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Expression of Carbonic Anhydrase Genes Under Dehydration and Osmotic Stress in *Arabidopsis Thaliana* Leaves

Yanyou Wu[1,2,*], Dick Vreugdenhil[3], Congqiang Liu[1], and Weiguo Fu[2]

[1]*State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang 550002, China*
[2]*Key Laboratory of Modern Agricultural Equipment and Technology, Ministry of Education and Jiangsu Province, Institute of Agricultural Engineering, Jiangsu University, Zhenjiang, Jiangsu 210213, P. R. China*
[3]*Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands*

Expression of the genes coding for carbonic anhydrase in leaves of *Arabidopsis thaliana*, as induced by dehydration and osmotic stress (polyethylene glycol, PEG) was analyzed, and the activities of carbonic anhydrase were determined, using three accessions, *Landsberg erecta* (*ler*), *Cape verde Islands* (*Cvi*) and *Antwerpen* (*An*). Carbonic anhydrase activities and the expression of genes encoding carbonic anhydrases in *Arabidopsis* leaves had different responses to dehydration and to polyethylene glycol treatment. The expression of the gene coding for the chloroplastic form of carbonic anhydrase (*ca1*) was little influenced by the treatments, as compared to that for the cytoplamic form (*ca2*). The expression of the genes *ca1* and *ca2* in *Ler* accession was stable. The dehydration had little influence on the expression of *ca1* and the carbonic anhydrase activities, and the osmotic stress induced by polyethylene glycol may decrease the expression of *ca2* in *Cvi* and *An* accessions, and the carbonic anhydrase activities in all of accession. The results obtained from the present study can partly account for the difference of adaptation-drought mechanisms among the three accessions.

Keywords: *Arabidopsis Thaliana*, Carbonic Anhydrase, Gene Expression, Dehydration, Polyethylene Glycol.

1. INTRODUCTION

Arabidopsis thaliana (L.) Heyhn. is a small weed plant belonging to the mustard family (*Brassicaceae*). This wild crucifer has become an important model system of choice for research in plant biology.[1] Its genome has been completely sequenced, and the genetic variation for traits of many accessions in nature reflects their adaptation to some stress conditions.[2] Different accessions of *A. thaliana* have different strategies to cope with drought stress. *A. thaliana Landsberg erecta* (*Ler*) with a short life cycle display an escape drought stress, *Cape verde Islands* (*Cvi*) withstands water stress by drought tolerance, and *Antwerpen* (*An*) exhibits dehydration avoidance to drought stress.[3–6]

Carbonic anhydrase (carbonic anhydrase, CA; carbonate hydrolyase, EC 4.2.1.1), a zinc-containing metalloenzyme that catalyses the reversible conversion of CO_2 to bicarbonate, is widely distributed in organism, where it is involved in various physiological processes.[7,8]

The CA's responses to various environmental stresses varied with species, ecotypes and the CA isoforms. Under polyethylene glycol-mediated water stress, the activities of CA in leaves of maize were enhanced.[9] Dehydration of leaves also caused an increase in CA activity in barley.[10] Large increase in CA activity was observed in the rice leaf under drought, salts and osmotic stress.[11–14] Nevertheless, when the leaf water potential was below −2.0 MPa, the activities of CA in leaves of maize were decreased significantly.[15]

Expression of genes coding for CA responded to environmental stresses. Expression of the gene coding for CA in leaves and roots of rice responded to salts and osmotic stress, and was related to stress tolerance in rice.[14] Chilling stress heavily suppressed the expression of the gene encoding a beta CA in leaves of mung bean seedlings.[16] The level of expression of the gene-encoding beta CA in leaves of rye when grown at 20 °C/50 μmol m^{-2} s^{-1} growth regime was significantly lower than grown under other (water- or light-stressed) conditions.[17] The level of expression of gene *cah-4b* encoding an alpha carbonic anhydrases CAH-4b in *Caenorhabditis elegans* in an alkaline environment (pH = 9) was five-fold higher than that in a neutral environment (pH = 7).[12] The expression of two CA isoforms in the blue crab was stimulated by salinity.[13]

Six distinct cDNAs encoding beta CA in *A. thaliana*, including *ca1* (locus AT3g01500) encoding chloroplastic CA, *ca2*

*Author to whom correspondence should be addressed.

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(AT5g14740) encoding cytoplasmic CA, *ca3* (AT1g23730), *ca4* (AT1g70410), *ca5* (AT4g33580), and *ca6* (AT1g58180) have been sequenced.[18,19] CA1, CA2 and CA4 were found in mesophyll cells, and CA1, CA4 and CA6 in guard cells.[20] CA1 and CA4 in guard cells can form intracellular bicarbonate, which participate in stomatal signaling.[20]

We can learn from the above fact that expression of genes coding for CA and the CA activity manifold responded to water stress and osmotic stress. The responses to water stress and osmotic stress varied with ecotypes and the CA isoforms. However, whether the CA2 in mesophyll cells can modulate the water and CO_2 supply was unknown. The present study aims to understand the regulation of CA induced by water stress through identifying the difference between *ca1* and *ca2* gene expressions and the CA activity under dehydration and polyethylene glycol treatment in different accessions of *A. thaliana*.

2. MATERIALS AND METHODS

Arabidopsis thaliana accessions, *Landsberg erecta* (*Ler*), *Cape verde Islands* (*Cvi*), and *Antwerpen* (*An*) were obtained from the Laboratory of Genetics at Wageningen University. All seeds were pre-sown in Petri dishes on water saturated filter paper, followed by cold treatment for 3 d at 4 °C. Then, they were transferred to a climate room at 25 °C and 16 h light for 2 d before planting in plates with soil for the experiment of drying treatments or in the tubes with agar in hydroponics on Hoagland nutrient solution[21] for the experiment of osmotic stress. The plants were grown in an air-conditioned greenhouse with 70% relative humidity, supplemented with additional light providing a day length of at least 16 h light (long day), with a light intensity of 125 μmol m^{-2} s^{-1}, and maintained at a temperature between 22–25 °C (day) and 18 °C (night).

The detached leaves from four-week-old soil-grown plants were dehydrated for 0, 0.5, 1, 2 and 4 hours on Whatman 3 MM paper at room temperature and approximately 60% humidity under a light intensity of 125 μmol m^{-2} s^{-1}. The percent of water loss was obtained by weighing.

Four-week-old hydroponically-grown plants were subjected to osmotic stress for three days in Hoagland nutrient solution, containing 0 (the control, osmotic potential: −0.08 MPa), 2% (slight, osmotic potential: −0.20 MPa), 4% (moderate, osmotic potential: −0.40 MPa) and 8% (severe, osmotic potential: −1.03 MPa) (w/v) polyethylene glycol (PEG) 6000, respectively, under a light intensity of 125 μmol m^{-2} s^{-1}. The leaves were used for gene expression analysis and CA activity analysis.

RNA was isolated from leaf tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) analysis was conducted using the Superscript III One-step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) based on the protocols provided in the kits. The thermocycler parameters for the reaction were: 55 °C for 30 min, 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 55 °C (for tubulin and carbonic anhydrase 1 gene-*ca1*) or 51 °C (for carbonic anhydrase 2 gene-*ca2*) for 1 min and 72 °C for 1 min, followed by 72 °C for 5 min. *A. thaliana* β-tubulin gene (GenBank accession number At5g23860) was used as the internal standard and amplified using primers: forward primer, 5′-GCCAATCCGGTGCTGGTAACA-3′, reverse primer, 5′-CATACCAGATCCAGTTCCTCCTCCC-3′. The *A. thaliana ca1* (GenBank accession number At3g01500) was amplified using primers: forward primer, CTCACTCTCTCTGATCTCCG CTTCTC; reverse primer, CCAATAAGGGGCAATGATAGGAG CAGG. The *A. thaliana ca2* (GenBank accession number AT5g14740) was amplified using primers: forward primer, ATCAACAAGAGGCGGAGATACG; reverse primer, AGCAGA TTGGAGAGTCGTC.

The measurement of the CA activity conforms to the modified Wilbur and Anderson's method.[22] The protein content of leaves was measured specrophotometrically at 280 nm and by the Coomassie brilliant blue method.[23]

All determinations were performed in quintuplicate. The mean and standard deviation were calculated for each treatment. One-way ANOVA and LSD tests were conducted for each group. All analyses were carried out with the Statistical Package for the Social Sciences (SPSS) version 9.0 (SPSS, Inc., Chicago, IL, USA).

A phylogenetic tree from six sequences cDNAs encoding beta CA in *A. thaliana*, including *ca1*(locus:AT3g01500, sequence identification:gi|145337936) encoding chloroplastic CA, *ca2* (AT5g14740, gi|79611323) encoding cytoplasmic CA, *ca3*(AT1g23730, gi|145336073), *ca4*(AT1g70410, gi|145337368), *ca5*(AT4g33580, gi|145352174), and *ca6*(AT1g58180, gi|334183406) was drawn using the DNAman software package (Version 5.2.2, Lynnon Biosoft, Canada) and CLUSTALX software (EMBL, Heidelberg, Germany).

3. RESULTS

The dehydration had no significant differences among accessions (Fig. 1). In all accessions, *ca1* gene expression of *A. thaliana* leaves, which were induced by a short period of dehydration stress, was stable, and the differences among treatments was not significant. However, *ca2* gene expression changed with the dehydration time (Fig. 2(A)). In *Ler* accession, the *ca2* gene expression levels did not alter much during the first two hours of dehydration, but the level decreased when the leaves was desiccated for 4 hours. In *Cvi* accession, the *ca2* gene expression levels increased during 0 to 2 hours dehydration, decreased thereafter. In *An* accession, the *ca2* gene expression levels decreased with the dehydration time.

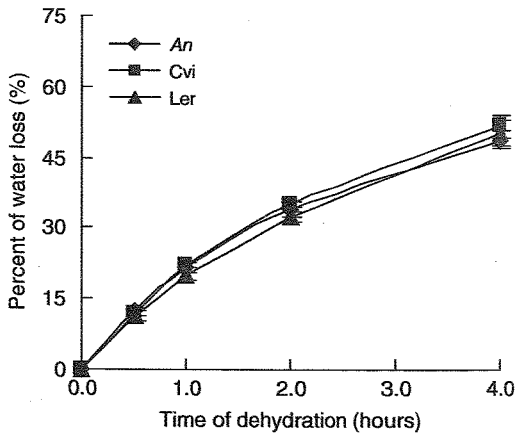

Fig. 1. The difference of leaves water loss induced by dehydration among *A. thaliana* accessions. *Ler*, *Cvi* and *An* are indicated *Landsberg erecta*, *Cape verde Islands* and *Antwerpen* accessions, respectively.

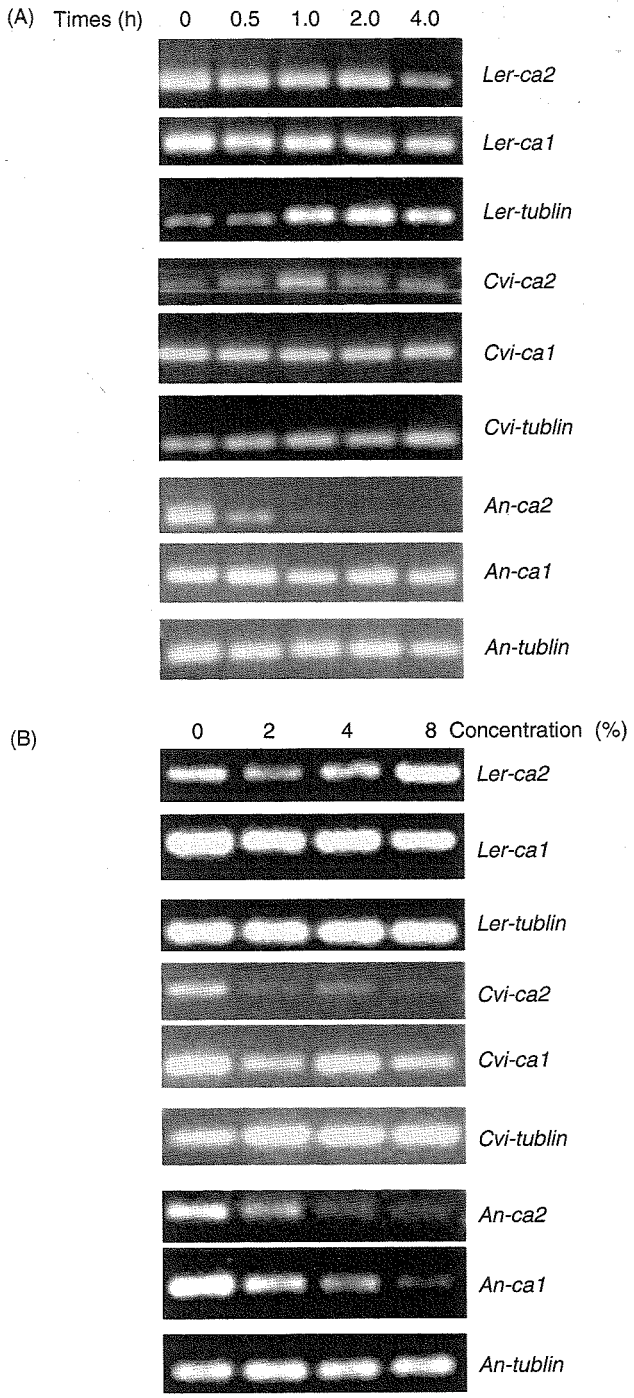

Fig. 2. The expression difference of CA gene induced by dehydration (A) or PEG treatments (B) among *A. thaliana* accessions (Fig. 1. A: From left to right, the values on the top of the figure are indicated the time of dehydration, 0, 0.5, 1.0, 2.0, and 4.0 hours, respectively. Figure 1B: From left to right, the values on the top of the figure are indicated the concentration of polyethylene glycol (%), 0, 2, 4, and 8%, respectively.). *ca1*, *ca2*, and *tublin* are indicated *A. thaliana* CA1 gene, CA2 gene, β-tubulin gene, respectively. *Ler*, *Cvi* and *An* are indicated *Landsberg erecta*, *Cape verde Islands* and *Antwerpen* accessions, respectively.

CA activity in the leaves was also examined under dehydration (Table I). CA activities did not differ significantly between treatments. However, the absolute levels of activity were different, and that in *Cvi* accession was the highest among accessions.

Table I. Effect of dehydration on CA activity in the leaves of *A. thaliana* (WAU mg^{-1} Protein) (WAU = Wilbur-Anderson Units). SE is shown in brackets ($n = 5$). No significant ($P < 0.05$) differences were observed. *Ler*, *Cvi* and *An* are indicated *Landsberg erecta*, *Cape verde Islands* and *Antwerpen* accessions, respectively.

	Time of dehydration (hours)				
Accession	0	0.5	1.0	2.0	4.0
Ler	51.84 a (17.30)	61.46 a (9.47)	59.40 a (13.73)	47.90 a (13.99)	63.84 a (24.68)
Cvi	97.80 a (12.06)	92.89 a (34.80)	90.05 a (24.63)	88.61 a (11.41)	119.92 a (36.56)
An	65.09 a (6.52)	68.37 a (15.59)	64.52 a (20.38)	39.10 a (5.29)	73.04 a (14.59)

The pattern of *ca1* gene expression induced by PEG in *A. thaliana* was somewhat different among accessions (Fig. 2(B)). In *Ler* and *Cvi* accessions, *ca1* gene expression level was hardly influenced by the treatments. In *An* accession, *ca1* gene expression levels decreased with increasing concentration of PEG. The pattern of *ca2* gene expression induced by PEG was also different among accessions (Fig. 2(B)). In *Ler* accessions, *ca2* gene expression levels did not differ significantly among treatments except for the 8% PEG treatment. In *Cvi* and *An* accessions, *ca2* gene expression levels decreased with the concentration of PEG treatment. CA activity was greatly influenced by PEG treatment, decreasing by 69 to 87%, depending on accessions (Table II). In *Ler* and *An* accessions, CA activity decreased rapidly with increasing concentration of PEG. But, In *Cvi* accessions, CA activity decreased slowly with the concentration of PEG treatment, and CA activity under the 8% PEG treatment was still up to 31% that under the control.

Figure 3 showed a phylogenetic tree from six sequences of beta-CA in *A. thaliana*. *ca1* and *ca2* were in the same cluster, *ca3* and *ca4* in another cluster, and *ca5* and *ca6* can also place in the same cluster.

4. DISCUSSION

Many studies had verified that CA activity was regulated by the level of *ca* mRNA, and there was a good correlation between the *ca* mRNA level and the CA activity.[8, 24, 25] However, in the present study, the CA activities did not change with the variation of levels of *ca1* and *ca2* gene expression induced by short dehydration in *Arabidopsis* leaves, especially taking into account the great variation in *ca2* gene expression. In *Ler* accessions, the reduction of CA activity did not correspond to the decreasing levels of gene expression under PEG treatment. It suggested that CA1 and CA2 partly contributed the activity of total CA in leaves, and other CA isoforms can account for the considerable contribution of the total CA activity.

In *Arabidopsis* leaves, CA activities and expression of genes encoding CA exhibited different responses to dehydration and PEG treatment. The dehydration had little influence on the expression of *ca1* and the CA activities, and the osmotic stress induced by PEG may decrease the expression of *ca1* in *An* accession, and the CA activities in all of accessions. Those results were dissimilar to those from rice and wheat.[11, 14, 16]

Our research had shown that the expression of *ca1* was stable compared to that of *ca2*, and that CA activities were more sensitive to PEG stress than to dehydration resulting from air-drying.

Table II. The difference of CA activity in the leaves of *A. thaliana* (WAU mg^{-1} Protein) (WAU = Wilbur-Anderson Units) induced by PEG. SE is shown in brackets ($n = 5$). The different letters following the figures in the same rows represent the significant difference ($P < 0.05$) among treatments. *Ler*, *Cvi* and *An* are indicated *Landsberg erecta*, *Cape verde Islands* and *Antwerpen* accession, respectively.

Accession		Concentration of PEG (%)			
		0	2%	4%	8%
Ler	WAU mg^{-1} Protein	48.31(4.19)d	21.16(5.05) c	15.27(2.49) b	6.84(1.28) a
	Percent %	100	45	32	14
Cvi	WAU mg^{-1} Protein	63.33(15.24)c	30.63(5.56) b	14.53(1.02) a	19.77(3.92) a
	Percent %	100	48	23	31
An	WAU mg^{-1} Protein	83.41(15.12)c	27.37(4.88) b	13.54(3.05) a	10.51(0.37) a
	Percent %	100	33	16	13

The pattern of *ca2* expression in response to the treatments was very different among the three accessions. For *Cvi* accession *ca2* expression under dehydration was firstly up-regulated, then down-regulated. For *An* accession *ca2* expression under dehydration was down-regulated. Under PEG treatment, *ca2* expression in *Cvi* and *An* accessions was down-regulated, and at the concentration of 8% PEG treatment, *ca2* expression in *Ler* accession was upregulated.

Our research had also shown that the expression of CA genes in *Ler* accession was stable compared with other two accessions. The expression of CA genes in *Ler* accession was not greatly affected by dehydration or osmotic stress. This study had also indicated that the variation of *ca2* expression did not result in parallel of CA activity. Therefore, we suggested that the *ca2* expression only marginally contributes to the total CA activity.

CA1 and CA2 have distinct roles. CA1, chloroplastic CA, functions to facilitate the supply of CO_2 from the stomatal cavity to the site of CO_2 fixation.[8] CA2, cytoplasmic CA, may function as the catalysis of HCO_3^- provided carbon skeletons for amino acid biosynthesis as well as replenishment of Krebs cycle intermediates.[1,9,26] Chloroplastic CA is abundant in plant mesophyll cells under normal growth conditions. The decline of CA1 activities in some plants has no significant negative effect on the photosynthetic assimilation of CO_2.[8,24,25,27] The response of CA2 was sensitive to the stress induced by dehydration and PEG treatment. The difference of the response of CA2, not CA1 to water stress may reflect the adaptation to environmental stress among plant species or accessions.

Carbonic anhydrase, in particular, CA2 can regulate internal water status when plant leaves were in water deficit.[22,28] Dehydration process of detached leaves was consistent in all accessions (Fig. 1). *Ler* accession had drought escape mechanism through a short life cycle, allowing it to reproduce before the environment becomes dry. It cannot regulate internal water status in a dry environment.[5,6] The present study showed that there is no obvious change in CA, in particular, *ca2* expression level and CA activity when leaves were in the short-term dehydration. Therefore, *Ler* accession cannot acclimate to the drought stress by *ca2* expression regulating internal water status.

Cvi accession had dehydration tolerance mechanism in a dry environment through increasing water use efficiency under drought stress.[4,5] The present study also inferred that the increasing of *ca2* expression could promote the ability of regulating the leaf internal water status when the leaves were in shorter time dehydration, but *ca2* expression and regulation ability decreased with the increasing time of dehydration thereafter.

An accession had dehydration avoidance mechanism in a dry environment by maximizing water uptake.[3,5] The detached leaves unable to acquire water from the environment were liable to subject to water stress. CA2 gene (*ca2*) expression in *An* accession would be inhibited when the detached leaves were dehydrated (Fig. 2(A)). Therefore, *An* accession can adapt to drought stress by increasing root biomass and leaf area,[3] not by CA2 regulating leaf internal water status.

Additionally, *ca2* expression and CA activity were seriously inhibited when the plants were treated by PEG for 3 days. Therefore, we speculated that *Ler*, *Cvi* and *An* accessions can not adapt to water stress for a longer period of time (Fig. 2(B)).

Overall, the findings of the present study provided partly explanation for the different strategies to adapt to drought among the three accessions. Nevertheless, in fact, the response of CAs on the water stress in leaves of *A. thaliana* had a grid-type regulation. Beta-CAs had six isoforms, which are localized in different tissues and organs, as well as different subcellular compartments, and their genetic distance is very small (Fig. 3). These isoforms were jointly in response to water stress like a network. CA1 and CA4 may be involved in the regulation of stomatal movements in guard cells,[20] CA2 in mesophyll cells may provide water and CO_2 for photosynthesis during the stomatal closures when the water stress came, while, CA6 may regulate the remove of carbon dioxide from the mitochondria.[18]

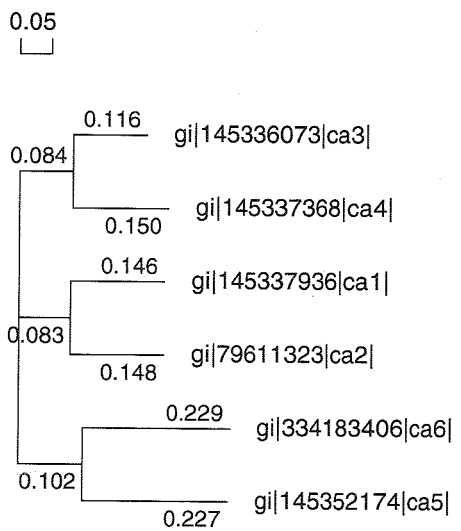

Fig. 3. Phylogenetic tree of *Arabidopsis* beta carbonic anhydrases. The scale bar indicates 0.05 substitutions per site.

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