanisms in the total photosynthetic electron chain take place. Thus, in Arabidopsis plants, the maximum stress effect from the changed CO₂ level developed on the 16th day.

One of the questions raised by investigations of the effect of CO₂ content changes is if a decrease in CO₂ content increases stress, or if the stress weakens the plants’ response to changes in the CO₂ level [48]. Our results imply the first conclusion. The dissipation of light energy per active reaction center (D_{l0}/RC) in both ICO₂ and hCO₂ plants was higher than in nCO₂ ones (Figure 1C). Changes in the expression of stress-induced genes showed that both ICO₂ and hCO₂ conditions are stressful for plants, wherein, in ICO₂ plants and in hCO₂ plants, apparently, different stress responses are induced. In ICO₂ plants, the expression levels of the ABA- and JA-induced genes were higher than in nCO₂ and hCO₂ plants (Figure 2A,B), while the expression levels of the marker genes of SA pathways were lower. It is important that the expression of the npr1 gene, which is the key regulator of SA-mediated SAR, was about seven times lower in ICO₂ plants than in nCO₂ and hCO₂ ones (Figure 2C). Since it is known that ABA and JA are synergists and both of them are antagonists of SA [39], these data mean that in plants at ICO₂, the ABA- and JA-induced SAR pathways are activated, while the SA-mediated pathway is suppressed.

Our data indicate that for Arabidopsis plants, not only the conditions of low levels but also the conditions of high levels of carbon dioxide in the air, are stressful, although to a lesser extent. This is demonstrated both by the intensity of expression of stress genes and by photosynthetic parameters (Figures 1 and 2). The response of stress marker genes to the acclimation to hCO₂ was less expressed than in ICO₂ plants; for most genes analyzed, there was a small, only 10–50% increase in their expression in the hCO₂ plants versus the nCO₂ plants (Figure 2A–C).

The lipophilic CO₂ molecule should easily diffuse across lipid membranes [54,55]. However, this diffusion through cell and organelle membranes is slowed down by a series of resistances due to a high content of protein and sterol molecules in these membranes [55,56].

The diffusion to the site of carboxylation can also be slowed down due to diffusion resistance in the aqueous phases of the cytoplasm and chloroplast stroma [57]. It has long been hypothesized that the CAs of the plasma membrane, cytoplasm, mitochondria, and chloroplasts are involved in Ci transport, in particular, in the supply of CO₂ to carboxylation centers [8,16,47].

The CA activity of mitochondria is determined by the presence of the complex of γCAs-subunits attached to the inner mitochondrial membrane [58] and of the presence of βCA6 in the matrix [1]. Soto et al. [4] showed that the expression intensity of the encoding γCAs genes decreased under conditions of high CO₂ content, and Fabre et al. [1], using
semi-quantitative PCR, demonstrated a higher intensity of the band of PCR products in plants grown at high CO$_2$ levels versus normal CO$_2$. In our experiments, the CA activity of the mitochondria preparations incubated with 1% Triton X-100 and the contents of the $\beta$cat transcripts were independent of the CO$_2$ level in the air (Figures 4B, 5E and 7A). One of the earlier hypotheses [59] about the role of mitochondrial CAs assumed their participation in Ci supply to chloroplasts under conditions of low CO$_2$ in the apoplast, for example, under conditions leading to stomatal closure. Our data do not support these assumptions. Participation in the processes of dark respiration in mitochondria seems to be more probable. This is indicated by a significant increase in the intensity of $\beta$ca6 gene expression after 48 h in the dark [48].

The CA activities of the cytoplasm and all analyzed fractions of chloroplasts, i.e., preparations of stroma and thylakoids, were higher in 1CO$_2$ plants, than in nCO$_2$ plants (Figure 4A,C–E). At that, the CA activity of the preparations of the stroma and Thyl.1.0 (CA activity of granal thylakoid membranes) in 1CO$_2$ plants turned out significantly higher than in hCO$_2$ plants. These data imply that CAs located in the cytoplasm of photosynthesizing cells and CAs located in the chloroplasts, both in stroma and in thylakoids, participate in Ci supply to carboxylation sites in higher plants. Participation in the conversion of bicarbonate into CO$_2$ to provide it to Rubisco in the stroma by several soluble CAs in parallel with thylakoid CAs seems to be even necessary. This would ensure the optimal rate of CO$_2$ supply to the carboxylation centers, which is the most important physiological process for plants. At that, the cooperative participation of stromal and thylakoid CAs in this process would improve plants’ ability to adapt to changing environmental conditions. It seems most likely that of all the thylakoid CAs, it is $\alpha$CA5, located on the stromal side of the stromal thylakoid membranes, which can be involved in this process [13]. The CA activity of the stromal thylakoids was the highest in ICO$_2$ (Figure 4D). Herein, the activity of the stromal thylakoids was 2.5 times higher in hCO$_2$ plants versus nCO$_2$ plants. Under hCO$_2$, the activity of the stromal thylakoids was even higher than the CA activity of the preparations of stroma (Figure 4C). These data imply that under high CO$_2$, the function of CO$_2$ supply to Rubisco is carried out, to a greater extent, by $\alpha$CA5 than by the stromal CAs.

Under the ICO$_2$ level, i.e., in conditions of a deficiency of a Calvin–Benson cycle substrate, the value of NPQ increases. This is confirmed by the increment of the DI$_{10}$/RC parameter in ICO$_2$ plants versus nCO$_2$ ones (Figure 1B). The CA activity of the granal thylakoids under ICO$_2$ is more likely determined by the need for the participation of $\alpha$CA4 located here [10,11] in the development of NPQ [30–32] than by the involvement of granal thylakoid CAs in Ci transport.

The expression level of CA-encoding genes in higher plants is daytime-dependent. That was shown for A. thaliana [48] and CAM plants, Sedum album, Ananas comosus, Kalanchoe fedtschenkoi, and Isoetes taiwanensis [60]. In our experiments, leaves were taken at the same time of the day for the measurements of the level of gene expression. The expression level of almost all CA genes in adult, 2-month-old plants, after 16 days of acclimation to the changed CO$_2$ concentrations in the air did not differ much from that in nCO$_2$ plants of the same age (Figures 5 and 6). The increase in the level of the $\beta$ca1.3+1.4 (Figure 5C), $\beta$ca2 (Figure 5B), $\beta$ca3 (Figure 5D), and $\beta$ca4 (Figure 5A) transcripts encoding cytoplasmic CAs as well as of the $\beta$ca1.1+1.2 (Figure 6A) and $\beta$ca5 (Figure 6E) transcripts encoding chloroplast CAs in a short-term range 1–2 days after the beginning of the acclimation was preceded to the high CA activity (Figure 4). This result is surprising due to the convincing data of the role of $\beta$CA4 and $\beta$CA1 in CO$_2$ transport into cells and in CO$_2$-dependent regulation of stomatal permeability [14,15].

$\beta$CA5, presumably located in the thylakoid lumen [8,9], was the only thylakoid CA of which the expression level responded to the CO$_2$ content in the air. The content of transcripts of the genes encoding thylakoid CAs, $\alpha$ca2 and $\alpha$ca4, was not increased in ICO$_2$ plants versus nCO$_2$ ones, whereas the CA activities of both preparations of thylakoids, Thyl.1.0 and Thyl.0.3, were the highest in ICO$_2$ plants (Figure 4D,E).
From two stromal Cas, only \(\beta\)ca1.1+1.2 transcripts showed an increase from exposure to l\(\text{CO}_2\) (Figure 6A). Thus, with a decrease in the concentration of carbon dioxide, it was intensified the synthesis of \(\beta\)CA1, i.e., of that CA, which participation in photosynthesis is constantly being questioned. The level of \(\alpha\)ca1 transcripts was independent of \(\text{CO}_2\) content in the air (Figure 6B). However, in plants with knocked-out genes encoding \(\alpha\)CA1, the number of indicators of photosynthetic activity, as well as the ability to accumulate starch, was decreased [61]. These data indicate that both stromal CAs, \(\beta\)CA1 and \(\alpha\)CA1, as well as the thylakoid \(\alpha\)CA5 (Figure 4D), play an important role in maintaining the Ci concentration close to carboxylation sites of Rubisco.

A significant decrease in the CA activity of the preparations of the chloroplast stroma and Thyl.1.0 in h\(\text{CO}_2\) plants versus n\(\text{CO}_2\) plants had no parallelism with the expression intensity of the corresponding genes. The expression levels of the genes encoding the stromal and thylakoid CAs in h\(\text{CO}_2\) plants were about the same or even slightly higher, against n\(\text{CO}_2\) plants (Figure 6). These data mean that the changes in the intensities of the synthesis of these CAs at the stage of transcription of the genes encoding them is not the main way of CA activity regulation, at least in the growth conditions used and at age of about two months. Apparently, this mechanism is associated, first of all, with the regulation of CAs’ activities, and to a lesser extent, with an increase in their biosynthesis at the level of the transcription of the genes encoding them. The mechanism of the regulation of CA activity was revealed for \(\beta\)CA1 by studying the action of high-temperature and water deficit stresses in the leaves of Helianthus annum [62] and Brassica napus [21]. The key regulation mechanisms of the CA activity in the chloroplasts in these plants were the nitration and phosphorylation of tyrosine residues in the active site of CA. The binding and dissociation of these nitrate and/or phosphate groups block and open, respectively, the passage of the substrate to the active site cavity according to plant needs. It is very likely that similar mechanisms of the regulation of CA activity exist for other CAs.

The additional studies on the effect of a decrease in the \(\text{CO}_2\) content in the air on the expression levels of CAs genes in Arabidopsis plants younger than two months have shown that in 26-days-old seedlings, these conditions caused a significant intensification of the synthesis of CA gene transcripts (Figure 7A). The expression levels of most cytoplasmic and chloroplast CAs genes were higher in 26-days-old plants after about two weeks of exposure to l\(\text{CO}_2\) versus n\(\text{CO}_2\) plants (Figure 7A). In 50 days-old plants after 16 days of acclimation to l\(\text{CO}_2\) an increase in the intensity of the transcription of CA genes was less pronounced (Figure 7B). Thus, the younger the plants exposed to low \(\text{CO}_2\), the more the effect of an increase in the expression level of the genes encoding cytoplasmic and chloroplast CAs in them. Apparently, in young Arabidopsis plants, the increased intensity of the synthesis of these CAs at the stage of transcription of the genes encoding them makes a greater contribution to the increase in the content of CAs in the plant cell than that in mature plants.

Studies conducted using CA mutants, especially those with single mutations, most often demonstrate little pronounced effects on photosynthesis [15,22,63], except mutation in the \(\beta\)ca5 gene, which leads to significant suppression of the growth of Arabidopsis plants [36]. In recent years, more data on the involvement of CAs in higher plant cells in protection against stress or in transmitting a response signal to a negative environmental impact have begun to appear. Initially, these data were obtained for only \(\beta\)CA1 [34,35,63]. The functioning of \(\beta\)CA1 in these processes was ascribed to the possibility of \(\beta\)CA1 participation in fatty acids (FA) biosynthesis [64] and/or to \(\beta\)CA1’s ability to bind salicylic acid (SA) [34]. These two assumptions, in fact, not only do not exclude, but also complement each other, since SAs and FAs are the key molecules of the stress-induced regulation of metabolic pathways. Recently, Hines et al. [37] have found that leaves of \(\Delta\beta\)-ca1ca5 tobacco double mutants developed abnormally, and their leaves were significantly damaged from necrosis even when supplied with sucrose. Apparently, all six \(\beta\)CAs participated in these processes. Medina-Puche et al. [36] have shown the association of \(\beta\)CAs 1–6 from Arabidopsis with NPR receptors, which are the main participants of SA-induced stress signals.
These authors concluded that βCAs are not involved in photosynthetic processes in higher plants. Wherein, our data show that under such stress as a low CO₂ concentration in the air, a significant increase in CA activity occurs (Figure 4), but the intensity of npr1 gene transcription, on the contrary, decreases (Figure 2C). These data show that at least under these conditions, an increase in the activity of CAs is not associated with their participation in the stress signal transmission through binding with the NPRI protein.

In fact, the involvement of CAs in stress signaling does not exclude the possibility of their participation in photosynthetic processes. Dabrowska-Bronk et al. [38] have shown that in Arabidopsis plants all six βCAs are involved in the uptake of HCO₃⁻ ions by roots, and their functioning is important for plant growth and cell homeostasis, especially under such stresses as lack of water and high light. The possible reason for the absence of dramatic effects of CAs mutations is that the CAs function together in plant cells, replacing each other in case of the suppression of the synthesis of any of them. In support of this hypothesis, it has been demonstrated that the addition of ethoxyzolamide, which is able to penetrate cell membranes and inhibit thus all cellular CAs, has led to a decrease in photosynthesis in the leaves of C3 and C4 plants at low CO₂ concentrations [65] and at the CO₂-dependent O₂ release by pea leaf protoplasts both at low and optimal CO₂ concentrations [66,67].

The described changes in the intensities of CAs gene expression, depending on the time of exposure to changed CO₂ in the air and on the age of plants (Figures 5–7), as well as the data of Hu et al. [15], DiMario et al. [2], Dabrowska-Bronk et al. [38], Medina-Puche et al. [36], and Hines et al. [37] indicate that the functioning of CAs in plants cells is carried out together, interdependently, and complexly. Our data show that this functioning depends significantly on the carbon dioxide content in the air, and this dependence is appreciably determined by the age of the plants.

4. Materials and Methods

4.1. Plant Material

Experiments were performed with Arabidopsis thaliana (L.) Heynh. ecotype Columbia-0 (Col). Three-week-old seedlings were planted into pots, one per pot, containing a commercially available soil mixture, and were grown in a growing chamber (CO₂ content of 450 ppm, temperature 19 °C, 8 h day/16 h night photoperiod, photosynthetically active radiation of 50–70 μmol quanta m⁻² s⁻¹) for 25 days and then exposed to conditions of the low and high CO₂ content, 150 and 1200 ppm under the same other conditions (Supplementary Materials Figure S1).

For the additional determination of the effects of age on the changes in the genes’ expression level after adaptation to low CO₂, young Arabidopsis plants were grown in the conditions described above. At the age of 10 days (Supplementary Materials Figure S2A) and 34 days (Supplementary Materials Figure S2B) the plants were exposed to conditions of the low CO₂ content (150 ppm). Redundant seedlings were removed from the pots of plants aged 10 days.

4.2. Measurement of Chlorophyll a Fluorescence

The maximum quantum yield of PSII (Fv/Fm), performance indexes (PIabs and PItotal), and the dissipation of light energy per active reaction center (Dl0/RC) were calculated according to Kalaji et al. [68] after the measurement of OJIP chlorophyll a fluorescence kinetics. The OJIP chlorophyll a fluorescence transient was measured using a Handy-PEA (Hansatech) fluorometer, with the leaves illuminated with a 1 s flash of red light of 3000 μmol quanta m⁻² s⁻¹. Before measurements, the plants were adapted to dark conditions for two hours.

4.3. Measurement of CO₂ Assimilation Rate

The CO₂ assimilation rate was measured in a leaf chamber using the LI-6800 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA) according to LI-6800 manual in the range of CO₂ concentration of 0–1200 ppm under a constant light intensity of 350 μmol quanta
m$^{-2}$ s$^{-1}$ (90% red, 10% blue light), 23 °C, and 50% relative humidity. Before measurement, the plants were pre-adapted to the illumination of 350 μmol quanta m$^{-2}$ s$^{-1}$ for 2 h. The leaf areas were measured using the Petiole application (Petiole LTD).

4.4. Determination of Starch and Soluble Carbohydrates Content

The starch content was analyzed by measuring the absorbance at 620 nm of leaf aqueous extracts supplemented with KI after thorough washing from pigments [69]. Prior measurements of leaves were kept on a wet filter paper for an hour in order to normalize a turgor of leaves. The content of soluble carbohydrates was determined in hydroalcoholic extract using phenol-sulfuric acid reaction according to Du Bois et al. [70].

4.5. Isolation of Cell Preparations

The isolation of the preparations of the cytoplasm, mitochondria, stroma, and thylakoids from Arabidopsis leaves was performed according to [7,41,42,71] with modifications (Supplementary Materials Figure S3). The leaves were homogenized in the Medium 1 containing 50 mM MES-Tris buffer (pH 8.2), 0.3 M sucrose, 40 mM NaF, 5 mM MgSO$_4$, 1.5% Polyclar AT (w/v), 5 mM EDTA, 0.5% bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM α-aminocaproic acid, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate filtered through nylon cloth was centrifuged for 1.5 min at 150× g for the sedimentation of large fragments of leaves. The supernatant was centrifuged for 5 min at 2500× g, yielding the precipitate of chloroplasts and the supernatant “a”.

The supernatant “a” was centrifugated for 10 min at 8000× g, yielding precipitate (preparations of mitochondria) and supernatant “a’”, enriched with the proteins of cytoplasm. The preparations of the mitochondria obtained were suspended in Medium 1 and were used for analysis after incubation for 20 min with 1% Triton X-100.

The precipitate of the chloroplasts was suspended in Medium 2’ (Medium 2 diluted to one-tenth) to break the chloroplast envelope. Medium 2 contained 0.4 M sucrose, 35 mM K$_2$HPO$_4$, 15 mM NaH$_2$PO$_4$, 3 mM MgSO$_4$, 10 mM KCl, 20 mM sodium ascorbate, 1 mM KHCO$_3$, and 0.5 mM EDTA-Na, 1 mM DTT, 1 mM benzamidine, 1 mM α-aminocaproic acid, and 1 mM PMSF. The mixture was centrifuged for 5 min at 2500× g, yielding a precipitate of thylakoids and the supernatant “b” enriched with the proteins of chloroplast stroma.

Supernatants “a’” and “b” were additionally centrifuged for 1 h at 175,000× g to remove the rest of the membranes yielding preparations of cytoplasm and stroma, respectively, which were used for analysis. The preparations of the stroma were enriched with Rubisco and the preparations of the thylakoids and mitochondria had no Rubisco that was checked by a Western blot assay, using antibodies against the large subunit of Rubisco (Agrisera) (not shown).

The thylakoids were washed three times by suspending the pellets in Medium 2 followed by centrifugation for 5 min at 2500× g. Part of the preparations of the thylakoids was used for analysis after incubation for 20 min with Triton X-100 at a Triton/Chl ratio of 0.3 (Thyl.0.3). The other part of the preparations of the thylakoids was used for analysis after incubation for 20 min with Triton X-100 at a Triton/Chl ratio of 1.0 (Thyl.1.0).

4.6. Determination of the Protein Content

The protein content in the supernatants was determined using a DC$^\text{TM}$ Protein Assay kit Bio-Rad according to Bio-Rad protocol.

4.7. Determination of the Chlorophyll Content

The chlorophyll content was determined in ethanol extracts according to Lichtenthaler [72].

4.8. Measurement of Carbonic Anhydrase Activity

Carbonic anhydrase activity was evaluated according to Khristin et al. [73] as the difference between the rates of pH decrease, measured with a pH electrode, from 8.3 to 7.8
in the course of CO₂ hydration at 2 °C in 13.6 mM Veronal buffer (pH 8.4) in the presence and in the absence of an aliquot of the preparation. The difference in the buffer capacities was taken into account to express the CA activity as the extent of the proton release. The CA activity was calculated as the difference between the rates of the pH decrease in the presence and the absence of the preparation and was expressed in µmol H⁺ per 1 mg of Chl or protein per 1 min.

4.9. Quantitative Reverse Transcription PCR

Leaves were taken at the same time of day, namely, at 11 a.m. Total RNA was extracted from frozen Arabidopsis leaves, using the Aurum total RNA Mini Kit (BioRad), and treated with DNase to eliminate any genomic DNA contamination. Complementary DNA synthesis was performed using a reverse transcription kit OT-1 (Sintol) with oligo (dT15) as a primer. A quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with qPCRMix-HS SYBR (Evrogen) and the primer pairs specific for genes coding COR414-TM1 (At1g29395) (forward 5'-GATAACCTAAGCGATTGAGCA-3' and reverse 5'-ATCTTCCACACTGTGACTAAATCTAACA-3'), ANAC019 (At1g52890) (forward 5'-CATAGAAACCAATCCTCAAACCTAAGC-3' and reverse 5'-AAAAATATCTGACGGAGAAGACAAAG-3'), LOX3 (At1g74710) (forward 5'-TCCAGCTGTTGTGCTTACACCTC-3' and reverse 5'-GTCCGTAACCAGTATTGAGCAAG-3'), AOS (At5g42650) (forward 5'-GAGATTCGTCGGAGAAGAAGGAGAGAA-3' and reverse 5'-AATCACAACAACCTCGGAGACGAGAGAAAGG-3'), ICSI (At1g74710) (forward 5'-AGGAGAAGACGACACTGCTGAGAAA-3' and reverse 5'-TCAATGCCCCAAGACCCTTT-3'), PAD (At3g01500.1 + At3g01500.2 (βca1.1+1.2), At3g01500.3 + At3g01500.4 (βca1.3+1.4), At3g12730 (βca2), At1g14480 (βca3), At1g70410 (βca4), At4g33580 (βca5), At1g58180 (βca6), At3g52720 (aca1), At3g52720 (aca2), At4g20990 (aca4). Primers sequences for the genes encoding CAs were used according to Rudenko et al. (2017), with primers for βca1.1+1.2 corresponding to βca1a primers, and primers for βca1.3+1.4 corresponding to βca1b primers. qRT-PCR data were normalized against Actin 7 gene. PCR reactions were performed in a LightCycler 96 Instrument, Roche Diagnostics GmbH.

5. Conclusions

In summary, this study demonstrated that in photosynthesizing cells, the conditions that require an increase in the intensity of inorganic carbon entry into cells lead to an increase in the activity of CAs located in the cytoplasm and CAs located in the chloroplasts, both stromal and thylakoid ones. Changes in the intensity of the expression of the genes encoding these CAs depended on the age of the plants. The increase in the level of CAs gene expression in lCO₂ plants versus hCO₂ plants was most pronounced in young 26-days-old plants. In 50 days-old plants, it was less noticeable, and in two-month-old plants, after 16 days of acclimation to the changed CO₂ concentrations in the air, it did not differ much from that in nCO₂ plants of the same age. The increase in the CA activities of the preparations of the cytoplasm, stroma, and thylakoids was preceded by an increase in the content of the transcripts of βca1.3+1.4, βca2, βca3, and βca4 encoding cytoplasmic forms of CAs as well as of βca1.1+1.2 and βca5, encoding chloroplast forms of CAs in a short-term range of 1–2 days after the beginning of the acclimation. The CA activity of the preparations of the mitochondria as well as the expression level of the gene encoding mitochondrial βCA6 was independent of the content of the carbon dioxide level in the air.

These data do not support the assumptions of the participation of mitochondrial CAs in Ci supply to chloroplasts under conditions of low CO₂.

Taken together, our data imply that CAs located in the cytoplasm and CAs located in the chloroplasts, both in stroma and in thylakoids, cooperatively participate in inorganic carbon supply to carboxylation sites in higher plants (Figure 8).
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much from that in nCO$_2$ plants of the same age. The increase in the CA activities of the preparations of the cytoplasm, stroma, and thylakoids was preceded by an increase in the content of the transcripts of βca1.3+1.4, βca2, βca3, and βca4 encoding cytoplasmic forms of CAs as well as of βca1.1+1.2 and βca5, encoding chloroplast forms of CAs in a short-term range of 1–2 days after the beginning of the acclimation. The CA activity of the preparations of the mitochondria as well as the expression level of the gene encoding mitochondrial βCA6 was independent of the content of the carbon dioxide level in the air. These data do not support the assumptions of the participation of mitochondrial CAs in $\text{Ci}$ supply to chloroplasts under conditions of low CO$_2$.

Taken together, our data imply that CAs located in the cytoplasm and CAs located in the chloroplasts, both in stroma and in thylakoids, cooperatively participate in inorganic carbon supply to carboxylation sites in higher plants (Figure 8).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11162113/s1, Figure S1: Scheme of Arabidopsis plants cultivation during the experiment. Figure S2: Scheme of Arabidopsis plants cultivation during the experiment on the effect of the low CO$_2$ level on the expression of CA genes. Figure S3: Scheme of isolation from Arabidopsis leaves of preparations of cytoplasm, mitochondria, stroma, and thylakoids.

Author Contributions: Conceptualization, N.N.R. and L.K.I.; Methodology, N.N.R., L.K.I., I.A.N. and N.S.N.; Writing—original draft preparation, N.N.R.; Review and editing, B.N.I. and L.K.I. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by The Ministry of Science and Higher Education of the Russian Federation, State Scientific Program, theme no. 122041100186-2.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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