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Original Research

Effect of Bicarbonate Stress on Carbonic Anhydrase Gene Expressions from *Orychophragmus violaceus* and *Brassica juncea* seedlings

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Abstract

Three β-type genes coding for carbonic anhydrase and CA activities from *Orychophragmus violaceus* L. and *Brassica juncea* L. leaves in response to $NaHCO_3$-induced bicarbonate stress were examined. Three full-length cDNA CDS sequences were designated as *OvCA1*, *OvCA3*, and *OvCA4* in *Orychophragmus violaceus*, and as *BjCA1*, *BjCA3*, and *BjCA4* in *Brassica juncea*; these genes encoding β-CAs were identified and characterized. In particular, *OvCA1* and *BjCA1* encode two putative chloroplast isoforms. *OvCA3* and *BjCA3* encode two putative cytosolic isoforms. *OvCA4* and *BjCA4* encode two putative plasma membrane isoforms. Quantitative real-time RT-PCR analysis revealed that *OvCA1* and *OvCA4* expressions in *Orychophragmus violaceus*, *BjCA1,* and *BjCA4* expressions in *Brassica juncea* changed synchronously with CA activities as bicarbonate stress was intensified. Bicarbonate stress synchronously stimulated *OvCA1* and *OvCA4* expressions along with CA activities in *Orychophragmus violaceus* at slight stress level; but it decreased CA activity, *BjCA1* and *BjCA4* expressions, and stimulated *BjCA3* expression in *Brassica juncea*. *Orychoophragmus violaceus* could better adapt to slight bicarbonate stress than *Brassica juncea* due to the former exhibiting higher *OvCA3* expression levels and CA activities than the latter. The responses of *CA1* and *CA4* in *Orychophragmus violaceus* and *CA3* in *Brassica juncea* to bicarbonate stress partly regulate HCO_3^- to water and CO_2 supplied to plants. Diverse CA gene expressions can partially account for different adaptation strategies of the two plant species subjected to different bicarbonate stress levels.

Keywords: bicarbonate, *Orychophragmus violaceus*, *Brassica juncea*, carbonic anhydrase, gene expression

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Introduction

Bicarbonate stress is considered the major abiotic stress that cause adverse effects on plant growth and crop productivity globally [1, 2], especially in karst regions, where the major anions from the soil solution of calcareous soils are HCO_3^- and CO_3^{2-}, which are the severe harmful factors for the plants [3]. Sodium bicarbonate, which brings to high levels of pH, Na^+, HCO_3^-, and CO_3^{2-}, lead to distraction of intracellular pH, hypertonic stress, and ultimately affect plant growth and development [4, 5]. Exposed to bicarbonate stress, the plants have to resist adverse bicarbonate stress because they do not avoid it, thus plants have to develop a series of complex systems of signal transduction to respond and adapt to bicarbonate stress [6, 7]. Such systems are correlated with reprogramming of downstream regulation and significant environmental stress-inducible genes expression [8, 9]. Recently, a number of genes have been discovered and demonstrated the correlation between bicarbonate stress and expression of these genes. Consequently, the production of osmolytes and proteins are induced to regulate the hyperosmotic of cytoplasm in plant tissues [10, 11]. However, some studies have suggested that adding a certain amount of bicarbonate to culture medium can improve the growth of the plants [12-14]. But excessive levels of bicarbonate often affect normal growth and development of some plants [15]. Furthermore, a great many studies have been devoted to exploring and selecting the ideal bicarbonate-resistant plants based on the wide adaptability of plants [16].

Orychophragmus violaceus (L.) and *Brassica juncea* (L.), which are both cruciferous plant species, and some studies indicated that *Orychophragmus violaceus* grew better in limestone soil in the karst region than *Brassica juncea* in correlation with higher photosynthetic activity and bicarbonate-use capacity of *Orychophragmus violaceus* [17, 18], demonstrating that *Orychophragmus violaceus* has developed physiological and molecular mechanisms to adapt to karst adversity. Therefore, *Orychophragmus violaceus* is an ideal plant for determining the physiological and molecular mechanisms of plant bicarbonate stress tolerance. However, *Brassica juncea* is widely grown in southwest china due to its tolerance of bicarbonate stress [19]; it was used as the control plant and compared with *Orychophragmus violaceus* in identifying the differences in response to bicarbonate stress.

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc-containing metalloenzyme in living organisms, with the key function as catalysing the reversible inter-conversion of HCO_3^- and CO_2 [20]. A number of studies have demonstrated that the CA family contribute to the regulation of various biochemical processes. In higher plants, CA can provide ample inorganic carbon for carboxylases, such as ribulose-bisphospate and phosphoenolpyruvate carboxylase, for photosynthetic assimilation [21-23]. However, CA activities and gene expression levels in response to different bicarbonate stresses varied in terms of plant species and the extent of stress.

A great many studies have found four types of CAs (i.e., α -, β -, γ - and δ), which are crucial to plant cells and are involved in various physiological and biochemical processes [20]. Recently, many CA family genes have been investigated; in particular, the β-CA class predominate most in all known CAs in higher plants [24]. The known model plant *Arabidopsis thaliana* and the roles and locations of CA have been reported in recent years [25, 26]. All *AtβCAs* (*AtβCA1-6*) and three *AtαCAs* of *Arabidopsis thaliana* have been reported to be expressed in all green tissues. Six *AtβCAs* are located in the chloroplast (*AtβCA1* and *AtβCA5*), mitochondria (*AtβCA6*), and plasma membranes (*AtβCA4*), and within the cytoplasm (*AtβCA2* and *AtβCA3*), respectively. Some studies have reported that the CA gene expressions in response to different atmospheric CO_2 supply changes to regulate the inorganic carbon utilization to maintain carbon metabolic process [21-26]. However, the functions of CAs in the C_3 plant photosynthesis are still unclear.

Arabidopsis thaliana, *Orychophragmus violaceus,* and *Brassica juncea* are all cruciferous C_3 plants. Numerous studies have reported the functions and localization of CAs in *A. thaliana* [25]. However, little information is known regarding the functions and localizations of CAs in the other two plant species. This study revealed three distinct cDNA sequences which, when encoding CA in *O. violaceus* and *B. juncea,* have been sequenced and aligned to the high homologous beta CA sequences in *A. thaliana*. These sequences include *CA1* (locus At3g01500) encoding chloroplast CA, *CA3* (locus At1g23730) encoding cytoplasmic CA, and *CA4* (locus At1g70410) encoding plasma membrane CA in *A. thaliana*. Furthermore, the experimental results showed that the CA enzyme is inducible and responds to increasing levels of bicarbonate in water culture conditions. Three distinct CA gene expressions are investigated. The aim of the present study is to elucidate the differences of CA genes expression in response to $NaHCO_3$-induced bicarbonate stress in *O. violaceus* and *B. juncea*.

Material and Methods

Plant Materials and Bicarbonate-Stress Treatments

*Orychophragmus violaceu*s (L.) O. E. Schulz and *Brassica juncea* (L.) Czern.et Coss. cv. Zangyou No. 8 were used as the experimental materials, which were grown in the laboratory of the Institute of Geochemistry, Chinese Academy of Sciences, Guizhou Province, China [26.35°N, 106.42°E]. The experiment was performed from July 14 to September 21. The seeds of two plant species were germinated in 12-hole trays

containing perlites in a greenhouse under a 12-h light cycle (400 μmol·m^{-2}·s^{-1}, PPFD), a day/night temperature range of 25/18°C, and a relative humidity range of 50-60%. After germination, the seedlings with uniform sizes were selected and cultured with half-strength Hoagland nutrient solution [27]. After two months, the two cruciferous plant species were exposed to bicarbonate treatments by adding sodium bicarbonate to modified Hoagland nutrient solution. The modified Hoagland nutrient solution contained 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 0.25 mM $NH_4H_2PO_4$, 0.75 mM NH_4Cl, 2 mM Fe(Na)EDTA, 2 μM KCl, 50 μM H_3BO_3, 4 μM $MnSO_4$, 4 μM $ZnSO_4$, 0.2 μM $CuSO_4$, and 0.2 μM $(NH_4)_6M_{O7}O_{24}$ at pH 8.30±0.05. The modified Hoagland nutrient solution included four concentrations of sodium bicarbonate (i.e., 0, 5, 10, and 15 mM $NaHCO_3$). The modified Hoagland nutrient solution was changed daily to maintain a constant extent of each stress. Measurements were performed after 7 d.

Measurement of Carbonic Anhydrase Activities of Leaves in the Two Plant Species

The third fully expanded leaf from the top of the two plant species was chosen for measuring carbonic anhydrase activity. About 0.1 g of leaves (fresh weight) were quickly frozen in liquid nitrogen and ground into power with a mill, then the sample was ground with 3 ml extracted buffer (0.01 M barbitone sodium with 0.05 M 2-mercaptoethanol, pH 8.30). The homogenate was centrifuged at 10,000×g and 4ºC for 10 min before being placed on ice for 20 min. Then the supernatant was used to measure the activity of carbonic anhydrase using the method with modifications [28]. CA activity was measured at 0-2°C in a container consisting of 4.5 ml 0.02 M baritone buffer (5, 5-diethylbarbituric acid; pH 8.30), 0.4 ml sample, and 3 ml CO_2-saturated H_2O. The carbonic anhydrase activity was defined as $WA = (t_0/t) - 1$, where t_0 and t were the time(s) taken from the enzyme-free buffer and the supernatant of the sample. At the same time, the protein content of leaves was determined by the Coomassie brilliant blue method [29].

Identifying and Characterizing cDNA Coding for β-CAs

Searches within public NCBI databases revealed the presence of three distinct nucleotide sequences in *O. violaceus* and *B. juncea* coding for β-CAs. The corresponding nucleotide sequences, which were aligned, and the complete nucleotide open reading frame (ORF) of the cDNA sequences were determined (NCBI, USA, *ncbi.nlm.nih.gov*) (Table 1). The deduced amino acid sequences suggested the presence of *O. violaceus* and *B. juncea* CA cDNAs, respectively. These distinct mRNA sequences of the two plant species were conducted for multiple alignments with ClustalX version 1.8, and the results of evolutionary trees were run with the Neighbour-Joining method [30].

Table 1. Basic information of the CA genes in *O. violaceus* and *B. juncea.*

S. No.	Genes	Full CDS length (bp)	Protein length	GenBank accession No.
1	*OvCA1*	996	331	KM586043
2	*OvCA3*	777	258	KM586044
3	*OvCA4*	777	258	KM586045
4	*BjCA1*	1014	337	KM586046
5	*BjCA3*	780	259	KM586047
6	*BjCA4*	777	258	KM586048

RNA Extraction and Real-Time RT-PCR Analysis

The same levels used as the experiment for measuring CA activities were harvested and ground in liquid nitrogen for gene expression analysis. They were harvested on the seventh day after the bicarbonate stress treatment. Extraction of total RNA was performed on 80 mg of each plant material according to the method [31] described by the plant rna purification reagent (Invitrogen). To determine the gene expression accurately and eliminate first the contaminating genomic DNA during the purification of RNA, all the total RNA samples were treated and reverse-transcribed according to the instructions of the manufacturer using a Prime SCRIPT RT reagent kit with gDNA eraser (Perfect Real Time) (TaKaRa code: DRR047S). Furthermore, the concentration of each RNA treatment and material was determined by measuring the sample absorbance of RNA (A260/A280) using a spectrophotometer. Afterward, these samples were stored at -80ºC for determining the expression of genes encoding CA using real-time RT-PCR analysis.

The three distinct genes whose expressions were to be studied on the influence by bicarbonate were *OvCA1* and *BjCA1*, coding for tow putative chloroplast isoforms CA; *OvCA3* and *BjCA3* coding for two putative cytoplasmic isoforms CA; and *OvCA4* and *BjCA4* coding for two putative plasma membrane isoforms CA. The *actin* genes in *O. violaceus* and *B. juncea* were used as internal standard in the quantitative RT-PCR reaction, termed as *Ovactin* (*GenBank* accession No. KC979147) and *Bjactin* (*GenBank* accession No. KC979151), respectively. *O. violaceus* and *B. juncea* gene-specific primers were designed with Primer 5.0 software (Premier Biosoft International, USA) (Table 2).

Quantitative RT-PCRs were run in a 48-well Applied Biosystems one-step real-time PCR system using a SYBR green master mix (Applied Biosciences, USA), 1 μg of the cDNA as template, and the gene-specific

Table 2. Lists of primers used for Quantitative RT-PCR.

S. No.	Genes	Forward primers (5′→3′)	Reverse primers (5′→3′)
1	*OvCA*1	CGGCGGAACTTAAGACAGGT	CTCCTTTGCCAGTCACCGTA
2	*OvCA3*	CTTCGACGATCAGTGCACCA	GTGAGCTCCTCTTATGGCGA
3	*OvCA4*	AGAAGGCAGATCTGGGGAAC	TTGATTCGTTCAACGGCGTC
4	*Ovactin*	CGTTGCCCTGAGGTTCTCTT	TTGAACCACCACTGAGGACG
5	*BjCA1*	ATCCGTAACGAGCCCATTCT	GGCCTCTTGGTATGATTCGCT
6	*BjCA3*	TCCAAGCGACACATTCACAG	AAGAACAGGGTCAAGCAGGAA
7	*BjCA4*	GAACCGAGCACTGTCCTTCAA	ATTCCAGCAACTCTGACGCC
8	*Bjactin*	GGAATGGTTAAGGCTGGTTTCG	GTTGTTGACGATGCCGTGTT

primers at a finial concentration of 0.5 μM. The PCR conditions were 40 cycles of 95ºC for 15 s and 60ºC for 1 min. A modification of the comparative threshold cycle method was used for the relative quantification of gene expression. In the case of different bicarbonate stress treatments, relative transcript levels of the target gene (X) were calculated as a ratio to the expressed actin gene transcripts (a), as $(1+E)^{-\Delta Ct}$; where ΔCt was calculated as $(Ct^x - Ct^a)$, and E value, which represented the PCR efficiency for each amplification, was calculated with the linear regression method [32]. All these real-time RT-PCRs were examined on six biological repeats.

The Log Relationship between Relative Gene Expression and Relative CA Activity

In order to identify the differences of three distinct beta-type CA genes expression in *O. violaceus* and *B. juncea*, we examined log equation to describe the log relationship between relative gene expression (RGB) and relative CA activity (RCA). The RGB and RCA were calculated as: RGB (RCA) = $\log(a_t/a_0)$, where a_t is the value of relative gene expression or CA activity at each bicarbonate treatment, and a_0 is the value of relative gene expression or CA activity at the control (0 mM $NaHCO_3$), respectively.

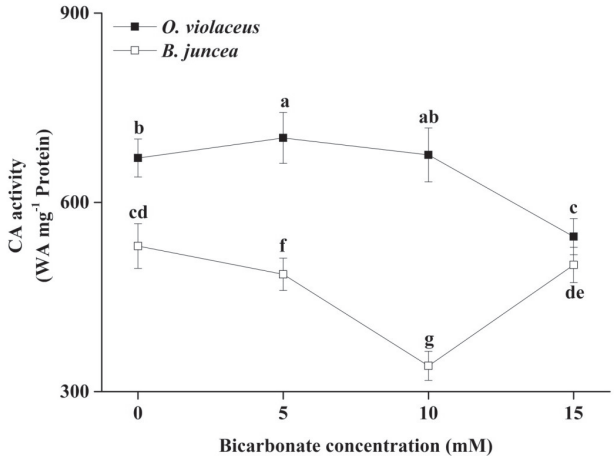

Fig. 1. CA activities of leaves in *Orychophragmus violaceus* and *Brassica juncea* seedlings subjected to bicarbonate treatment. Values show means ± SE (n = 6), which are presented using different letters showing significant differences in these characteristics at P<0.05 between group means, according to one-way ANOVA and t-test.

Data Analysis

The overall measurement data were subjected to ANOVA to determine significant differences (defined as P<0.05) between group means. Data were shown as mean ± standard error (SE) using a factorial analysis of SPSS software (version 20.0). The mean results were compared by Duncan post hoc test at 5% significance level (P<0.05). All the diagrams were constructed using Origin software (version 8.5).

Results

CA Activities and Protein Content in *O. violaceus* and *B. juncea* Leaves

The CA activities in leaves of the two cruciferous plant species under $NaHCO_3$-induced bicarbonate stress were examined and described as WA mg^{-1} protein (Fig. 1). Obviously, CA activities did vary in terms of plant species and the extent of bicarbonate treatments. CA activities were higher in *O. violaceus* than in *B. juncea* among the bicarbonate treatments. The highest CA activity in *O. violaceus* was 5 mM $NaHCO_3$ and reached 106%, and the lowest CA activity was 96% of the control at 15 mM $NaHCO_3$, respectively. However, CA activities in *B. juncea* were significantly lower in $NaHCO_3$-treatment than the control, but the CA activity at 15 mM $NaHCO_3$ was higher than the control. Similarly, the protein content of seedlings in the two plant species under bicarbonate treatments were examined (Table 3). With the increasing concentration of bicarbonate treatments, the protein content changed synchronously with CA activities in the two plant species.

Table 3. Protein contents of leaves in the in *O. violaceus* and *B. juncea*.

Bicarbonate treatments (mM)	Plant species	Protein content (mg g^{-1} FW)
0	*O. violaceus*	5.53±0.02
	B. juncea	5.18±0.01
5	*O. violaceus*	5.68±0.05
	B. juncea	4.89±0.02
10	*O. violaceus*	5.99±0.05
	B. juncea	4.50±0.03
15	*O. violaceus*	5.40±0.04
	B. juncea	4.80±0.01

Identification and Characterization of cDNA Coding for CA

The coding of three cDNAs for CA in the two plant species were obtained in our laboratory, and the three putative CA domain-containing protein sequences in the two cruciferous plant species were aligned, and the deduced amino acid sequences suggested the presence of cDNAs, which represented three full-length complete open reading frames (ORF) coding for CA, respectively. The cDNAs of *O. violaceus*, namely *OvCA1*, *OvCA3*, and *OvCA4*, and those of *B. juncea*, namely *BjCA1*, *BjCA3*, and *BjCA4*, were subsequently determined. To identify these CA gene families, we conducted a similarity search of sequences using the BLAST algorithm. The corresponding sequences were also aligned and characterized. Sequence alignments showed that these CA sequences are considerably similar to the βCA gene families. Multiple sequence alignments of the CA gene products of the two plant species are shown in Fig. 2. The relationship is indicated by a high E value score. The three cDNA sequences of the two plants were compared with previously characterized β-CAs. Our results revealed that these sequences – *OvCA1* and *BjCA1* – are highly similar to chloroplast isoforms CA (β-CA1); *OvCA3* and *BjCA3* are highly similar to cytoplasmic isoforms CA (β-CA3); and *OvCA4* and *BjCA4* are highly similar to plasma membrane isoforms CA (β-CA4).

Relative Expression of the Three CAs in the Two Plant Species

After the presence of the CA activities and three CA isoforms in the two cruciferous plants have been experimentally established and characterized in the previous sections, the relative transcript levels of the three CA forms in the two plant species in solution cultures grown under different bicarbonate stress treatments were then estimated using real-time quantitative PCR. First, total RNA was isolated from fresh leaf at each treatment in the two plants species. Figure 3 show the relative transcript levels of *OvCA1*, *OvCA3*, and *OvCA4* in *O. violaceus* leaves, whereas those of *BjCA1*, *BjCA3*, and *BjCA4* were in *B. juncea* leaves. These three CA gene transcript levels varied with plant species and bicarbonate stress levels. The levels of *OvCA1* and *OvCA4* expressions performed a synchronous trend with extended bicarbonate stress, and the highest values were also examined at 5 mM $NaHCO_3$; the levels of *OvCA3* were also lower than the control among the $NaHCO_3$-treatments; and the three gene expressions in *O. violaceus* had the lowest values at 10 mM $NaHCO_3$ than the control. However, the levels of *BjCA1* and *BjCA4* expression of *B. juncea* exhibited a synchronous trend with extent of bicarbonate stress, and the values of expression were gradually dropped with increasing $NaHCO_3$ levels. But *BjCA3* expression levels performed a similar trend to those of *OvCA1* and *OvCA4*, with the highest and lowest values of *BjCA3* at 5 and 15 mM $NaHCO_3$, respectively.

Discussion

A great many studies have indicated that bicarbonate stress is the major abiotic stress that causes threats via hypertonic stress to plant growth and productivity [1-7]. During bicarbonate stress, water and CO_2 supply in some tissues, especially in the photosynthetic tissues, will be even lower than normal conditions due to the hyperosmotic of cytoplasm in plant tissues [12-14]. Therefore, the lower water and CO_2 supply to photosynthetic tissues will have a bad affect on the fate of plants. Hence, how to improve and efficient use of water and CO_2 inside the plant tissues are crucial to plant survival in bicarbonate stress. Some studies have suggested that some mechanisms participate in the regulation of CO_2 and water, including the activity of CA enzyme [21-23], especially in this study. CA is a key enzyme that has been known to regulate various biochemical processes among the plant species under different conditions [20-24]. Furthermore, the crucial role of β-CAs could participate in the inter-conversion of HCO_3^- to CO_2 and water in higher plants, which can provide partial CO_2 and water as photosynthetic substrates for photosynthesis [25, 26]. The expression of carbonic anhydrase genes in response to $NaHCO_3$-induced bicarbonate stress has been reported. However, previous studies about the expression of carbonic anhydrase genes and CA activities varied in terms of plant species and the extent of bicarbonate stress [2, 16, 21, 23-26].

This study revealed that CA activities and gene expression levels of leaves in the two cruciferous plant species performed distinct responses to $NaHCO_3$-induced bicarbonate stress. Furthermore, the phylogenetic relationship studies suggested that three distinct β-CAs were highly similar to β-CA gene families (Fig. 2). The bicarbonate stress had

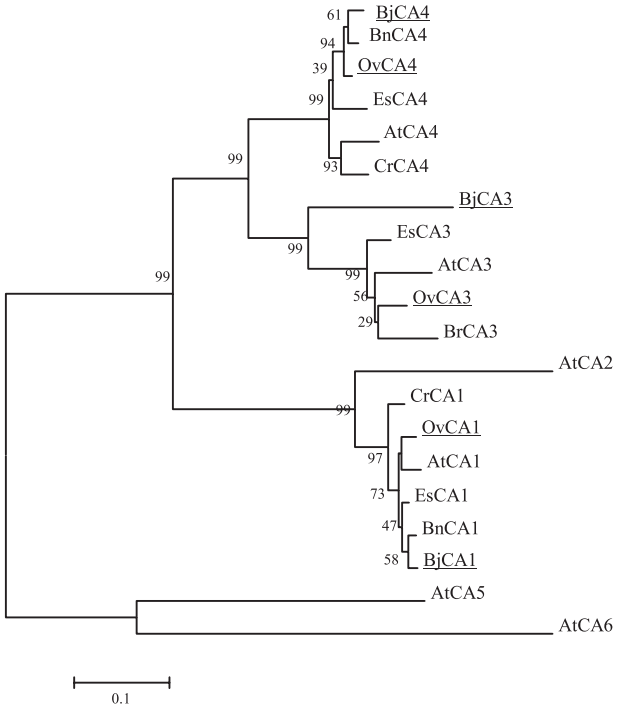

Fig. 2. Phylogenetic relationship of three *OvCA* (*OvCA1*, *OvCA3,* and *OvCA4*) and three *BjCA* (*BjCA1*, *BjCA3,* and *BjCA4*) gene sequences to other CAs, respectively. The evolutionary history was inferred using the neighbour-joining method. The phylogenetic tree distances were computed using the Poisson correction method and expressed in the units of the number of sequences. Phylogenetic analyses were conducted in MEGA4. *Arabidopsis thaliana* (*CA1* AT3G01500, *CA2* AT5G14740, *CA3* AT1G23730, *CA4* AT1G70410, *CA5* AT4G33580, *CA6* AT1G58180); *Eutrema salsugineum* (*CA1* XP006408513, *CA3* XP006416059, *CA4* XP006390894); *Brassica napus* (*CA1* ADI52861, *CA4* CDY57304); *Capsella rubella* (*CA1* XP006298106, *CA3* XP006301888); and *Brassica rapa* (*CA3* XP009103160).

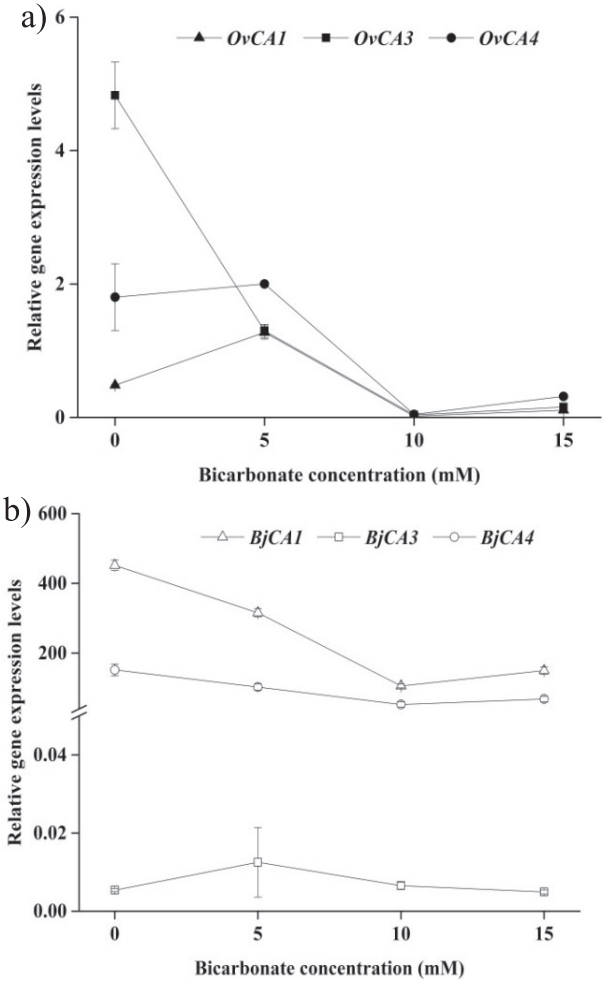

Fig. 3. Relative expression levels of the three carbonic anhydrase genes in *Orychophragmus violaceus* a) and *Brassica juncea* b) in response to bicarbonate stress, respectively. Total RNA was isolated from leaves of the two cruciferous plant species subjected to reverse-transcription and followed real-time PCR analysis. Relative transcript levels were calculated relative to the levels of *actin* gene expression. Values show mean ± SE (n = 5).

significant influence on CA activities between the two plants, which have a significant impact on *B. juncea* larger than *O. violaceus*. CA activities of *O. violaceus* performed significant lower values than the control (except at 5 mM $NaHCO_3$), whereas that of *B. juncea* exhibited the lower values than the control (expect 15 mM levels). The lowest value of CA activity was at 15 mM $NaHCO_3$ levels in *O. violaceus*, but that was at the 10 mM $NaHCO_3$ level in *B. juncea*. These results revealed that CA may partly participate in regulating the intercellular hyperosmotic effect induced by a high level of bicarbonate anion.

Studies indicated the CA expressions regulated CA activity [33-34]; and CA expression levels and CA activities varied from plant species, types, and extent of treatments [21-26]. In this study, CA expression of three isoforms in *O. violaceus* and *B. juncea* were reported to respond to bicarbonate stress induced by $NaHCO_3$, respectively. Furthermore, multiple sequence alignments suggested that the three β-CAs were identified and characterized, which were highly similar to chloroplast isoforms CA (*OvCA1* and *BjCA1*), cytoplasm isoforms CA (*OvCA3* and *BjCA3*), and plasma membrane isoforms CA (*OvCA4* and *BjCA4*), respectively. *CA1*, which was localized at chloroplast and abundant in plant mesophyll cells, has the key function to facilitate CO_2 fixation [33]. The decrease in *CA1* expression had no significant effect on photosynthetic CO_2 fixation. *CA3* and *CA4*, which were localized at the cytoplasm and plasma membranes, function as the inter-conversion of HCO_3^- to CO_2, respectively [24]. Studies had reported increased expression of cytosolic carbonic anhydrase (*CA3*) as a result of a significant increase in total CA activity and little impact on CO_2 assimilation, but plasma membrane CA (*CA4*) gene expression only represented a small proportion of total CA activity in plant cells. In this study, the expression of three distinct CA genes and CA activity varied from plant species and extended

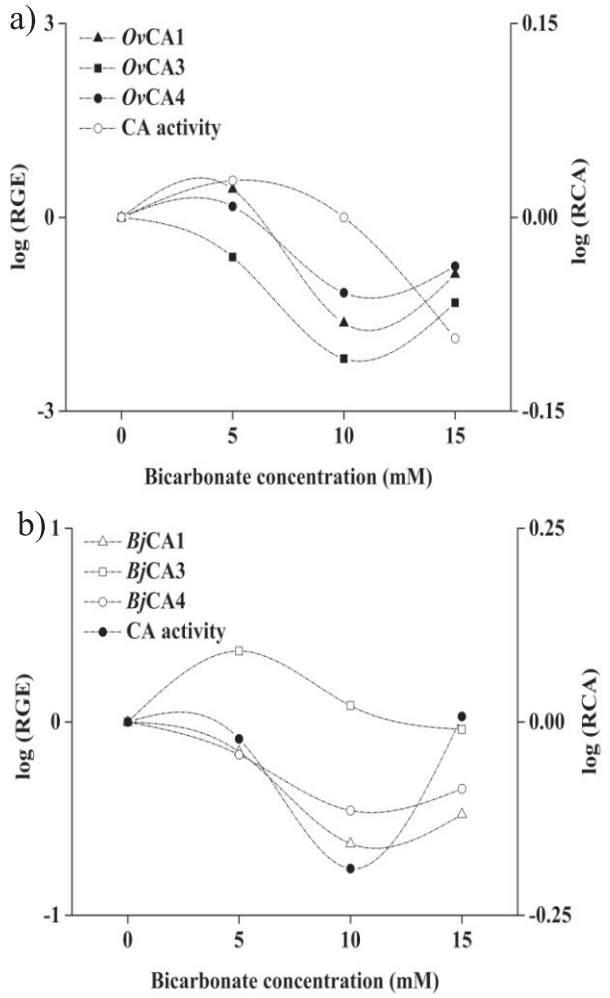

Fig. 4. The log relationship between relative gene expression (RGE) and relative CA activities (RCA, WAU mg $^{-1}$ Protein) in *Orychophragmus violaceus* L. a) and *Brassica juncea* L. b). Values show means on each bicarbonate stress treatment (n = 5).

bicarbonate stress. Thus, the presence of three distinct expressions of CA genes in response to $NaHCO_3$-induced bicarbonate stress between *O. violaceus* and *B. juncea* suggested that the two cruciferous plants exhibited different regulating mechanisms under bicarbonate stress.

We also studied the relationship between the expression of three distinct β-CA genes and the CA activities in response to bicarbonate stress in the two cruciferous plant species (Fig. 4). The expression patterns of the three CA genes during the bicarbonate stress were different between the two plant species. For *O. violaceus*, the expression levels of *OvCA1* and *OvCA4* in the leaves changed synchronously with CA activities as bicarbonate stress was intensified (Figs 1 and 3a); and the relative gene expression (RGE) levels of *OvCA1*, *OvCA3*, and *OvCA4* changed synchronously, but did not change synchronously with relative CA activities (RCA) among bicarbonate-treatments (Fig. 4a). However, in *B. juncea*, *BjCA1* and *BjCA4* expression levels were significantly higher than that of *BjCA3*, and dropped synchronously with increasing bicarbonate levels, but *BjCA1*exhibited the highest value at 5 mM $NaHCO_3$ levels, then dropped among bicarbonate treatments (Fig. 3b). Furthermore, relative CA activities (RCA) changed synchronously with relative *BjCA1* and *BjCA4* expressions (RGE) of *B. juncea* in responses to bicarbonate stress (Fig. 4b).

With increasing concentrations of bicarbonate stress, the relative expression levels of *OvCA1* and *OvCA4* changed synchronously with CA activities in the two plant species. However, the contribution of *CA4* to total CA activity was small, and its regulation on the intercellular HCO_3^- can be ignored; the contribution of *CA1* to total CA activity was abundant and relatively stable in abnormal environmental conditions. Therefore, we deduced that the responses of *CA1* and *CA4* of *O. violaceus* and *CA3* of *B. juncea* to bicarbonate stress partly regulate HCO_3^- into water and CO_2 supply to plants, which partly explain that *O. violaceus* could more adapted to $NaHCO_3$-induced bicarbonate stress than *B. juncea*.

Conclusions

Our study partially elucidated the differences in gene expression responses and adaptation mechanisms of the two cruciferous plant species exposed to different bicarbonate stress levels. These results suggested that *O. violaceus* exhibited higher *OvCA3* expression levels and CA activities than *B. juncea*. Therefore, *O. violaceus* could better adapt to slight bicarbonate stress than *B. juncea*. Distinct CA gene expression levels could partly explain these different adaptation strategies of the two cruciferous plants exposed to different bicarbonate stress levels.

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Conflict of Interest

The authors declare no conflict of interest.

Abbreviations

CA: Carbonic anhydrase
qPCR: Quantitative polymerase chain reaction
OvCA: *Orychophragmus violaceus* CA gene
BjCA: *Brassica juncea* CA gene

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