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Features of Photosynthesis in *Arabidopsis thaliana* Plants with Knocked out Genes Encoding Chloroplast Carbonic Anhydrases αCA1 and βCA1

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Abstract—The knockout of either *At3g01500* or *At3g52720* gene encoding *Arabidopsis thaliana* βCA1 and αCA1 carbonic anhydrase (CA), respectively, led to a lower CA activity of the chloroplast stroma preparations from the knockout mutant plants (αCA1-KO and βCA1-KO) compared to such preparations from the wild-type (WT) plants. To identify the differences in the photosynthetic characteristics of mutant and WT plants, they were grown in low light (LL; 50-70 μmol quanta·m^{-2}·s^{-1}, natural conditions) and high light (HL; 400 μmol quanta·m^{-2}·s^{-1}, stressful conditions). The rate of CO_2 assimilation measured at 400 μmol quanta·m^{-2}·s^{-1} in plants grown under LL was lower in αCA1-KO and βCA1-KO mutants compared to WT plants. The rate of photosynthetic electron transport was lower in αCA1-KO plants and higher in βCA1-KO plants than in WT plants; the content of CO_2 in chloroplasts was lower in βCA1-KO plants than in both WT and αCA1-KO plants, where it differed little. The value of the proton-motive force was higher in βCA1-KO plants and lower in αCA1-KO plants than in WT plants due to changes in ΔpH value. The obtained results suggest that βCA1 facilitates the intake of inorganic carbon into chloroplasts, while αCA1 ensures the conversion of bicarbonate into CO_2 in the chloroplast stroma for its use in the reaction catalyzed by Ribulose bisphosphate carboxylase/oxygenase (RuBisCO). In both αCA1-KO and βCA1-KO mutants, the expression levels of genes encoding other chloroplast CAs differed markedly from those in WT plants; the pattern of the changes in the genes expression depended on the light intensity during cultivation. The content of hydrogen peroxide in the leaves of both αCA1-KO and βCA1-KO mutants was higher in LL and lower in HL than in WT plants. The expression levels of stress marker genes changed similarly in both types of mutant plants. A possible involvement of the chloroplast stroma CAs in the transmission of stress signals in higher plants is discussed.

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INTRODUCTION

Carbon dioxide (CO_2) molecules from the air enter cells of terrestrial plants, where photosynthesis takes place, and are converted into bicarbonate (HCO_3^-) ion in the aqueous medium of the cells. In the light, the stroma of chloroplasts in C3 plants is weakly alkaline (pH 7.7-8.0) [1]. Under these conditions, bicarbonate makes 96-98% of the total inorganic carbon (C_{inorg}), i.e., its level is almost 30-50 times higher than the level of CO_2. Ribulose bisphosphate carboxylase/oxygenase (RuBisCO), a key enzyme of the Calvin–Benson–Bassham (CBB) cycle, which is localized in the stroma of chloroplasts, incorporates C_{inorg} into organic compounds in a form of CO_2. Therefore, it is important that C_{inorg} in converted to CO_2 in close proximity to the carboxylation centers. According to the existing concepts, a relatively slow spontaneous conversion of

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bicarbonate into CO_2 against the background of fast C_{inorg} consumption in its reaction with ribulose phosphate can limit photosynthesis [2]. Carbonic anhydrase (CA; carbonate hydrolase EC 4.2.1.1) accelerates interconversion of C_{inorg} forms. The role of CAs located in the chloroplast stroma has been traditionally discussed in regard to their potential involvement in the transport of C_{inorg} to carboxylation centers (RuBisCO) or in the conversion of bicarbonate, the predominant form of C_{inorg} in the alkaline stroma, into CO_2 that serves as a substrate for RuBisCO [3-5].

Higher C3 plants contain about twenty genes encoding CAs that belong to the α, β, and γ families. According to the TAIR database, the genome of *Arabidopsis thaliana* contains 8 genes of CAs from the α-family (αCAs), 6 genes of βCAs, 3 genes of γCAs, and 2 genes for γCA-like proteins [6]. Often, the same cell compartment contains several CA isoforms, both membrane-bound and soluble, that belong to different families. βCA1 is a long-known and well-studied protein [7]; its content in the cells is exceeded only by the content of RuBisCO [8], the most abundant protein in plant leaves. Similar to RuBisCO, this enzyme is located in the stroma of chloroplasts [9]. *A. thaliana* contains 4 βCA1 isoforms formed by alternative splicing; one of them was found in the chloroplast envelope [10]. These isoforms differ in both their structure and cellular localization [10].

αCA1 was found in 2005 [11], and the data on its localization are still contradictory. The presence of this enzyme in the stroma of Arabidopsis chloroplasts had been shown by using GFP fusion proteins and labeling with anti-αCA1 antibodies with attached gold particles [11]. Later, Hines et al. [12] found αCA1 in the plasma membrane of *Nicotiana tabacum* leaves. However, the study conducted in 2023 showed that in rice plants, this protein localized to the stroma of chloroplasts [13]. The authors demonstrated that the CA mutants lagged behind the wild type (WT) plants in growth, contained less starch, and exhibited a lower rate of CO_2 assimilation (A_{CO2}) and reduced water use efficiency.

Arabidopsis chloroplasts also contain βCA5 [6] located in the chloroplast stroma (similar to βCA1 and αCA1) [14]. The thylakoid membrane of *A. thaliana* chloroplasts was found to include αCA4 [15] located apparently in the grana [16]. αCA5 was identified in the preparations of *A. thaliana* stromal thylakoid membranes [17]. The lumen of thylakoids from *Arabidopsis* and pea plants contained CA belonging to the β-family (according to its properties) [18, 19]; however, the amino acid sequence of this protein is yet to be determined. The shutdown of αCA2 synthesis in *Arabidopsis* produced multiple effects on the functioning of its photosynthetic apparatus [20, 21], which also suggests the presence of αCA2 in chloroplasts. A large number of CAs catalyzing the same reaction considerably complicates the studies of individual functions of each enzyme.

The studies on the role of CAs in the chloroplast stroma showed that reducing the level of βCA1 to 1% in tobacco plants by using the corresponding antisense RNA (which bound to the βCA1 mRNA and induced its degradation, resulting in the reduced enzyme synthesis) caused no changes in the phenotype or photosynthetic parameters [22]. Studying the effects of single mutations in βCA1 and simultaneous shutdown of two chloroplast CAs (ΔβCA1/βCA5) [12] led to the assumption that the function of stromal CAs is not associated with photosynthesis. It has been shown that βCA1 is important at the stage of appearance and growth of cotyledon leaves in *Arabidopsis* [23], as well as involved, together with βCA4, in the regulation of stomatal movements [24]. In rice leaves, this mechanism seems to involve only one βCA, which is structurally similar to βCA4 but localizes to chloroplasts [25]. In contrast, βCA4.1 and βCA4.2 from *Arabidopsis* have been identified in the plasma membrane [6, 26] and cytoplasm [26], respectively. Tobacco ΔβCA1/βCA5 mutants demonstrated a lower rate of seed germination, reduced mass, and growth retardation compared to WT plants [12] because of the reduced rate of fatty acid synthesis in chloroplasts. The involvement of stromal CA in fatty acid synthesis was hypothesized as early as in 2002 [27]. Beside playing an important structural role, fatty acids serve as precursors in the synthesis of jasmonates, including jasmonic acid (JA) [28]. Jasmonates are a group of plant hormones involved in the triggering of plant response to various stress factors. It was also found that CAs bind salicylic acid (SA), another stress hormone in plants [29]. This ability has been shown for a number of CAs, especially βCA1 [30]. The authors demonstrated that the binding of βCA1 with SA and major cognitive receptor proteins, including NPR1 (nonexpressor of pathogenesis-related genes), promote transduction of stress signals to the nucleus.

Exposure of plants to stress conditions promote the manifestation of effects caused by the absence of particular CA proteins in mutant plants [31-33]. Expression of CA genes varies depending on the growth conditions, thus demonstrating that the needs of plants for CAs depends on external conditions [34, 35]. So far, the direct involvement of stromal CAs (βCA1 and αCA1) in the photosynthesis has not been proven experimentally. Here, we studied the properties of βCA1- or αCA1-deficient *Arabidopsis* plants were grown under natural and stress (excess light) conditions. The goal of this study was to further elucidate the physiological role of these CAs and investigate their involvement in the photosynthesis and stress signaling in higher plants.

MATERIALS AND METHODS

Plants and growth conditions. The plants used in the study were *A. thaliana* WT plants (Columbia ecotype), plants with the knocked out *At3g52720* gene encoding αCA1 (αCA1-KO; homozygous lines 082 and 029) that were derived from the SALK_082033C and SALK_029393C lines, respectively, and plants with the knocked out *At3g01500* gene encoding βCA1 (βCA1-KO; homozygous line) obtained from the SALK_106570 line. The plants were grown to the age of 36 days in a climate chamber at a low light (LL) intensity corresponding to the natural conditions (50-70 µmol quanta·m^{-2}·s^{-1}) with an 8 h/16 h day/night photoperiod at the atmospheric CO_2 concentration (450 ppm) at 19°C. Next, some plants were left under the control conditions described above (LL), while the other plants were adapted to high light (HL, 400 µmol quanta·m^{-2}·s^{-1}) for 14-28 days. The seeds of WT *Arabidopsis* plants were kindly provided by Professor R. Scheibe from the Collection of the Plant Physiology Department at the University of Osnabrueck, Germany. The seeds of homozygous mutant plants were kindly provided by Professor J. V. Moroney (Louisiana State University, United States).

Isolation of stromal preparations from the leaves of WT and mutant *Arabidopsis* plants was performed as described by Rudenko et al. [35]. The leaves were homogenized in the isolation medium containing 0.4 M sucrose, 35 mM K_2HPO_4, 15 mM NaH_2PO_4, 3 mM $MgSO_4$, 10 mM KCl, 20 mM Na ascorbate, 1 mM $KHCO_3$, 0.5 mM Na ethylenediaminetetraacetate, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM α-aminocaproic acid, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was filtered through a nylon tissue and centrifuged at 150*g* for 1.5 min at 2°C to remove large leaf fragments. The supernatant was centrifuged at 2500*g* for 5 min at 2°C; the resulting pellet (chloroplasts) was resuspended in the shock medium (isolation medium diluted 10-fold with distilled water) to destroy chloroplast envelopes. The chloroplast suspension was centrifuged at 2500*g* for 5 min at 2°C to obtain a pellet of thylakoids and a supernatant enriched with proteins of the chloroplast stroma. The supernatant was additionally centrifuged at 175,000*g* for 1 h at 4°C to remove membrane debris, resulting in the stromal protein preparation for further analysis.

Protein assay was performed according to Bradford [36].

CA activity assay. The activity of CAs was assessed as a difference in the rates of pH decrease measured with a pH electrode (from 8.4 to 7.9) during CO_2 hydration at 2°C in 13.6 mM Veronal buffer (pH 8.4) in the presence and absence of stromal protein preparation and expressed in µmol H^+ per mg protein per min [37].

Measurement of gas exchange in plants. The rate of A_{CO2} and stomatal conductance were measured in unseparated leaves using an LI-6800 portable system for the analysis of photosynthetic processes (Li-Cor, USA) at 400 ppm CO_2 in a leaf chamber at a light intensity changing discretely from 50 to 1600 µmol quanta·m^{-2}·s^{-1} (90% red light, 10% blue light) at 23°C and 50% relative humidity. The leaf area was measured with the Petiole mobile app (Petiole LTD).

Electrochromic shift (ECS) of carotenoid absorption for determining the values of the proton motive force (pmf) components was measured at 515 nm in unseparated leaves using a Dual-PAM-100 Fluorescence Measuring System equipped with a P515/535 emitter-detector module (Heinz Walz, Germany) [38]. The measurements were made using 2.5-µs flashes alternating with 5-s dark periods for the excitation of electron transfer in the reaction centers of photosystems I and II (PSI and PSII). The averaged signal of 50 flashes was accumulated in the fast kinetics mode; the amplitude of the averaged signal was calculated in OriginPro and used as the ECT standard (ECSst) value. The values of pmf and its components (ΔpH and ΔΨ) were determined in leaves after illumination for 5-min with the actinic light of increasing intensity (84, 155, 279, 364, 577, and 904 µmol quanta·m^{-2}·s^{-1}). The pmf value was determined by the difference between the ECS values at the moment immediately before the light was turned off and the minimum of the inverted signal in the dark after the light was turned off. The difference was normalized to the ECSst determined for a given leaf [39]. The ΔpH value was found as the difference of ECS values corresponding to the minimum inverted dark signal and the maximum signal after relaxation of the inverted signal. The ΔΨ value was determined by the difference between the pmf and ΔpH values.

Photosynthetic parameters were determined by simultaneously measuring the gas exchange parameters in plants and chlorophyll *a* (Chl *a*) fluorescence in leaves using an LI-6800 portable system for the analysis of photosynthetic processes. Before the measurement, the plants were adapted in the dark for 1.5 h. After a saturating flash in the dark, the actinic light (400 µmol quanta·m^{-2}·s^{-1}) was turned on, followed by light flashes every 30 s. The measurements were made for 10 min at the CO_2 concentration of 400 ppm in the chamber; then the concentration of CO_2 was reduced to 150 ppm and the measurements were continued for another 5 min. Next, the CO_2 concentration was increased to 1200 ppm for 5 min and then returned again to the initial value of 400 ppm for 2.5 min. The electron transport rate (ETR), A_{CO2}, and number of closed PSII reaction centers (1-qL) were determined by the formulas (1-3):

$$\mathrm{ETR} = \mathrm{Y(II)} \times 0.50 \times \mathrm{PPFDa}, \qquad (1)$$

where PPFDa is the rate of light absorption by a sample, μmol $m^{-2} \cdot s^{-1}$;

$$YII = (Fm' - Fs)/Fm', \quad (2)$$

where Fm′ is the maximum yield of fluorescence in response to the saturated impulse upon illumination with the actinic light; Fs is the stationary level of fluorescence upon illumination with the actinic light [40, 41];

$$1 - qL = 1 - [(Fm' - Fs)/(Fm' - F_0')] \times F_0'/Fs, \quad (3)$$

where F_0' is the minimum level of fluorescence in the light-adapted state.

The content of CO_2 in the chloroplasts (Cc, μmol $CO_2 \cdot mol^{-1}$ of air) was calculated by the formula (4):

$$Cc = G^* \times [ETR + 8(A + Rd)]/[ETR - 4(A + Rd)][13], \quad (4)$$

where G* is the CO_2 compensation point in the absence of respiration (μmol CO_2 mol^{-1} of air); Rd is the rate of CO_2 release from the mitochondria during respiration (μmol CO_2 $m^{-2} \cdot s^{-1}$) and does not depend on illumination. For G* and Rd, the following empirical values were used: 49 μmol CO_2 mol^{-1} [42] and 1 μmol CO_2 $m^{-2} \cdot s^{-1}$ [43], respectively.

Starch content measurement. The leaves were cut off in the morning after 3-h illumination under the growing conditions. The content of starch was determined by measuring light absorption at 620 nm in the aqueous extracts of leaves after incubation with 0.12% KI [44].

Measurement of Chl and carotenoid levels. The content of carotenoids and Chls was determined after extraction from plant leaves with 96% ethanol and expressed in mg pigment per wet weight of leaves [45].

Hydrogen peroxide content in leaves was determined by the luminol-peroxidase reaction [46]. Leaves (50-100 mg) were frozen in liquid nitrogen, transferred into 0.4 mL 2 M trichloroacetic acid, and homogenized. Hydrogen peroxide was extracted by adding 3 mL of 0.05 M K-phosphate buffer (pH 8.5). To remove Chls and carotenoids, the extract was incubated with 5% polyvinylpyrrolidone (PVP) and then centrifuged at 10,000*g* for 10 min. The supernatant was collected and its pH was adjusted to 8.5 with 2 M KOH. Luminol (1 mL; 2.26×10^{-4} M) and peroxidase (1×10^{-6} M) mixture (1 mL) was added to 50 μL of the extract directly in the measurement cuvette to determine the H_2O_2 level. Chemiluminescence was recorded with a Lum-100 luminometer (DISoft, Russia) using solutions with the known H_2O_2 concentration for calibration.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the frozen *Arabidopsis* leaves using an Aurum Total RNA Mini kit (Bio-Rad, USA) and treated with DNase I to eliminate any contamination of genomic DNA. cDNA was synthesized using an RT-1 reverse transcription kit (Syntol, Russia) using oligo(dT) as a primer in a LightCycler 96 Instrument (Roche Diagnostics, Switzerland). RT-qPCR was performed with a ready-to-use qPCRmix-HS SYBR reagent mixture (Evrogen, Russia), using the primers for the CA-encoding genes, as well as *COR414-TM1* (*At1g29395*), *ANAC019* (*At1g52890*), *NPR1* (*At1g64280*), and *JAZ1* (*At1g19180*) genes. Gene expression was calculated with the formula $2^{-(Ct\ sample\ -\ Ct\ control)}$, where C_t is the threshold number of PCR cycles. The *At5g09810* (actin 7) gene was used as the C_t control [34]. The content of βCA1 transcripts was determined jointly in pairs (*βCA1.1* + *βCA1.2* and *βCA1.3* + *βCA1.4*), as these alternative splicing forms possess identical sequences at the 3′end. The sequences of primer (Table S1 in the Online Resource 1) were from Rudenko et al. [34, 35] and Borisova-Mubarakshina et al. [47].

Statistical analysis was performed with OriginPro. The data are presented as mean values with standard errors of the mean (SEM). Statistical significance was evaluated by ANOVA with the paired comparison plot using the Holm–Bonferroni method.

RESULTS

The effect of light intensity on the pigment content in the leaves of WT plants and plants deficient by stromal CAs. No phenotypic differences were observed between the WT, αCA1-KO, and βCA1-KO plants grown under HL and LL (natural for *Arabidopsis*) conditions. The levels of Chl *a*, Chl *b*, and carotenoids in the leaves of αCA1-KO grown under LL were 10% lower compared to WT plants, while the content of these pigments in βCA1-KO mutants was close to that in WT plants (Table 1). At the same time, the Chl *a*/Chl *b* ratio in the leaves of WT, αCA1-KO, and βCA1-KO plants was very similar. These findings demonstrate that under LL conditions, the absence of αCA1 or βCA1 had no significant effect on the pigment biosynthesis. After the three-week adaptation to HL, the amount of both Chl forms decreased similarly in βCA1-KO and WT plants, while αCA1-KO plants demonstrated a minor (7%) increase in the Chl *a* content (Table 1). At the same time, the Chl *a*/Chl *b* ratio increased by 20-25% in all studied plants, although in WT and βCA1-KO plants, it was due to a decrease in the Chl *b* levels, while in αCA1-KO plants, the increase resulted from the elevation of the Chl *a* content. Although the level of carotenoids decreased

Table 1. Effects of light intensity on the pigment content and Chl *a*/Chl *b* ratio in the leaves of *Arabidopsis* WT, αCA1-KO, and βCA1-KO plants grown at 50-70 μmol quanta·m^{-2}·s^{-1} (LL) and 400 μmol quanta·m^{-2}·s^{-1} (HL)

Light intensity, μmol quanta, m^{-2}·s^{-1}	Plant	Pigment content, mg/g wet weight				Chl *a*/Chl *b*
		Chl *a*	Chl *b*	Chl *a* + Chl *b*	Carotenoids	
50-70 (LL)	WT	0.80 ± 0.05	0.38 ± 0.04	1.17 ± 0.08	0.17 ± 0.01	2.04 ± 0.02
	αCA1-KO	0.71 ± 0.06	0.34 ± 0.01	1.06 ± 0.10	0.15 ± 0.01	2.08 ± 0.12
	βCA1-KO	0.82 ± 0.12	0.40 ± 0.07	1.22 ± 0.19	0.17 ± 0.03	2.05 ± 0.01
400 (HL)	WT	0.62 ± 0.05 (78%)	0.24 ± 0.02 (63%)	0.86 ± 0.08 (74%)	0.12 ± 0.02 (71%)	2.55 ± 0.06 (125%)
	αCA1-KO	0.76 ± 0.02 (107%)	0.31 ± 0.01 (91%)	1.07 ± 0.03 (101%)	0.14 ± 0.02 (93%)	2.50 ± 0.02 (120%)
	βCA1-KO	0.56 ± 0.01 (68%)	0.22 ± 0.01 (55%)	0.78 ± 0.02 (64%)	0.10 ± 0.01 (58%)	2.49 ± 0.02 (121%)

Note. The table shows the data of a typical experiment ($n \geq 4$). Similar results were obtained in six independent experiments. The values in parentheses represent a percentage of the respective value under LL.

in WT plants and both mutant lines, it remained the highest in αCA1-KO plants.

The CA activity in the stroma of WT and mutant plants. The soluble fractions enriched in stromal proteins were obtained from the leaves of WT, αCA1-KO, and βCA1-KO plants grown under LL. The CA activity in the preparations from WT plants was the highest, since it was determined by the presence of at least three CAs (αCA1, βCA1, and βCA5) (Fig. 1), while knocking out either αCA1 or βCA1 led to a 5-fold decrease in the CA activity, indicating that both βCA1 and αCA1 are soluble stromal enzymes.

The effects of the knockout of stromal CAs on the expression levels of genes coding for chloroplast CAs in the plants grown at LL and after acclimation to HL. No αCA1 expression was observed in αCA1-KO mutants, while βCA1-KO plants lacked *βCA1.1* + *βCA1.2* and *βCA1.3* + *βCA1.4* transcripts (data not shown). The relative expression of βCA1 was high in both WT and αCA1-KO plants under both LL and HL (Fig. 2, a and b), with the content of *βCA1.3* + *βCA1.4* transcripts being the highest. Under the LL conditions, expression of the *βCA1.1* + *βCA1.2* and *βCA1.3* + *βCA1.4* transcripts in αCA1-KO mutants was approximately 2 times higher than in WT plants (Fig. 2, a and b). Under the same conditions, expression of *αCA2* in these mutants was 3 times higher and expression of *βCA5* was 4.5 times lower compared to WT plants (Fig. 2, d and e). Illumination of βCA1-KO plants with LL led to the 3- and 9-fold downregulation of the *αCA1* and *βCA5* genes, respectively (Fig. 2, c and e).

After adaptation to HL, WT plants showed an increase in the expression levels of all studied CA genes (Fig. 2) except for *βCA5* (similar effect was described previously by us in [34]). At the same time, in βCA1-KO plants, adaptation to HL caused upregulation of all studied CA genes, including *βCA5* (Fig. 2e). On the contrary, in αCA1-KO plants, expression of most investigated CA genes was downregulated under HL (Fig. 2, a, b, d, and f), this decrease being especially noticeable (30-fold) for the *αCA2* gene transcripts (Fig. 2d).

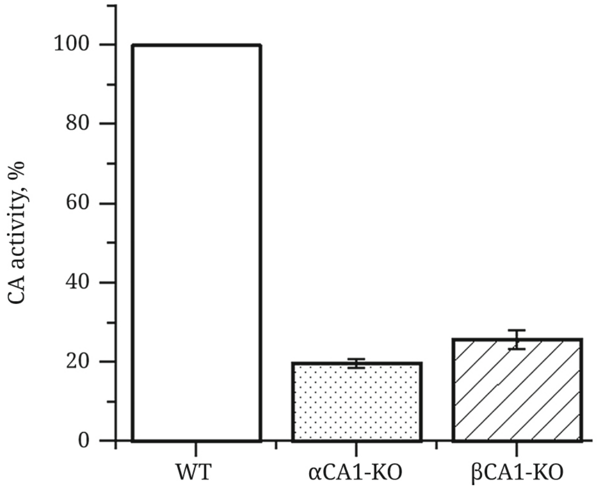

Fig. 1. The CA activity in the preparations of soluble chloroplast stromal proteins from the leaves of WT, αCA1-KO and βCA1-KO plants grown under LL. The activity of stromal proteins isolated from WT plants (1060 and 3560 μmol H^{+}·mg protein^{-1}·s^{-1} in two experimental replicates, $n \geq 6$) was taken as 100%.

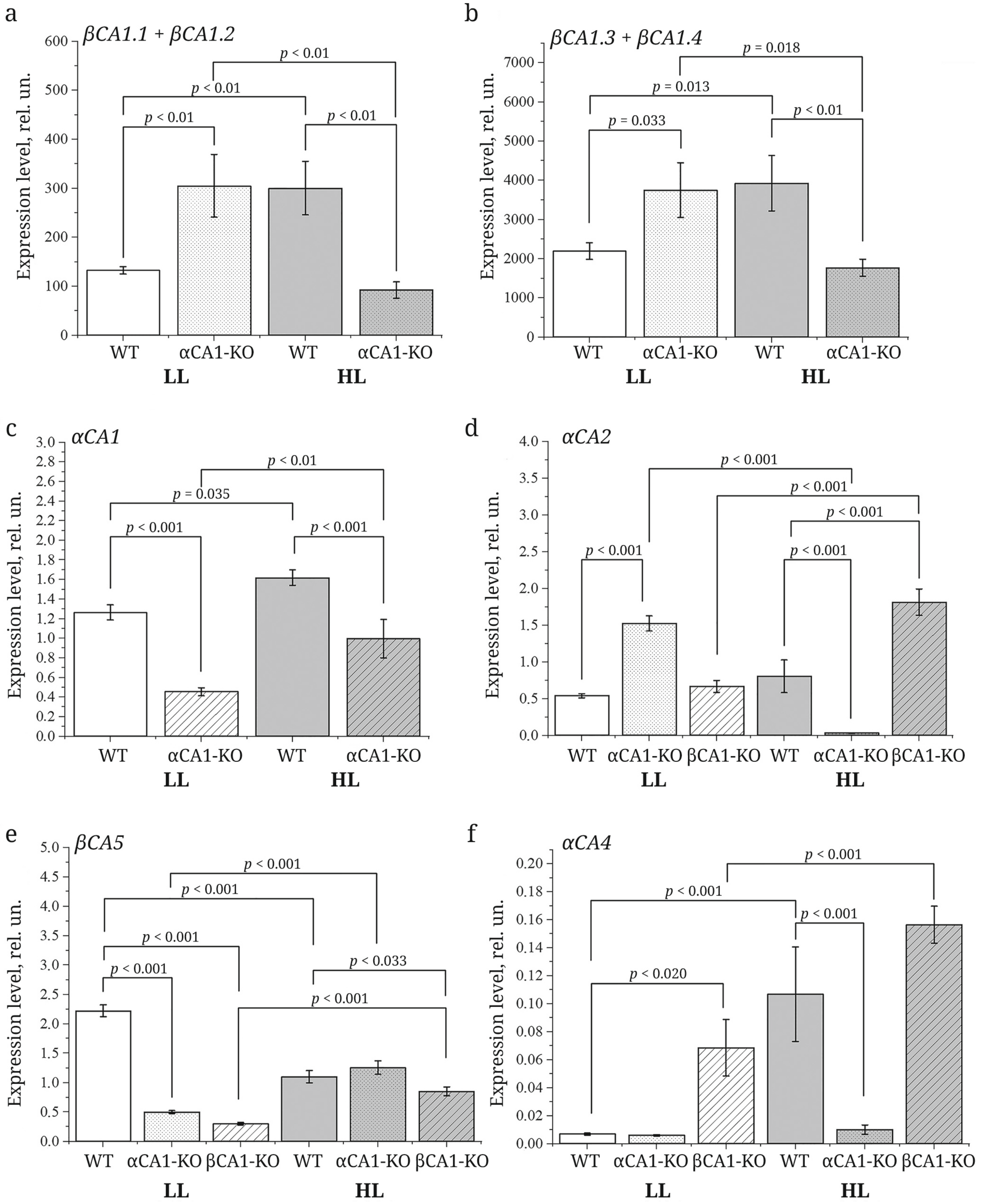

Fig. 2. Expression levels of genes coding for the chloroplast enzymes βCA1 (a, b), αCA1 (c), αCA2 (d), βCA5 (e), and αCA4 (f) in WT, αCA1-KO (line 082), and βCA1-KO plants grown at LL (50-70 μmol quanta·m^{-2}·s^{-1}; white columns) and HL (400 μmol quanta·m^{-2}·s^{-1}; gray columns). Expression of four alternative splicing transcripts of the *βCA1* gene were determined pairwise: *βCA1.1* + *βCA1.2* (a) and *βCA1.3* + *βCA1.4* (b). Significance of differences was determined by the Holm–Bonferroni method ($n \geq 9$). Similar results were obtained in three experiments (3 biological and 3 analytical replicates).

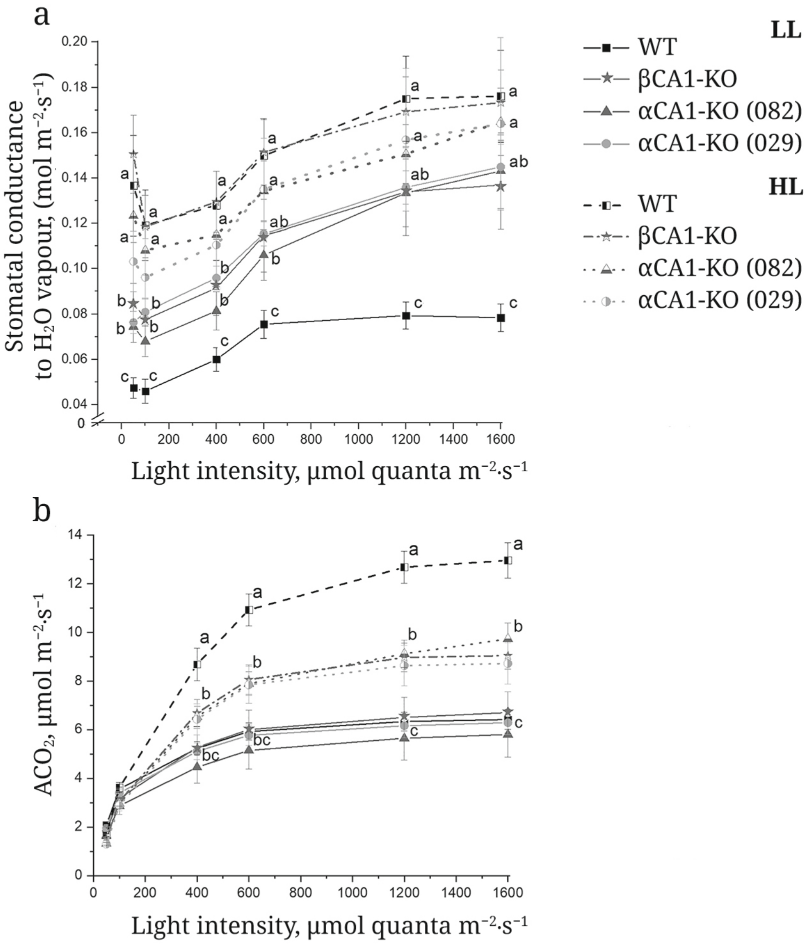

Fig. 3. Dependence of stomatal conductance (a) and CO_2 assimilation rate (A_{CO2}) (b) on the light intensity in the measurement chamber in WT, αCA1-KO, and βCA1-KO plants grown at 50-70 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1} (closed symbols, solid curves) and 400 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1} (semi-closed symbols, dashed curves). The measurements were performed at 400 ppm CO_2. The differences were considered significant at $p \leq 0.05$ as determined by the Holm–Bonferroni method ($n \geq 14$). The figure presents the results of 6-7 biological replicates from four independent experiments.

The effect of the lack of stromal CAs on the gas exchange parameters and starch metabolism. The assessment of gas exchange parameters in WT and mutant plants at a discretely increasing light intensity (from 50 to 1600 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1}) has shown that stomatal conductance in the leaves of both αCA1-KO and βCA1-KO mutants grown under LL was higher than in WT plants grown at any light intensity (Fig. 3a). No such differences were observed for the HL-adapted plants. The A_{CO2} values in the plants grown under LL were the same in WT and mutant specimens (Fig. 3b). In the HL-adapted WT plants, A_{CO2} was found to be significantly higher than in WT plants grown under LL already at 400 μmol quanta$\cdot$m$^{-2}\cdot$s^{-1}, whereas in HL-adapted αCA1-KO and βCA1-KO mutants, the increases in A_{CO2} and stomatal conductance were much less pronounced than in WT plants (Fig. 3, a and b). Therefore, both parameters in the HL-exposed αCA1-KO and βCA1-KO plants were lower compared to WT plants.

In αCA1-KO and βCA1-KO mutants exposed to LL, the content of starch (reserve polysaccharide and one of the main products of photosynthesis) determined at 57 days of age was 25-30% lower than in WT plants of the same age (Table 2). At the age of 64 days, the content of starch was ~60% lower in αCA1-KO plants and 85% lower in βCA1-KO compared to WT plants, i.e., the lack of any of these stromal CAs significantly reduced the formation of starch under LL. After three weeks of adaptation to HL, the content of starch in the leaves of WT plants increased. A much more significant elevation of the starch content was found in αCA1-KO and βCA1-KO plants compared to the plants grown under LL (Table 2). At the same time, the content of starch in the leaves of αCA1-KO and βCA1-KO mutants was 1.5-2.5 times higher than in WT plants, despite a lower A_{CO2} value (Fig. 3b). However, after another week of exposure to HL, the starch content in the mutant plants was only insignificantly higher than in WT plants (Table 2).

Table 2. The effects of light intensity on the starch content in the leaves of WT, αCA1-KO, and βCA1-KO plants grown at 50-70 μmol quanta·m^{-2}·s^{-1} (LL) and 400 μmol quanta·m^{-2}·s^{-1} (HL)

Light intensity, μmol quanta·m^{-2}·s^{-1}	Plant	Starch content in leaves, mg/g wet weight	
		Age	
		57 days	64 days
50-70 (LL)	WT	1.54 ± 0.05 (100%)	1.28 ± 0.81 (100%)
	αCA1-KO	1.31 ± 0.21 (85%)	0.47 ± 0.30 (37%)
	βCA1-KO	1.10 ± 0.25 (71%)	0.18 ± 0.04 (15%)
400 (HL)	WT	2.70 ± 0.21 (100%)	24.23 ± 1.98 (100%)
	αCA1-KO	6.21 ± 0.10 (230%)	31.06 ± 1.20 (128%)
	βCA1-KO	4.39 ± 0.43 (163%)	26.24 ± 2.41 (108%)

Note. The table shows representative results of one experiment ($n \geq 4$). The content of starch in HL-adapted plants was determined after three (57-day old plants) and four (64-day old plants) weeks of adaptation. The value for WT plants under the corresponding growing conditions was taken as 100%. Similar results were obtained in six growing of plants.

Assessment of pmf components in unseparated leaves of WT, αCA1-KO, and βCA1-KO plants grown under LL was carried out at the increasing light intensity. Already at 364 μmol quanta·m^{-2}·s^{-1}, the pmf value was higher in βCA1-KO plants and lower in αCA1-KO plants compared to WT plants (Fig. 4a) due to the corresponding changes in ΔpH (Fig. 4b). At the light intensities below 904 μmol quanta·m^{-2}·s^{-1}, ΔΨ did not differ significantly between the mutant and WT plants. At 904 μmol quanta·m^{-2}·s^{-1}, ΔΨ was higher in αCA1-KO mutants than in WT plants (Fig. 4c), which allowed to partially compensate for the differences in the pmf value under these conditions.

Photosynthetic parameters of WT plants and mutant plants deficient by stromal CAs. Despite a pronounced effect caused by the knockout of genes coding for stromal CAs on the CO_2 assimilation (Fig. 3b) and starch synthesis (Table 2), there were no differences between the ETR values in the leaves of WT and mutant plants grown under LL and HL (data not shown). However, when the illumination of plants grown under the LL conditions was increased to 400 μmol quanta·m^{-2}·s^{-1} and the CO_2 level in the measurement chamber was decreased from 400 to 150 ppm, the ETR in αCA1-KO plants was lower and the ETR in βCA1-KO plants was higher compared to WT plants (Fig. 5a). It was also shown that under these conditions, the value of 1-qL parameter characterizing the proportion of closed PSII reaction centers and relative reduction in the plastoquinone pool was higher in αCA1-KO mutants and lower in βCA1-KO mutants compared to WT plants (Fig. 5b). The differences between WT and βCA1-KO plants persisted even when the CO_2 concentration in the measurement chamber was increased to 1200 ppm (saturating concentration), while the differences between the WT and αCA1-KO plants disappeared.

The rate of CO_2 assimilation measured at 400 ppm CO_2 under the same conditions (after the dark adaptation of plants) was lower in αCA1-KO and βCA1-KO

Table 3. The effect of light intensity on the H_2O_2 content in the leaves of WT, αCA1-KO (line 082), and βCA1-KO plants grown at 50-70 μmol quanta·m^{-2}·s^{-1} (LL) and 400 μmol quanta·m^{-2}·s^{-1} (HL) after 3 weeks of acclimation to HL

Light intensity, μmol quanta·m^{-2}·s^{-1}	Plant	H_2O_2 content, μmol H_2O_2/g wet weight
50-70 (LL)	WT	0.94 ± 0.06
	αCA1-KO	1.29 ± 0.09**
	βCA1-KO	1.60 ± 0.10***
400 (HL)	WT	1.0 ± 0.10
	αCA1-KO	0.32 ± 0.01***
	βCA1-KO	0.26 ± 0.01***

Note. The table shows the data of a representative experiment; *** $p \leq 0.001$ and ** $p \leq 0.01$, significant difference according to the Holm–Bonferroni test. In each case, the mutants were compared to WT plants grown under the same experimental conditions. Similar results were obtained in 3-4 biological replicates from three growing of plants.

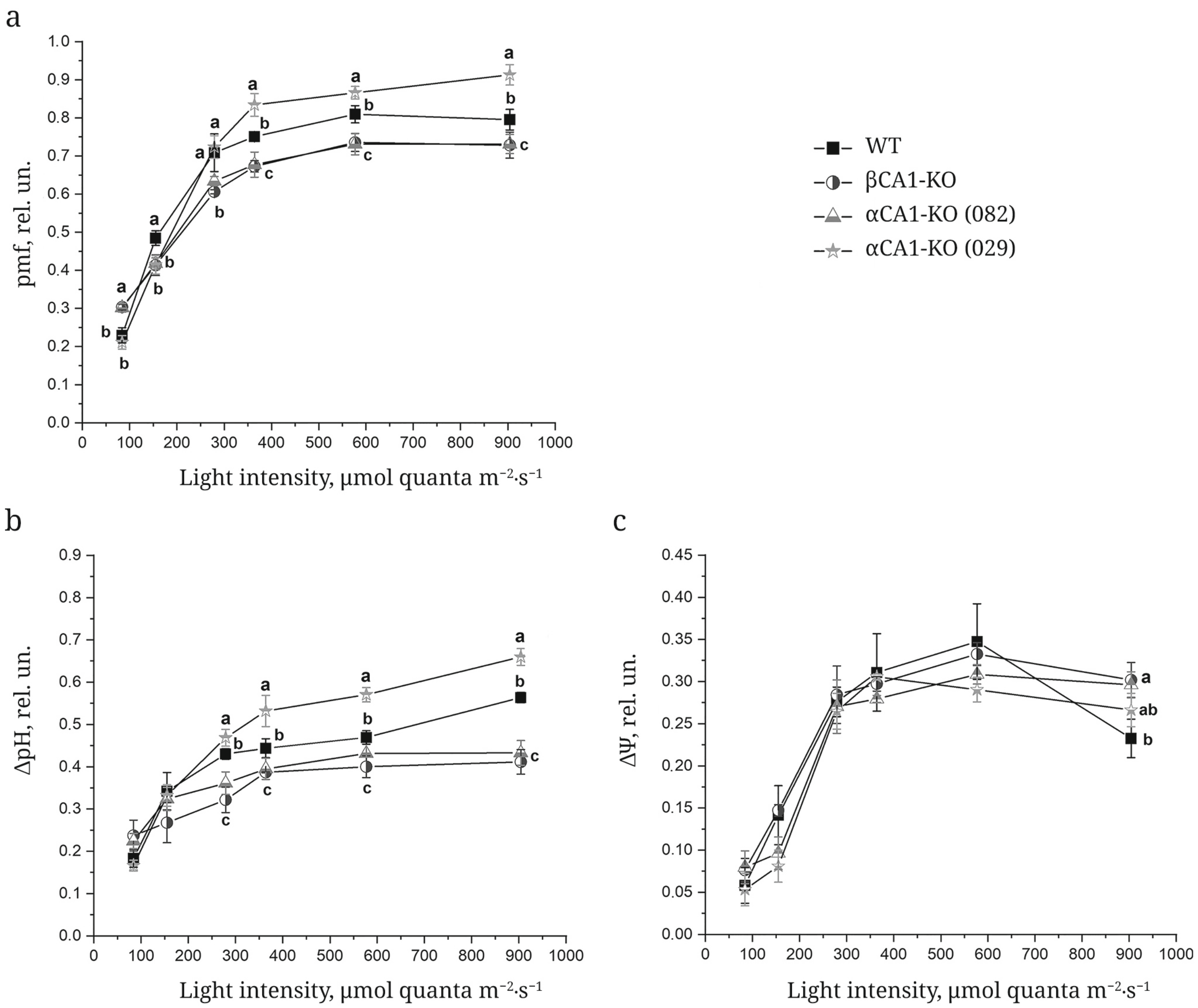

Fig. 4. The effects of the lack of αCA1 and βCA1 on the pmf (a), ΔpH (b), and ΔΨ (c) on the thylakoid membrane in the leaves of *A. thaliana* plants grown under LL. The differences were considered significant at $p \leq 0.05$ as determined by the Holm–Bonferroni method ($n \geq 14$). The results were obtained in 7-8 biological replicates in four independent growing.

mutants than in WT plants (Fig. 5c); this difference was even more pronounced at 1200 ppm CO_2 in the measurement chamber. The Cc value determined at 400 ppm CO_2 (atmospheric concentration of carbon dioxide) as two times lower in βCA1-KO mutants than in WT and αCA1-KO plants (Fig. 5d).

The effects of illumination on the hydrogen peroxide concentration and expression of stress markers in the leaves of WT and mutant plants. High light intensity, salinization, drought, temperature changes, and other stress factors promote generation of reactive oxygen species (ROS) [48, 49]. In response to stress, plants trigger adaptation mechanisms including the so-called ROS wave, i.e., the propagation of stress signal activating multiple physiological, molecular, and metabolic responses necessary for the plant acclimation to stress [49]. The intensity of the ROS wave can be increased by various stress hormones, such as SA, JA, abscisic acid (ABA), and others, that can affect expression of specific stress response genes [50]. In our experiments, the levels of H_2O_2 in αCA1-KO and βCA1-KO mutants under LL were 40-60% higher than in WT plants (Table 3). The acclimation to HL for 21 days caused no changes in the H_2O_2 content in WT plants, while the H_2O_2 concentration in αCA1-KO and βCA1-KO mutants decreased 4 and 6 times, respectively, compared to the LL conditions.

The expression levels of the ABA-induced genes *COR414-TM1* (*At1g29395*) and *ANAC019* (*At1g52890*), the SA-induced gene *At1g64280* (*NPR1*), and the JA-induced gene *At1g19180* (*JAZ1*; JA pathway repressor) were determined after 21 days of exposure to HL [50, 51]. Under LL, expression of all four stress genes in αCA1-KO and βCA1-KO mutant plants

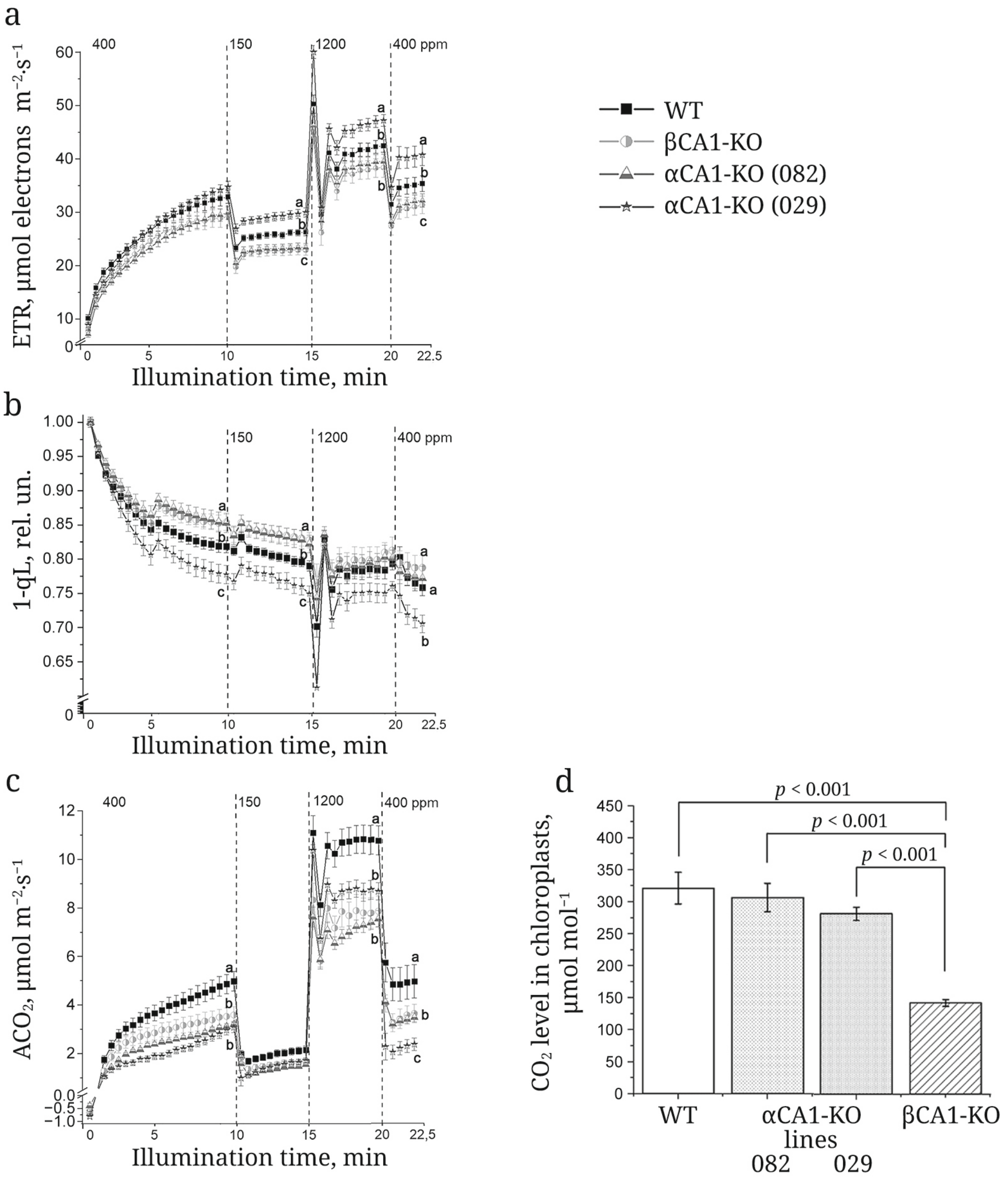

Fig. 5. The effects of the lack of αCA1 and βCA1 on the ETR (a), proportion of closed PSII reaction centers (1-qL) (b), A_{CO2} (c), and CO_2 level in chloroplasts (Cc) (d) in WT, αCA1-KO, and βCA1-KO plants grown at 50-70 µmol quanta·m^{-2}·s^{-1}. The Cc values are shown for the stationary conditions (400 ppm CO_2). The differences were determined the Holm–Bonferroni method separately for each region of the curve corresponding to particular CO_2 concentrations and were considered significant $p < 0.05$ ($n \geq 14$). The results were obtained in 6-7 biological replicates in four independent cultivations are presented. The measurements were performed at 400 µmol quanta·m^{-2}·s^{-1}.

was 3-5 times higher than in WT plants (Fig. 6), except for *NPR1*, whose expression was 12 times higher in αCA1-KO mutants than in WT plants (Fig. 6b). After acclimation to HL, the expression of *At1g52890* and *JAZ1* increased 3-fold (Fig. 6, a and c) and expression of *NPR1* increased 17-fold in WT plants (Fig. 6b). On the contrary, in αCA1-KO and in βCA1-KO mutants exposed to HL, the expression levels of all studied stress marker genes decreased 3.5-10 times and, accordingly, were found to be lower than in WT plants. Hence, the changes in the expression levels of genes induced by stress hormones in WT plants and mutants deficient by stromal CAs corresponded to the relative changes in the hydrogen peroxide level in these plants both under LL and HL.

DISCUSSION

The assumptions on the physiological role of CAs in the cells of higher C3 plants mostly consider their potential involvement in C_{inorg} transport to chloroplasts

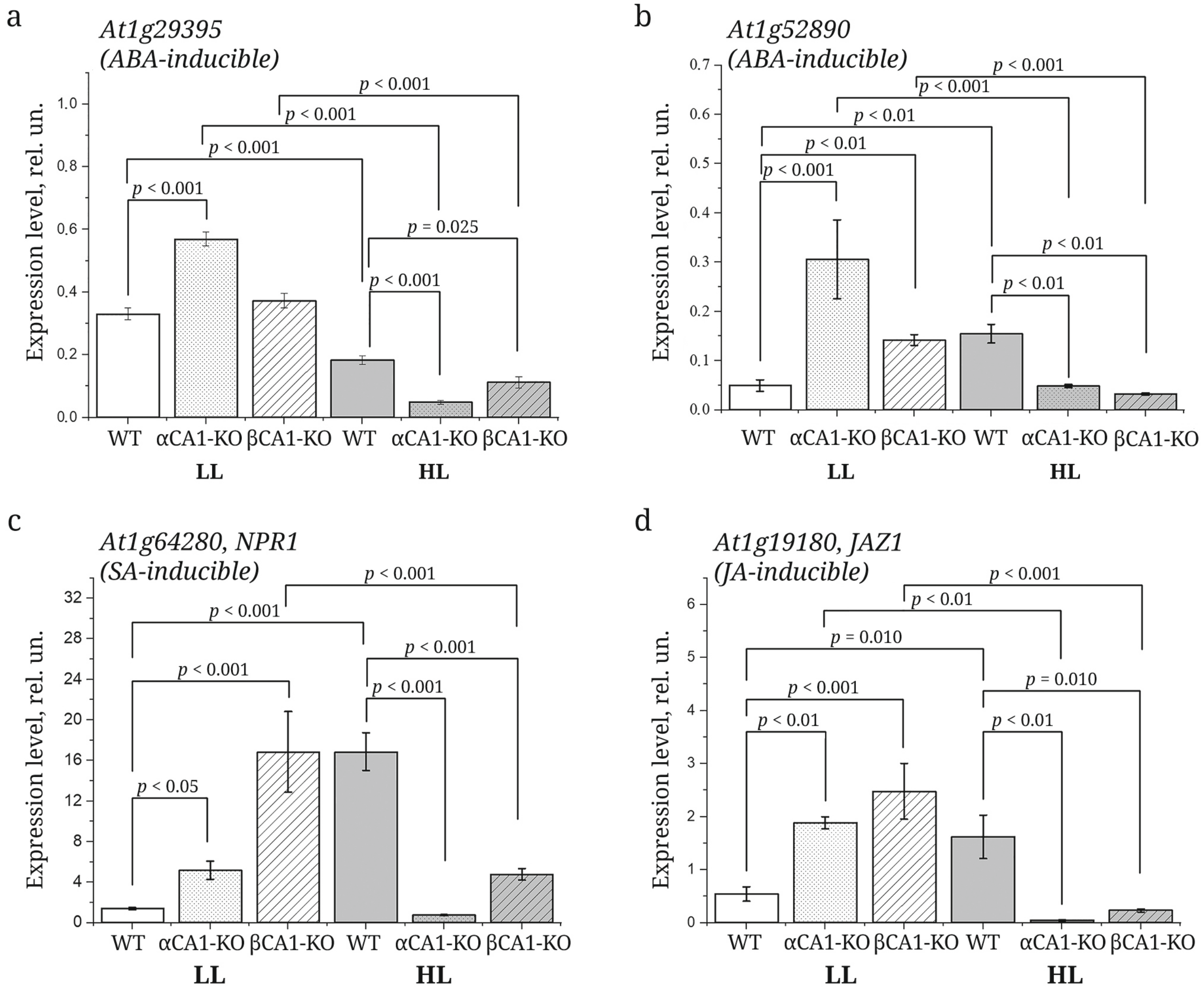

Fig. 6. Expression levels of genes induced by phytohormones in the leaves of *Arabidopsis* plants grown at LL (50-70 μmol quanta·m^{-2}·s^{-1}; white columns) and plants of the same age after 14-21 days of acclimation to HL (400 μmol quanta·m^{-2}·s^{-1}; gray columns). a) *At1g29395* (*COR414-TM1*) and b) *At1g52890* (*ANAC019*) induced by ABA; c) *At1g64280* (*NPR1*) induced by SA; d) *At1g19180* (*JAZ1*) induced by JA. The data were normalized to the actin gene expression. Significant differences were determined by the Holm–Bonferroni method, $n \geq 9$. Similar results were obtained in three growing of plants in 3 biological and 3 analytical replicates.

or the necessity of HCO_3^- conversion into CO_2 (direct substrate for RuBisCO) [3-5]. At the same time, it was found that RuBisCO and stromal CAs are functionally related and colocalize in the chloroplasts of different plant species [52, 53]. However, the attempts to study the effects of single mutations in CAs [22, 24] or simultaneous shutdown of two and more CAs [12, 26] have not led to the development of generally accepted concepts on the functions of stromal CAs.

The knockout of both αCA1 and βCA1 resulted in a 5-fold decrease in the CA activity of stromal preparations (Fig. 1), which confirmed the presence of both enzymes in the stroma of chloroplasts. The effects of mutations in αCA1 and βCA1 were more pronounced when the plants were exposed to a higher light intensity compared to the conditions under which they had been grown. When exposed to HL, αCA1-KO, and βCA1-KO plants grown under LL demonstrated a lower A_{CO2} value at 400 ppm CO_2 (compared to WT plants) and even a more pronounced difference at 1200 ppm CO_2 (Fig. 5c) after exposure in the dark, followed by turning on the light, i.e., upon activation of the CBB cycle. At the same time, the Cc value in βCA1-KO mutants was two times lower than in WT and αCA1-KO plants (Fig. 5d), suggesting that the decrease in the A_{CO2} in βCA1-KO mutants (Fig. 3b and 5c) was due to the insufficient rate of C_{inorg} inflow into chloroplasts, while in αCA1-KO plants that had the same Cc value as WT plants (Fig. 5d), the observed reduction in A_{CO2} (compared to WT plants) was due to the insufficient intensity of stromal HCO_3^- conversion to CO_2 for providing the CBB cycle. This was also evidenced by the differences between the ΔpH values in these mutants: in βCA1-KO plants, ΔpH was higher than in WT plants (Fig. 4), which was apparently determined by a higher (compared to WT) pH value of the stroma in the case of insufficient CO_2 inflow. Under normal conditions, CO_2 entering a chloroplast is converted to HCO_3^- with the release of H^+, which shifts stromal pH to a more acidic region. In the βCA1-deficient mutants, the inflow of CO_2 can be impaired and, accordingly, the generation of H^+ in the stroma is reduced, leading to the pH increase. At the same time, the absence of CA supplying CO_2 for RuBisCO through its conversion from bicarbonate should lead to a slight decrease in stromal pH and, therefore, to a decrease in ΔpH on the thylakoid membrane. This was observed in αCA1-KO plants, in which ΔpH was lower than in WT plants and, accordingly, even lower than in βCA1-KO plants (Fig. 4).

The convergence of A_{CO2} values in βCA1-KO and WT plants at 1200 ppm CO_2 (Fig. 5c), compared to the respective values at 400 ppm, can be explained with regard to the presumptive function of βCA1 (see above): if βCA1 is responsible for the CO_2 inflow into chloroplasts, then the increased CO_2 concentration in the air can partially compensate for its absence.

In αCA1-KO plants, the ETR was lower than in WT and βCA1-KO plants at all CO_2 concentrations (Fig. 5a), while the A_{CO2} value at 400 ppm CO_2 was lower than in WT plants but higher than in βCA1-KO mutants (Fig. 5c). The increase in the CO_2 concentration to 1200 ppm caused no convergence of A_{CO2} values for αCA1-KO and WT plants, but rather "distanced" them due to the reduction of this parameter in αCA1-KO plants. The latter fact can also be explained by the hypothetical function of αCA1 (see above): if αCA1 catalyzes bicarbonate dehydration coupled with supplying CO_2 directly to RuBisCO, then its absence at a high CO_2 concentration in the air, which increases the level of bicarbonate in the stroma, is capable even to limit even to a greater extent (compared to its absence at lower levels of CO_2 and, accordingly, bicarbonate) this supply compared to WT plants.

No differences between A_{CO2} values in the leaves of mutant and WT plants grown under LL were observed under the stationary photosynthesis conditions (Fig. 3b), probably because the CA mutants lacking stromal CAs compensated for the insufficient supply of chloroplasts with C_{inorg} via enhanced stomatal conductance (Fig. 3a). An increase (compared to WT plants) in the stomatal conductance of mutants deficient by both αCA1 and βCA1 has been shown previously in rice and tobacco plants [13, 25]. For some reason, plants grown under HL were unable to maintain such compensatory mechanism (Fig. 3a), which might be due to the fact that in αCA1-KO and βCA1-KO mutants, expression of stress-related genes induced by ABA (Fig. 6, a and b), the known regulator of stomatal closure, was reduced, possible because of the decreased ABA content in mutant plants.

A convincing evidence of the involvement of stromal CAs in the mechanisms of C_{inorg} supply for photosynthetic processes has been obtained in the experiments on the knockout of both the *αCA1* gene [13] (see "Introduction") and gene encoding stromal βCA in rice plants [25]. The abolishment of βCA synthesis led to a decrease in the plant biomass and net photosynthesis rate, which was compensated by the increased stomatal conductance, stomatal pore opening ratio, water loss rate, and RuBisCO activity [25]. The lack of pronounced effects of single mutations in CAs in dicotyledonous plants might be due to the cooperative function of CAs in the cells of these plants.

Physiological manifestations of CA knockouts in *Arabidopsis* and tobacco plants were observed only when the synthesis of two or more CAs was simultaneously shut down. Thus, the knockout of βCA1 and plasma membrane βCA4.1 has shown that these enzymes are jointly involved in the regulation of stomatal conductance for carbon dioxide [24]. Suppression of plant growth at low CO_2 levels was observed upon the simultaneous shutdown of at least two out of five genes coding for mitochondrial CAs [54], as well as two genes for cytoplasmic CAs in *Δβ-CA4.2/β-CA2* mutants [26]. The absence of pronounced effect of the abolishment of stromal CAs under typical growth conditions (such effect was observed only upon dramatic changes in illumination and CO_2 level in the air; Figs. 3-5) can also be explained by the cooperative function of CAs in plant cells.

Both expression of CA genes and CA activity varied depending on the growth conditions, thus demonstrating different needs of plants for CAs under different external conditions. Growth of plants at a low carbon dioxide level (150 ppm) led to a significant increase in the CA activity in the stromal and thylakoid preparations isolated from the leaves of WT plants compared to the plants grown at the normal (450 ppm) or elevated (1200 ppm) CO_2 concentrations [35]. In rice seedlings, expression of gene encoding CAs of the β-family, as well as the total CA activity in leaf extracts, increased after exposure to the osmotic stress [55]. Previously, we have shown that exposure of plants to HL upregulated expression of most genes coding for chloroplast CAs, with the exception of the *βCA5* gene [34]. Here, we confirmed this effect in WT plants (Fig. 2) by demonstrating that in the HL-exposed βCA1-KO mutants, the changes in the content of transcripts were the same as in WT plants, while in αCA1-KO mutants, these changes were the opposite, i.e., the amount of *βCA1.1* + *βCA1.2*, *βCA1.3* + *βCA1.4*, and *αCA2* transcripts decreased, the content of the *βCA5* transcript increased, while the content of the *αCA4* transcript remained the same (Fig. 2). The levels of *βCA1.1* + *βCA1.2*, *βCA1.3* + *βCA1* and *αCA2* transcripts in the mutants grown under LL was initially higher than in WT plants. The only gene with a higher expression level was *αCA4* in βCA1-KO plants. Hence, the knockout of αCA1 and βCA1 influenced the synthesis of other CAs in chloroplasts and this effect depended on the light intensity at which the plants were grown. The changes in the expression of some CA genes with the knockout of genes coding for other CAs have also been shown by Sharma et al. [14], Nadeeva et al. [21], and Rudenko et al. [56].

The effects of the αCA1 and βCA1 abolishment are either directly caused by consequences of the absence of these enzymes in chloroplasts (e.g., changes in ΔpH, decrease in A_{CO2}, reduction of Cc in chloroplasts) or are associated with the compensation for their absence (changes in the expression of other CA genes and increase in the stomatal conductance of leaves). Apparently, these compensatory mechanisms enable plants to successfully compensate for the lack of stromal αCA1 and βCA1. In αCA1-KO plants, the ETR was only 10% lower and the proportion of closed reaction centers of PSII was approximately 10% higher than in WT plants (Fig. 5, a and b). In βCA1-KO plants, the ETR was higher and, accordingly, the value of 1-qL was lower than in WT plants.

Despite the presence of mechanisms compensating for the absence of αCA1 and βCA1, the content of starch, the main structural carbohydrate and product of photosynthesis, in the mutants deficient by these enzymes was lower than in WT plants when the plants grown under LL conditions (Table 2): by 20-30% at the age of less than 2 months it and by 60 and 85% in αCA1-KO and βCA1-KO plants, respectively, that were older than 2 months. After 3 weeks of adaptation to HL, the starch content, on the contrary, was 1.6-2.3 times higher in αCA1-KO and βCA1-KO plants than in WT plants grown under the same conditions. These differences could be caused by the reduced starch degradation resulting from the increased ABS content, which was evidenced by the downregulated expression of ABA-inducible genes under HL conditions (Fig. 6, a and b). ABA promotes starch degradation [57, 58], which enhances stress resistance of plants. After one more week under HL, the starch content increased approximately 9-fold in WT plants vs. 5-6-fold in the mutants (Table 2), so it became almost the same in αCA1-KO, βCA1-KO, and WT plants.

The number of chloroplasts in a cell increases under HL [59] while the size of the PSII light-harvesting antenna PSII decreases, which is accompanied by the reduction in the Chl content and changes in the composition and amount of carotenoids. After adaptation to HL, the content of both Chl forms decreased in WT and βCA1-KO plants originally grown under LL, i.e., these plants showed similar changes in the content of pigments during adaptation to HL (Table 1). In αCA1-KO plants, the level of Chl *a* slightly increased (by 7%). As a result, there was a similar 20-25% increase in the Chl *a*/Chl *b* ratio; however, in αCA1-KO plants, it was due to the elevation in the Chl *a* content, while in WT and βCA1-KO plants, it was caused by the decrease in the Chl *b* amount. The content of carotenoids decreased in WT plants and both mutant lines, but still remained the highest in αCA1-KO plants. Thus, in the absence of αCA1 and with initially lower levels of pigments under LL, αCA1-KO plants demonstrated the ability to maintain a higher content of pigments under HL compared to WT and βCA1-KO plants.

There is a growing body of data on the involvement of βCA1 in stress signaling [30, 33] due to its ability bind both SA [29], as well as NPR1 and NRB4, the key proteins of the SA-mediated stress defense pathway [30]. In addition, a convincing evidence has been obtained that βCA1 participates in plant protection against stress as an enzyme of JA biosynthesis (see “Introduction”). The knockout of the *βCA1* and *αCA1* genes resulted in similar changes in the H_2O_2 content and expression levels of ABA-, SA-, and JA-inducible stress marker genes. In the mutant plants, the above parameters were higher under LL and lower under HL compared to WT plants (Table 3; Fig. 6), suggesting the involvement of not only βCA, but αCA1 as well. One of the indications of insufficient synthesis of JA under LL in the absence of stromal CAs is higher (compared to WT) expression levels of the *JAZ1* gene (Fig. 6d) coding for the JA pathway inhibitor, *NPR1* gene (Fig. 6c) encoding a major inducer of the SA pathway, and marker genes involved in the induction of the ABA pathway (Fig. 6, a and b). The action of these genesis antagonistic to the JA pathway; they are activated when this pathway is suppressed [60]. In addition, SA promotes the ROS wave, whereas JA suppresses it [49], which can explain a higher level of H_2O_2 in the mutants deficient by the stromal Cas under LL compared to WT plants (Table 3). After adaptation of plants to HL, as well as upon exposure to other stress factors, plants activate generation of ROS [49, 50], including H_2O_2, a molecule that induces changes in the expression of many genes. The increase in the H_2O_2 levels was observed only at the very beginning of adaptation to HL; even a few days later, the H_2O_2 concentration decreased to the level observed under LL conditions [61]. This explains the same content of H_2O_2 in WT plants exposed to LL and HL (Table 3). At the same time, the expression levels of stress marker genes in WT plants under HL were several times higher than under LL (Fig. 6). In αCA1-KO and βCA1-KO mutants, the expression of these genes under HL proved to be lower than in WT plants, probably due to the higher activation of antioxidant systems under normal conditions and greater "readiness" to stress, which might explain the absence of need for further enhancement of the antioxidant response to HL in these plants.

Abbreviations. αCA1-KO, homozygous plants with knocked-out *At3g52720* gene encoding αCA1; βCA1-KO, homozygous plants with knocked-out *At3g01500* gene encoding βCA1; ABA, abscisic acid; A_{CO2}, CO_2 assimilation rate; CA, carbonic anhydrase; Cc, CO_2 level in chloroplasts; Chl, chlorophyll; C_{inorg}, inorganic carbon; ECS, electrochromic shift of the absorption band of carotenoids in the thylakoid membrane; ETR, electron transport rate; HL, high light; JA, jasmonic acid; LL, low light; RuBisCO, ribulose bisphosphate carboxylase/oxygenase; pmf, proton motive force; PSI and PSII, photosystem I and II; ROS, reactive oxygen species; SA, salicylic acid; WT, wild type.

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