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Beta-carbonic anhydrase gene expression levels change depending on the drought severity in both the leaves and roots of *Arabidopsis thaliana*

Sertan ÇEVİK[1*], Oğuzhan KURT[2], Ayşin GÜZEL DEĞER[3], Ferit KOCAÇINAR[4], Rıza BİNZET[2]

[1]Department of Molecular Biology and Genetic, Faculty of Science and Letters, Harran University, Şanlıurfa, Turkiye
[2]Department of Biology, Faculty of Science, Mersin University, Mersin, Turkiye
[3]Department of Food Processing, Vocational School of Technical Sciences, Mersin University, Mersin, Turkiye
[4]Department of Forest Engineering, Faculty of Forestry, Kahramanmaraş Sütçü İmam University, Kahramanmaraş, Turkiye

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Abstract: In this study, it was aimed to determine the relationship between β carbonic anhydrase (βCA) gene expression levels and drought stress severity. For this purpose, the expression levels of six βCA genes in the roots and leaves of *Arabidopsis thaliana* were investigated under mild, moderate, and severe drought conditions. In addition, changes in the biomass, leaf water content, photosynthetic efficiency, CA enzyme activity, free proline content, and lipid peroxidation were also determined. The results showed that all of the β CA gene expression levels and CA enzyme activity increased in the leaf under mild drought conditions; however, the expression levels (except for βCA-6) and enzyme activity decreased as the severity of drought increased. In the roots, the gene expression levels were very low compared to the leaves and all of the βCA gene expressions decreased under drought stress. Moreover, depending on the severity of the drought stress, the biomass, leaf water content, and photosynthetic efficiency decreased, while the content of proline and lipid peroxidation increased. A positive correlation was determined between the photosynthetic efficiency and high βCA expression level. The results indicated that βCA genes may play important roles, especially in the onset of drought stress in *A. thaliana*. When the findings were evaluated together, βCA genes may be considered to be important candidate genes for increasing or maintaining photosynthetic yield of C_3 plants under drought stress conditions.

Key words: Beta-carbonic anhydrase, Gene expression, Drought, *Arabidopsis thaliana*

1. Introduction

The effects of drought stress in plants have been studied extensively for a long time using morphological, physiological, biochemical, and molecular methods. Various genes have been determined by these studies and affiliated with increasing or at least maintaining plant productivity under stress conditions (Zhou et al., 2016). Recently, transcriptomic studies have frequently been used to identify specific genes involved in providing resistance to drought stress (Iquebal et al., 2019).

Carbonic anhydrase (CA), a metalloenzyme, catalyzes the conversion reaction of diffused carbon dioxide (CO_2) into bicarbonate (Khalifah, 1971) and its concentration varies between 1% and 20% in the leaves (Wieczorek and Jelonek, 2017). CA proteins have been detected in the chloroplast, cytosol, plasma membrane, and mitochondria (Ignatova et al. 2019). There are a total of 19 genes belonging to three CA gene families, namely alpha (α), beta (β), and gamma (γ), in *Arabidopsis thaliana* (Rudenko et al., 2017). In an examination of the expression profiles of these CA gene families in *A. thaliana* leaves, it was determined that the expression rate of βCA genes was several times higher than that of αCA, and no γCA was expressed (Ignatova et al., 2019). These results indicate that βCAs are the most active CA group in *A. thaliana* leaves.

Early studies on the role of CAs in C_3 plants showed that these proteins did not play important roles in the C_3 photosynthetic mechanism (Majeau et al., 1994; Price et al., 1994; Williams et al., 1996). However, later studies have provided some evidence on the fact that there may be a strong relationship between CA activities and the C_3 photosynthesis mechanism (Studer et al., 2014; DiMario et al., 2016; Osborn et al., 2017). These contradictions may have been related to the inadequacies of the methods used and the presence of stress. Many studies have shown that CA activity or gene expression levels do not change significantly without a stress factor; however, in the case of stress, the amount of CA protein and gene expression

* Correspondence: srtncvk@gmail.com

levels change significantly in C_3 plants (Kravchik and Bernstein, 2013; Perez-Martin, 2014; Sun et al., 2016, Çevik et al., 2019).

In recent years, many studies have been carried out to investigate the roles of CA enzyme in C_3 plants. Strong clues have been obtained that this enzyme may play important roles under stress conditions (Rudenko et al., 2021). When different studies on this subject were examined, many conflicting results were found on both the activities and expression levels of CAs under various conditions in C_3 species (Majeau et al., 1994; Price et al., 1994; Badger and Pfanz, 1995; Williams et al., 1996; Studer et al., 2014; DiMario et al., 2016; Osborn et al., 2017). Moreover, most of the studies have focused on the role of CAs in the photosynthetic mechanism of C_4 plants and this mechanism was elucidated in detail (Ludwig, 2012). Until recent years, CAs were thought to only have active roles in C_4, but not in C_3 photosynthetic mechanism, but studies have shown that CAs are abundant in all plant leaves, regardless of the photosynthetic pathway and their activity or expression status changes under various conditions. These findings suggest that CAs may also play significant roles in C_3 plants (Studer et al., 2014; DiMario et al., 2016; Osborn et al., 2017).

Understanding the roles of βCAs in C_3 plants is still unclear. Most of the studies conducted, especially those using CA mutants, usually yielded little information about the roles of βCAs in C_3 plants, because most of these studies were conducted under nonstressed conditions, which made it difficult to understand the roles of CAs under stress. Therefore, the main aim of this study was to investigate the changes of βCA gene expression levels in the roots and leaves of *A. thaliana* under mild, moderate, and severe drought conditions. These changes were also supported by comprehensive morphological, physiological, and biochemical analyses. In addition, photosynthetic parameters were also analyzed and compared with βCA gene expression profiles. Thus, the possible correlation between βCA and photosynthetic efficiency under drought conditions was also investigated.

2. Materials and methods

2.1. Plant material and drought treatment

A. thaliana (Ecotip Columbia) seeds were obtained from the Department of Agricultural Biotechnology, Akdeniz University, Türkiye. The plants were grown in a climate chamber in 7.5-cm^2 pots at a 22/16 °C day/night temperature, 16/8 h day/night photoperiod, 55% humidity, and 120 µmol photons m^{-2} s^{-1} light intensity conditions (Clauw et al., 2015). A mixture of soil, perlite, and vermiculite (2:1:1) was used as a growth medium after sterilization in an autoclave (Yao et al., 2018). After planting one plant in each pot, the pots were watered well and covered with parafilm, and kept in the refrigerator at 4 °C for 2 days before being taken to the climate room (Harb et al., 2010; Su et al., 2013). The plants were irrigated with water in equal volumes every 2 days (Boyes et al., 2001).

The plants were grown until the 6th rosette leaf was larger than 1 mm (Boyes et al., 2001). At this phase of growth, half of the pots were subjected to drought stress by withholding water and the other half was irrigated every 2 days as a control group. The leaf water potential (LWP) of three randomly selected plants was measured at the same time every morning, three days after the initiation of drought stress. According to the LWP data, the plants with about –0.4 MPa were considered as the control group, the plants with approximately –0.8 MPa, –1.2 MPa, and –1.6 MPa were regarded as the mild, moderate, and severe drought stress groups, respectively. These LWP values were also compatible with the literature (Haswell and Verslues, 2015; Osmolovskaya et al., 2018). After biomass, plant-water status, and morphological analyses, the leaves and roots were collected, immediately frozen in liquid nitrogen, and stored at –80 °C for biochemical and gene expression analyses.

2.2. Biomass analysis

In order to determine the drought stress effects on plant biomass, 5 plants were randomly chosen from each group. Their roots were washed with tap water and excess water was carefully removed with a paper towel before the fresh weight (FW) measurements were performed. After measuring the root and shoot FWs, the samples were kept in an oven at 70 °C for 48 h for dry weight (DW) determination (Morel et al., 2010).

2.3. Water content analysis

LWP and leaf relative water content (RWC) analyses were performed to determine the changes in the water status of *A. thaliana* under mild, moderate, and severe drought conditions. Five plants from each group were used for these analyses. The LWP was measured on excised leaf samples using a Scholander type pressure chamber (PMS Instrument Company Model 1000, Oregon, USA) (Scoffoni et al., 2018). The RWC of the plants in each group, FW, turgid weight (TW), and DW of the leaves were determined and used to calculate the RWC, as RWC = [(FW-DW) / (TW-DW)] × 100 (Bouchabke et al., 2008).

2.4. Leaf gas-exchange and water use efficiency (WUE) analyses

Photosynthesis, transpiration, intercellular CO_2 concentration (Ci), stomatal conductance (Gs), quantum yield of dark-adapted leaves (variable fluorescence/maximum fluorescence (Fv/Fm), quantum yield of illuminated leaves during gas exchange measurements (Yield), and plant WUE were measured on six different plants from the control and drought groups using a

portable GFS-3000 photosynthesis-fluorescence system (Walz, Effeltrich, Germany). The WUE was calculated as the assimilation rate divided by the transpiration rate. The measurements were conducted during the day between 08:00 and 16:00 h assuming active transpiration. During the measurements, the light intensity was set as 250 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR), 55% cuvette relative humidity (vapor pressure deficit (VPD) ~ 15 Pa/kPa), 25 °C leaf temperature, and 410 ± 10 ppm ambient CO_2 (Ca). The plants were kept in complete darkness for 30 min before measuring the Fv/Fm, that is, the quantum yield of photosystem II (Akgün et al., 2018).

2.5. Biochemical analysis

2.5.1. Determination of the CA enzyme (EC 4.2.1.1.) activity

In order to determine the CA activity, 0.5 g of leaf tissue was homogenized in an extraction buffer (0.02 M Tris-HCl (pH = 8), 0.01% Triton X-100) and the obtained homogenate was used as a source of CA enzyme after centrifugation. The CA activity was determined by calculating the time it took for the pH of the water saturated with CO_2 to drop from 8.3 to 6.3. The CA activity was calculated as: activity (U) = 2(T0–T) / T (Dąbrowska-Bronk 2016).

2.5.2. Determination of the free proline content

The amount of free proline, which is one of the good indicators of oxidative damage due to the severity of drought, was determined according to the method of Bates et al. (1973). First, 0.5 g of leaf and root tissue were homogenized with 10 mL of 3% sulfosalicylic acid. After that, supernatant, ninhydrin, and acetic acid were mixed well and incubated in a water bath at 95 °C for 1 h. Following incubation, the reaction was terminated by placing the tubes on ice. Next, 4 mL of cold toluene was added to the mixture and the toluene phase was measured in a spectrophotometer at 520 nm and the results were expressed as µmol proline/gram using a standard curve.

2.5.3. Determination of the malondialdehyde (MDA) content

By measuring the MDA content, the extent of membrane damage (lipid peroxidation) due to the severity of drought was determined. For this purpose, 0.2 g of leaf and root tissue were homogenized in 5% trichloroacetic acid (TCA) solution (1 mL) and then centrifuged at 16,000 *g* for 15 min. The supernatant was carefully collected and transferred to a tube with 20% TCA containing equal volumes of 0.5% thiobarbituric acid (TBA). The tubes were incubated at 96 °C for 25 min, then kept in an ice bath for 5 min, and then centrifuged at 12,000 *g* for 5 min. The supernatant was measured in a spectrophotometer at 532 and 600 nm. TCA containing TBA was used as a blank and the MDA content was calculated using the extinction coefficient of 155 mM (Ohkawa et al., 1979).

2.6. Gene expression analysis

2.6.1. RNA isolation

For RNA isolation, 100 mg of the root and leaf tissues were placed into MagNA Lyser Green Beads tubes and 600 µL of Qiagen RNA extraction buffer (Hilden, Germany) was added. Then, isolation was performed according to the Qiagen RNAeasy Plant Mini Kit (Cat: 74904) protocol (Ren et al., 2011).

2.6.2. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

cDNA synthesis was carried out according to the protocol of the cDNA synthesis kit (Thermo Cat no: 4368814) following the manufacturer's instructions. First, the mixture [(10X RT buffer (2 µL), 25X DNTP 100 mM (0.8 µL), 10X RT random primer (2 µL), MultiScribe reverse transcriptase (1 µL), nuclease fee water (4.2 µL)] was prepared and reactions were carried out at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min in a thermal cycler.

The amount of synthesized cDNA was determined using a nanodrop spectrophotometer and the second step following the RT-PCR protocol was performed using the SYBR Green I (Cat no: 4707516; Roche Diagnostics, Basel, Basel-Stadt, Switzerland) master kit. The mixture of cDNAs containing samples of each primer and the contents of the SYBR Green I (Roche Diagnostics) Master Kit was placed on the 96th layer, covered with Roche light cycler sealing foil and centrifuged at 1500 rpm for 2 min. After centrifugation, the samples were run by selecting optimized temperatures for each primer in a Roche 480 II light cycler.

By adding the mixture prepared according to the optimized conditions for each primer, amplification reactions were performed on the Roche 480 II light cycler under the conditions of the PCR program in accordance with the protocol. During the reactions, actin gene was used as a housekeeping gene or as a control (Table 1).

The RT-PCR results were calculated using the 2-ΔΔCT method. According to this method: Control ΔCT = Control CT(c-gen) – Control CT(housekeeping gene)

Application ΔCT = Example CT(c-gen) – Example CT(housekeeping gen) ΔΔCT = [(Sample ΔCT)avg. – (Control ΔCT)mean] Amount of target gene (c-gene) mRNA = 2-ΔΔCT. The mRNA levels were determined according to these given equations.

2.7. Statistical analysis

The experiment was planned according to the randomized plot design with one factor (drought application). Data were collected in at least three replications with three plants in each replication. The homogeneity of the data was checked using the Levene test. The data were statistically tested using JMP package program (JMP, Version 13. SAS

Table 1. Sequences of the forward and reverse primers used in this study. Actin gene (ACT1) was used as a control gene.

Locus	Gene	Forward primer	Reverse primer
AT3G01500.2	(ATβCA1)	ATGACTTCGTCAAGGGTGCT	CTAGTTTCGGAGAGGCCAAA
AT5G14740.1	(AtβCA2)	AGCTTTGGGAGCTCCAGTTT	CGATGGTGATGGTGATGTGT
AT1G23730.1	(AtβCA3)	TGTCCTTGGGGAATCTTTTG	GAGCTCCTCTTATGGCAAGC
AT1G70410.2	(AtβCA4)	CATTCGTGAGAGCTGAGGTG	TCCCAGAGATCAAACGTTCC
AT4G33580.2	(AtβCA5)	CTTGGGTACCCGTGGATAGA	TCCACCATGGAGAGACAGTG
AT1G58180.2	(AtβCA6)	CAATTGTGGAGGAATTGCAG	TCCCATTCATAACCCACCTT
Reference gene			
AT2G37620	ACT1	ATGGCTGATGGTGAAGACATTCAA	TCAGAAGCACTTCCTGTGAACAAT

Institute Inc., Cary, NC, 1989–2021). Mean values of all of the parameters were compared using a one-way analysis of variance followed by the least significance difference (LSD) test ($p < 0.05$).

3. Results

3.1. Biomass

Drought stress decreased both the leaf and root biomass of the *A. thaliana* plants when compared with the control (except for the mild stress treatment) (Figures 1 and 2). Mild drought stress did not cause any reduction in the leaf and root FW or DW. However, the leaf biomass was more affected than the root biomass by the drought treatments. The reduction in biomass for both the leaves and the roots was more pronounced as the severity of the stress increased. Severe drought caused about 50% biomass reduction in both the leaves (Figures 1A and 1B) and the roots (Figures 1C and 1D).

3.2. Plant-water content analysis

The LWP and RWC decreased significantly under every drought stress level in *A. thaliana* (Table 2). The decrease in the LWP and RWC increased depending on the drought severity; thus, the greatest decrease was experienced under severe drought conditions (Table 2 and Figure 2). Severe drought treatment caused a significant decrease in the LWP (–1.6 MPa) and approximately 50% reduction in the RWC (Table 2).

3.3. Leaf gas–exchange and WUE

The effects of the drought stress treatments on the photosynthetic parameters in *A. thaliana* are presented in Table 3. The net photosynthetic rates were the same between the control and mild stress treatment. However, where moderate stress caused a 20% reduction in the net assimilation rate compared with the control (3.64 versus 4.6, respectively), severe drought stress caused approximately 50% reduction (Table 3). There was a slight decrease in the Ci under mild stress (~8%) and under moderate and severe stress (~15%) compared with the control. Although mild stress did not affect the assimilation rate, it reduced the transpiration and Gs by about 20%, and therefore, increased the WUE by 27%. Moderate stress (LWP: –1.25 MPa) significantly reduced the transpiration rate and Gs by 46% and 57%, respectively (Table 3). Severe drought stress caused the highest reduction in both the transpiration rate and Gs. There was an approximately 70% decline in both the transpiration and Gs under severe drought stress. Even though statistically not significant compared with the control, both moderate and severe drought stress conditions resulted in a 50% increase in the WUE (Table 3). Although statistically not significant, the moderate and severe drought reduced the quantum yield by about 10%. However, the stress treatments did not cause any change in the Fv/Fm (Table 3).

Photosynthesis (A), transpiration (E), intercellular CO_2 concentration (Ci), stomatal conductance (Gs), vapor pressure deficit (VPD), quantum yield of a dark-adapted leaf (variable fluorescence/maximum fluorescence (Fv/Fm), quantum yield of an illuminated leaf during gas exchange measurements (Yield), and plant water use efficiency (WUE).

3.4. CA enzyme activity

The highest CA enzyme activity was found in the plants subjected to mild drought stress (Figure 3). The enzyme activity had more than doubled under mild stress compared with the control. As the drought severity increased, the enzyme activity significantly decreased compared with the control. When compared with the control, the enzyme activity did not change statistically under moderate drought conditions; however, it decreased sharply in the plants subjected to severe drought conditions (Figure 3).

3.5. Free proline content

Drought stress significantly increased the free proline content in both the roots and leaves of *A. thaliana*. It was determined that as the severity of drought increased, the proline content also increased. The increases in the leaves were much more pronounced than those in the roots (Figure 4).

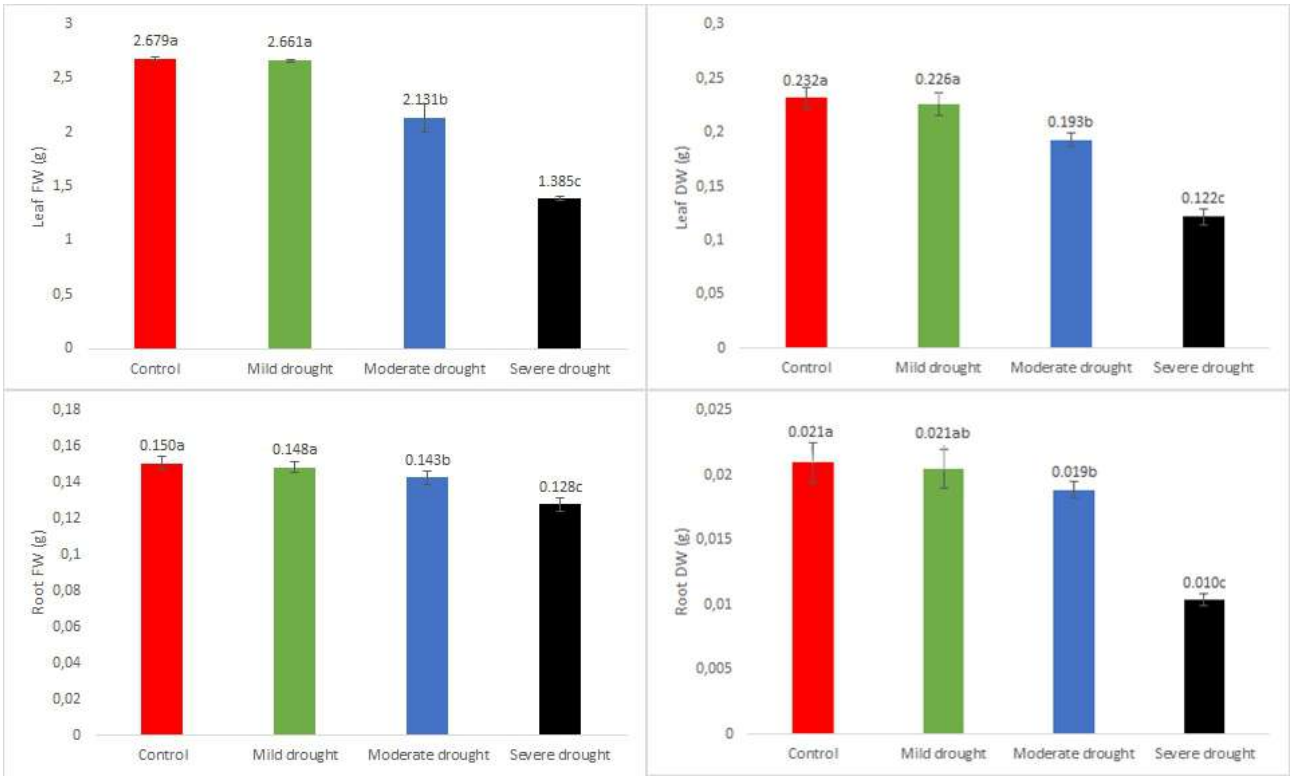

Figure 1. Effects of different levels of drought stress on the leaf and root biomass of *A. thaliana*. There was no statistically significant difference between the groups indicated with the same letters. Each bar represents the mean of five different plants. Vertical lines represent ±1 standard error (SE) of the mean.

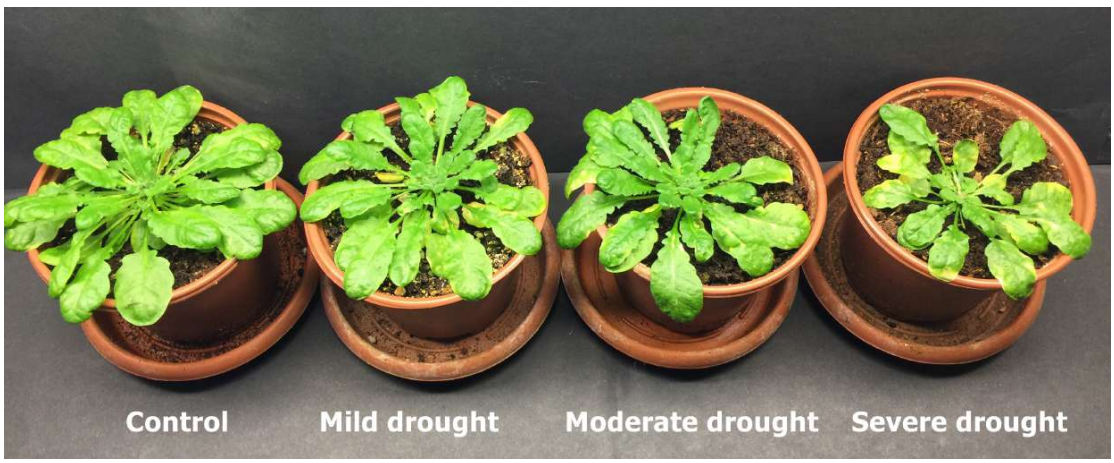

Figure 2. Representative images of the phenotypic changes in the *A. thaliana* seedlings under increasing drought stress treatments. Severe drought treatment caused the greatest effect on the plants, which is visible by the stunted growth and leaf chlorosis.

3.6. MDA content

The MDA content increased in the roots and leaves of *A. thaliana* under increasing drought stress levels, except under mild stress (Figure 5). The MDA content of the leaves was about an order of magnitude higher than the MDA content of the roots. The highest MDA level was found under severe drought conditions in both the leaves and roots. (Figure 5).

Table 2. Changes in the LWP and RWC in *A. thaliana* under different drought stress conditions. Different letters indicate significant differences among the treatments. Values are the means of five different plants at ±1 SE of the mean. ***p < 0.001.

	LWP (MPa)	RWC (%)
Control	–0.42 ± –0.10a	89.80 ± 1.60a
Mild drought	–0.87 ± –0.11b	76.92 ± 1.45b
Moderate drought	–1.25 ± –0.08c	60.52 ± 3.92c
Severe drought	–1.60 ± –0.06d	45.40 ± 2.98d
LSD	0.118***	3.545***

3.7. βCA gene expressions

The 6 isogenes of the βCA were found to be expressed in both the roots and leaves (Figures 6 and 7). When the expression levels of βCA in the roots and the leaves were compared, it was determined that they were expressed much more clearly in the leaves. Moreover, different expression profiles were obtained in the roots and leaves (Figures 6 and 7). According to the results, the expression levels of all of the isogenes increased significantly under mild drought conditions when compared with the control and with the other stress levels in the leaves (Figure 6). Under moderate and severe drought stress conditions, the gene expressions decreased significantly when compared with the control groups, except for βCA 6 under severe drought. Especially under severe drought conditions, the decreases were very evident (Figure 6).

The results obtained from the root tissue were completely different from the gene expression profiles obtained from the leaves (Figure 7). The gene expression levels were quite low in the roots compared to the leaves, and where the highest expression was under mild stress in the leaves, the untreated control plants showed the highest expression in the roots (Figure 6 and 7). In the roots, the expression levels of all of the βCA genes decreased under all of the drought stress levels when compared with the untreated control plants (Figure 7). When mild, moderate, and severe drought conditions were evaluated among themselves, the highest expression level was determined under severe drought conditions, except for βCA 6 (Figure 7).

4. Discussion

Drought stress is one of the most devastating abiotic stress factors affecting approximately 26% of agricultural land worldwide, either temporarily or continuously. However, under persisting global warming and changing climatic conditions, drought stress will continue to affect more arable lands much more frequently and more severely in the future (Eziz et al., 2017, Seleiman et al., 2021). Drought stress causes yield losses by impairing many important metabolic pathways, especially photosynthetic mechanisms and, therefore, the growth of plants (Çevik et al., 2019). In this study, the leaf and root biomass of *A. thaliana* decreased significantly under drought stress, especially with severe drought stress. Many studies have previously reported significant biomass losses in *A. thaliana* under drought stress (Harb and Pereira, 2011; Zolla et al., 2013). Eziz et al. (2017) analyzed 164 studies in a metaanalysis and revealed that drought stress significantly reduced the leaf and stem biomass, but not the root biomass. Pinheiro and Chaves (2011) showed that yield losses of photosynthetic metabolism as a result of drought are important reasons for the decrease in biomass. In this study, it was observed that the photosynthetic efficiency decreased, respectively, by 20% and 50% under moderate and severe drought conditions, which may have caused the significant reduction in leaf and root dry mass.

Drought stress decreased the LWP and RWC of *A. thaliana*. It was reported that a decrease in the plant water content affects the photosynthetic efficiency of plants by causing a turgor loss of leaves, resulting in a slowdown in growth and development, and a decrease in the chlorophyll content (Harb and Pereira, 2011). Maintaining a relatively higher water potential and turgor pressure under water stress was attributed to the plant's tolerance to water stress (Maréchaux et al., 2015). Some genes might play a significant role in maintaining a plant's tolerance to water deficit. A few studies have shown that CAs play a role in the opening and closing mechanism of the stomata (Hu et al., 2010; Hong et al., 2014). Under mild drought conditions, the expression of the βCA genes was very high when compared with the control, moderate, and severe drought conditions. This may be an indication that plants benefit from efficient water use as a result of better stomatal regulation at the early stages of drought stress.

Drought generally shows two types of effects on plants, which are water deficiency (loss of turgor) and metabolic impairment due to drying (Takahashi et al., 2020). With a lack of water, the stomata tend to close and thus gas exchange is limited. Drought stress negatively affects the photosynthetic rate, transpiration rate, Gs, and WUE (Akgün 2018). As the LWP decreases, photosynthesis and

Table 3. Changes in the leaf gas–exchange parameters under different levels of drought stress in *A. thaliana* plants. There was no statistically significant difference among the treatments indicated with the same letters. Values are the means of at least 6 different plants for each treatment at ±1 SE of the mean. *p < 0.05, ***p < 0.001. ns: not significant.

Treatment	A ($\mu mol\ m^{-2}\ s^{-1}$)	Ci (ppm)	Ca (ppm)	E ($mmol\ m^{-2}\ s^{-1}$)	Gs ($mmol\ m^{-2}\ s^{-1}$)	VPD (Pa/kPa)	Fv/Fm	Yield	WUE
Control	4.60 ± 0.54a	319.95 ± 16.37	413.78 ± 0.32	1.27 ± 0.17a	85.96 ± 12.15a	14.82 ± 0.04	0.81 ± 0.01	0.26 ± 0.04	3.68 ± 0.71
Mild drought	4.73 ± 1.55a	296.50 ± 41.64	397.36 ± 37.19	1.04 ± 0.15a	69.91 ± 10.59b	14.85 ± 0.06	0.81 ± 0.01	0.27 ± 0.03	4.70 ± 1.80
Moderate drought	3.64 ± 1.14ab	274.91 ± 23.04	413.93 ± 0.34	0.69 ± 0.35b	36.78 ± 7.81c	14.86 ± 0.05	0.81 ± 0.01	0.23 ± 0.04	5.66 ± 1.00
Severe drought	2.36 ± 1.05b	272.71 ± 31.34	414.18 ± 0.17	0.41 ± 0.11b	27.18 ± 7.99c	15.04 ± 0.32	0.82 ± 0.01	0.24 ± 0.01	5.68 ± 1.35
LSD	1.494*	ns	ns	0.294***	12.94***	ns	ns	ns	ns

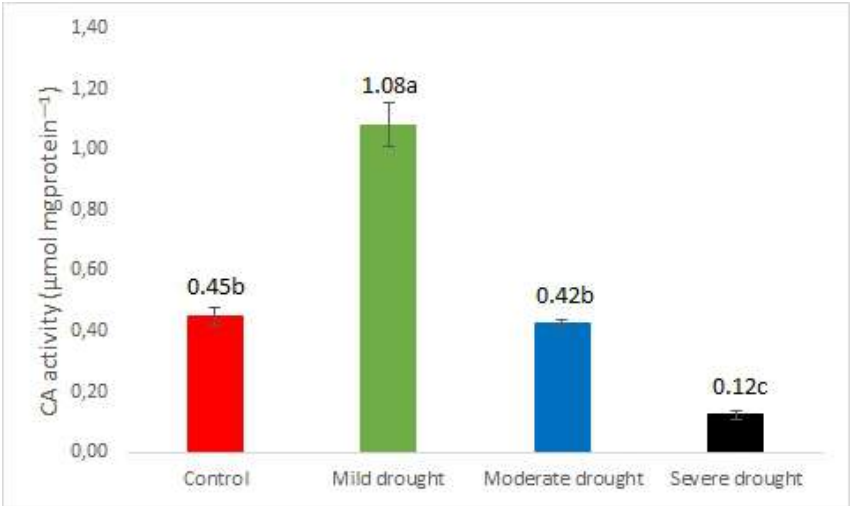

Figure 3. CA enzyme activity under drought stress in *A. thaliana* leaves. There was no statistically significant difference between the treatments indicated with the same letters. Each bar represents the mean of nine different plants. Vertical lines represent ±1 SE of the mean.

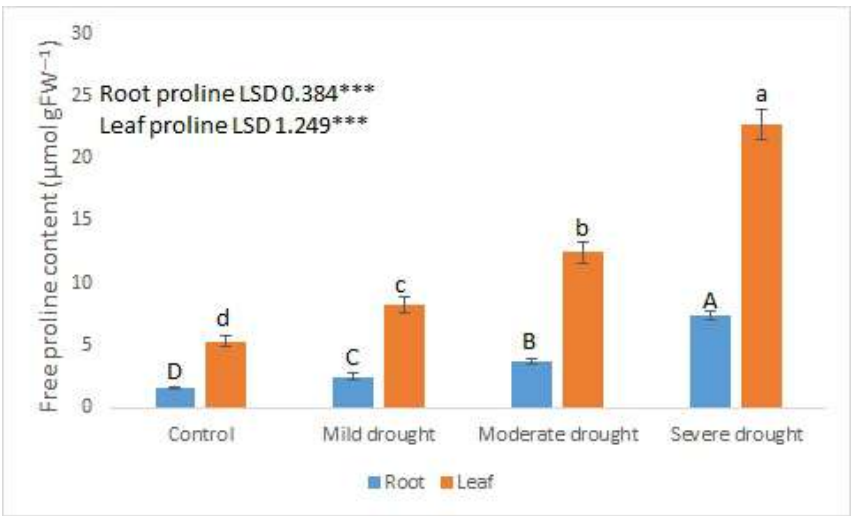

Figure 4. Free proline content under different levels of drought stress in the roots and leaves of *A. thaliana* plants. Different letters for each organ represent significant differences. Each bar represents the mean of nine different plants. Vertical lines represent ±1 SE of the mean.

Gs also decrease. When the values of the photosynthetic gas exchange parameters were examined in this study, it was determined that the assimilation rate, Gs, and transpiration rate decreased significantly in the stressed plants when compared with the well-watered control plants. The loss in the gas exchange parameters was more pronounced as the severity of stress increased. These data were also supported by the LWP and RWC values and phenotype of the treated plants (Figure 2).

In this study, unlike the other photosynthetic parameters, the WUE increased in parallel with the severity of stress. This happened because the decrease in the transpiration rates was much higher than the decline in the assimilation rates, resulting in an increased WUE. Flexas et al. (2016) reported that efforts to improve the WUE often lead to yield reductions. Improving the WUE of crops is considered beneficial under severe and deadly drought conditions, regardless of a yield loss; however, any growth

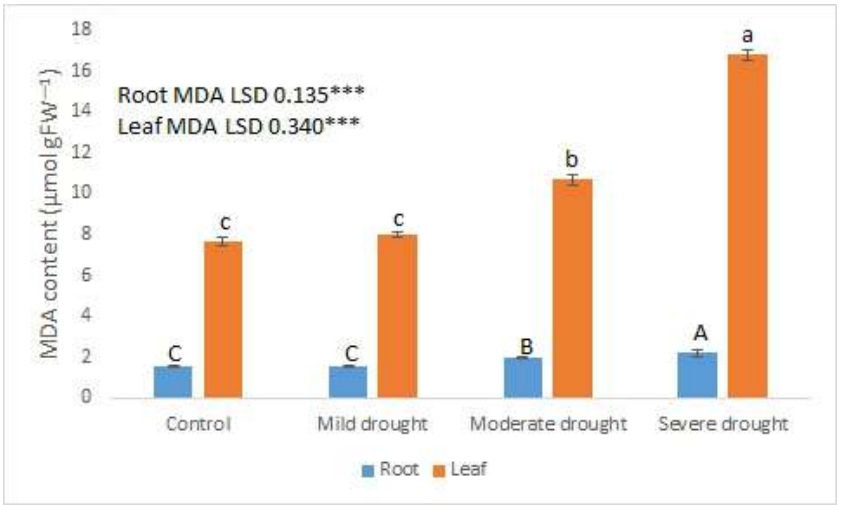

Figure 5. Effect of the increasing levels of drought stress on the MDA content in the roots and leaves of *A. thaliana* plants. Different letters for each organ represent significant differences. Each bar represents the mean of nine different plants. Vertical lines represent ±1 SE of the mean.

sustaining a WUE increase is advantageous under mild drought conditions (Tardieu et al., 2018). In order to increase the sustainability of agriculture, there is a need for high yielding varieties with an increased WUE (Blankenagel et al. 2018). C_3 plants can moderately increase the WUE by restricting transpiration, resulting in a higher intrinsic WUE. However, reduced CO_2 uptake in exchange for reduced transpiration also impairs photosynthesis and possibly growth and yield (Togawa-Urakoshi and Ueno, 2022). It was found herein that the assimilation rate decreased by about 20% and 50% under moderate and severe drought stress, respectively. Therefore, there is, in general, a negative correlation between photosynthesis or growth and the WUE. In the current study, there was also a slight drop in the Ci under drought stress when compared with the control group. Water stress showed no effects on the Fv/Fm and slightly decreased the yield of light-adapted leaves. Similar results were reported in wheat plants, where water stress had no effects on the primary photochemistry of PSII (Fv/Fm) but reduced the quantum yield of the PSII electron transport of light-adapted leaves (Lu and Zhang, 1999).

CO_2 uptake and water loss are controlled by stomatal movements. Therefore, the control of efficient stomatal movement is very important for regulating water loss and simultaneously maintaining photosynthetic efficiency, especially under drought conditions (Lawson and Vialet-Chabrand, 2019). The effects of CAs on the mechanism of stomatal regulation in C_3 plants remain an important research question.

Decreasing the cellular water content under drought stress enables plants to develop strategies to reduce water loss. One of these strategies is to increase the amount of intracellular osmolytes (Chun et al., 2018). It has been shown that proline increases under different abiotic stresses, and the increase in proline is related to stress tolerance, depending on the species, variety, and type of stress (Hayat et al., 2012). Fu et al. (2017) reported a significant rise in the proline content in parallel with the severity of drought in *A. thaliana*. In the current study, the proline content increased in roots and leaves under drought conditions. The increase was more pronounced under severe drought conditions. Similar to these findings, Sperdouli and Moustakas (2012) reported an increase in the proline content in *A. thaliana*, especially under severe drought conditions, emphasizing that the increase in proline content is important for the preservation of photosynthetic yield. In addition to being a good osmotic protector, proline was reported to play an indirect or direct role in the scavenging of free radicals (Szabados and Savouré, 2010). It is well known a fact that free radicals increase under drought conditions (Yildizli et al., 2018; Çevik et al., 2019); therefore, the accumulation of proline under drought stress may give the plant an advantage for the protection of cellular components against radicals.

MDA is the end product of lipid peroxidation and is one of the good indicators of membrane damage due to oxidative stress. In the present study, drought stress increased the MDA content in both the leaves and roots. It was previously emphasized that the main cause of MDA increases is free radicals, especially hydrogen peroxide and H_2O_2 (Ibrahim and Jaafar, 2012; Hasanuzzaman et al., 2020). Cao et al. (2020) determined an increase in the CA activity, βCA gene expression level, antioxidant enzyme

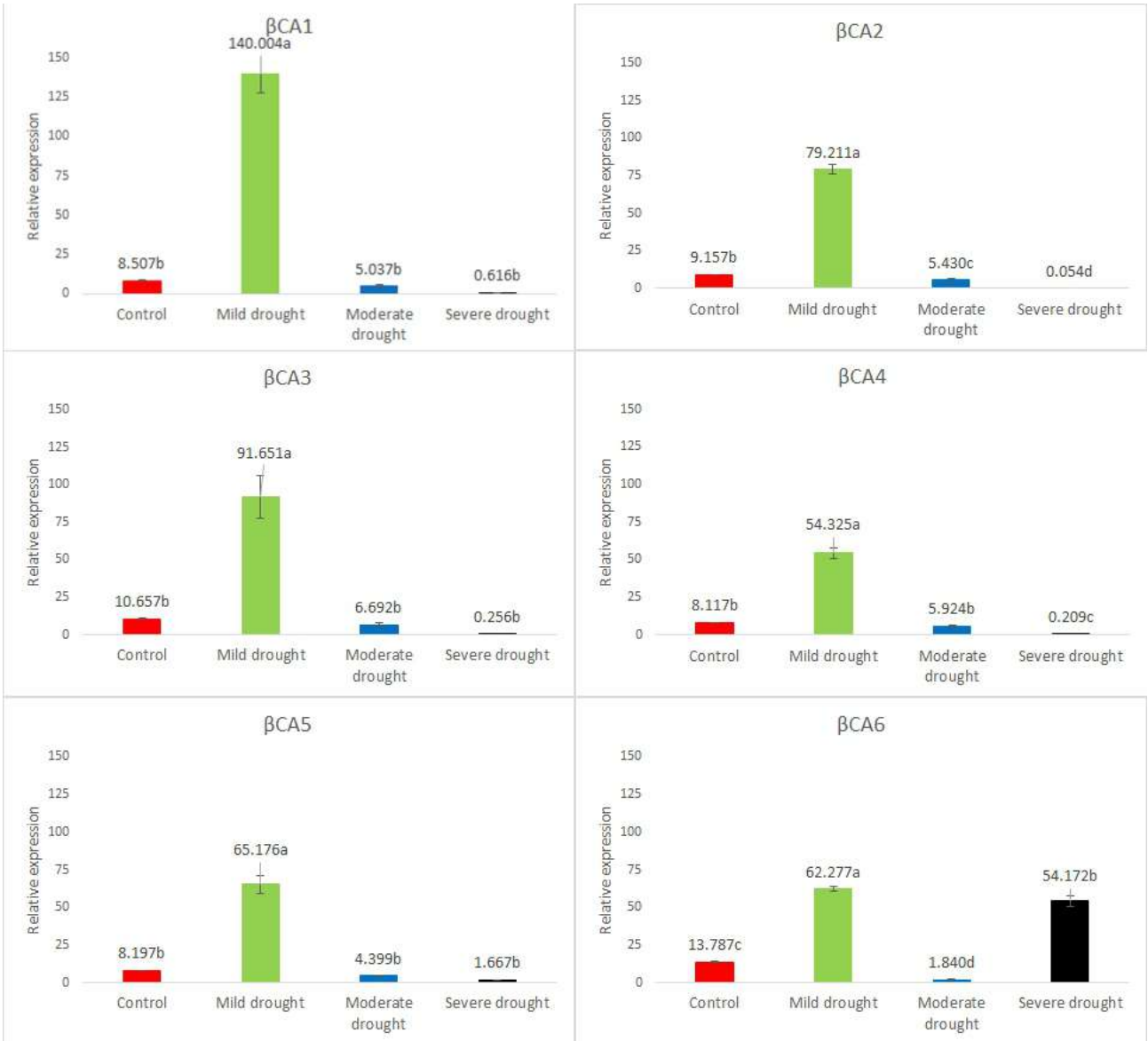

Figure 6. Expression levels of the 6 isogenes of βCA in the leaves of *A. thaliana* under different drought stress conditions. Data are presented as the number of copies.

activities and a decrease in the MDA content with exogenous zinc application. These results might indicate that CAs indirectly stimulate the antioxidant defense system in plants. Therefore, CAs may provide some advantages to plants, especially under stress conditions. The exact roles of CAs in C_3 plants still deserve further research and remain a popular topic. Any new information obtained provides a different perspective.

When the CA enzyme activity was examined herein, a significant increase was observed under mild drought conditions compared with the well-watered control plants. There are several reports in the literature examining the change in the total CA enzyme activity under drought conditions. In some of these studies, the CA activity increased under drought stress (Popova et al., 2000; Yu et al., 2007; Guliyev et al., 2008; Sun et al., 2016), while the activity decreased in others (Hayat et al., 2008; Wu et al., 2012; Gu et al., 2013; Xing et al., 2015; Wang et al., 2016). These conflicting results may have arisen from the different stress conditions applied. Based on the data obtained from the present study, the CA enzyme activity significantly increased under mild stress but decreased to control values under moderate stress, and further decreased under severe water stress (Figure 3). Since the analysis of

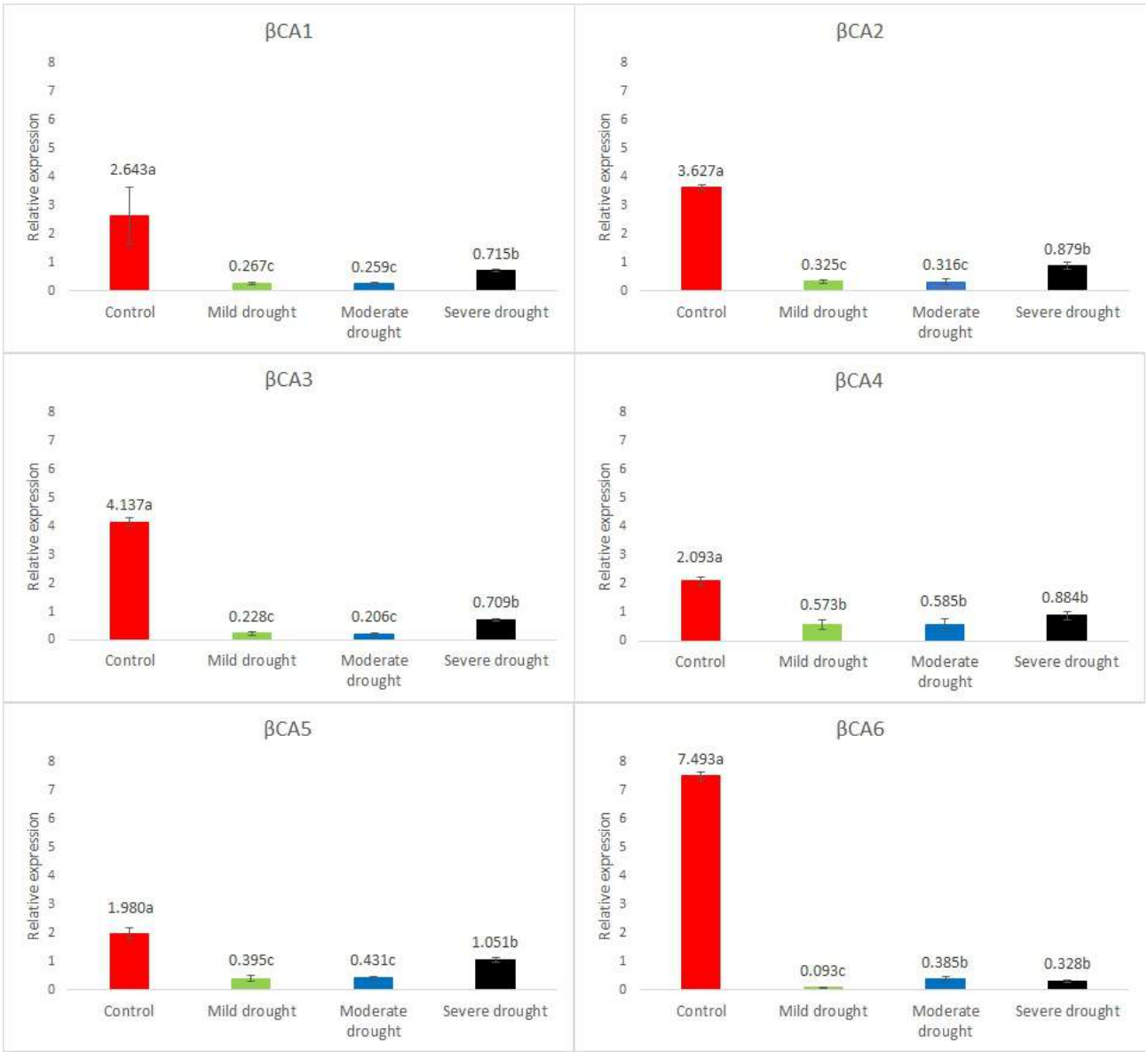

Figure 7. Expression levels of the 6 isogenes of βCA in the roots of *A. thaliana* under drought stress. Data are presented as the number of copies.

the total CA enzyme activity under drought conditions may lead to different results, it was thought that examining the CA isoenzymes on native gel may provide more consistent results. However, it was found that the correlation between the gene expression profile and enzyme activity was not high. Although only βCA gene expression levels were determined in this study, the total CA activity was analyzed. This may have affected the correlation between the gene expression and enzyme activity. In addition, there are many studies in the literature showing that the correlation between the gene expression and enzyme activity is not compatible in many cases (Arvas et al., 2011; Yin et al., 2017).

The βCA gene expression levels were found to be very low in the roots compared with the leaves and under severe stress when compared with the control plants. Polishchuk (2021) reported that βCAs are mostly expressed in photosynthetic parts. In the current study, the expression level of the βCA genes increased significantly as a result of mild drought treatment when compared with the control group leaves, but the expression level decreased in parallel with the severity of drought. Various researchers

have obtained many different results on this subject in the literature. For example, Kaul et al. (2011) found that the expression of βCA 1 increased within 24–72 h of drought application. Wu et al. (2012) reported that the expression of βCA 2 decreased as a result of polyethylene glycol application. Likewise, Perez-Martin et al. (2014) found that the βCA gene expression decreased, while Wang et al. (2016) reported an increase in expression. Li et al. (2020) emphasized that CA gene expressions may vary depending on their location in the cell. Many physiological and biochemical roles of CAs have been described, but the most well-known role is their metabolic activities in the photosynthesis mechanism. In C_4 plants, CAs have important catalytic functions in the photosynthetic metabolism of the CO_2 concentrating mechanism, capturing CO_2 in low concentrations, and converting it to carbonic acid (Polischuk, 2021). However, the roles of CAs in C_3 plants have recently been intensively investigated. When the literature was examined, contradictory results were found, which make it difficult for researchers to understand the role of these genes. The results that were obtained in the present study may contribute to the understanding of the conflicting results in the literature. According to the results herein, the increase in the expression of these genes only under mild drought conditions may be an indication that these genes are highly functional at the initial phase of stress. Studies to understand the roles of these genes using transgenic plants support these predictions (Majeau et al., 1994; Price et al., 1994; Gu et al., 2013).

The fact that photosynthesis was high under mild drought conditions where the βCA expression was also high in the current study may be an indication that this increase in gene expression may give the plant an advantage in maintaining photosynthetic efficiency at the onset of water stress. Herein, the gene expression of βCAs 1 and 5, which are reported to be especially localized in chloroplasts, increased (DiMario et al., 2017), indicating that these genes may play a significant role in maintaining photosynthetic efficiency at the early stages of the stress. It is important to investigate in detail the relationship between βCA 1 and 5 under stress conditions, especially with the rubisco enzyme. Although it has been shown that the βCA 1 gene plays a direct role in the stomatal opening and closing mechanism (Hu et al., 2015; Kolbe et al., 2018), the effects of the CA 1 and CA 2 genes on the overall photosynthetic efficiency may be realized by a much more complex mechanism.

One of the interesting results in this study was that the expression level of the βCA 6 gene, which is known to be localized in mitochondria, increased significantly under severe drought conditions, unlike the other 5 genes. This may indicate that this gene has a role within energy metabolism. However, it is also possible that this increase occurred in response to oxidative stress. Some findings in the literature have shown that increases in antioxidant enzyme activities due to oxidative stress are associated with CAs (Chao et al., 2020).

The results obtained in the study showed that βCAs may play important roles, especially at the initial stages, of drought stress. The obtained results provide important clues for future gene engineering studies. It is important to understand the roles played by βCA genes in C_3 plants under stress by silencing or increasing their expression. However, the inability to completely silence these genes may necessitate the use of different approaches. The determination of isoenzyme activity under stress conditions may also contribute to the understanding of the roles of these genes. In summary, it is important to carry out more precise studies to understand the roles of βCAs, especially in C_3 plants. Although it has not been fully revealed yet, we predict that these genes may have important roles in maintaining photosynthetic efficiency under drought stress conditions.

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Conflict of interest
The authors declare that there are no conflicts of interest.

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