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Tissue-Specific Differential Expression of Two β-carbonic Anhydrases in *Leucaena leucocephala* Under Abiotic Stress Conditions

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Abstract

Besides carbon fixation in plants, β-carbonic anhydrases (β-CAs) have been shown to be involved in plant adaptation against some abiotic stress conditions. The objective of this research was to determine the expression levels of two isoforms of β-CAs under various stress conditions in different tissues of *Leucaena leucocephala*, a highly stress-tolerant tree-legume of tropics. The cDNAs for two β-CAs isoforms, one chloroplastic (*cacp*) and other cytoplasmic (*cacyt*) were isolated. These isoforms have 79% similarities at amino acid level, and their secondary structures, active site residues including zinc ligands, and the three-dimensional (3D) quaternary structures were found to be highly conserved and similar to those of the β-CA from *Pisum sativum*, which is structurally well-characterized. The 3D-model of the two leucaena β-CAs predicted them as octameric proteins like the β-CA from *P. sativum*. The transcripts for *cacp* was found to be more abundant than the transcripts for *cacyt* in leaf and stem tissues, however the transcripts level of both β-CAs were similar in root tissues. The drought, salt, and light conditions caused up-regulation, and dark resulted down regulation of the *cacp* and *cacyt* transcripts. However, the changes in the expression

levels of *cacp* was more pronounced than those of *cacyt*, except in salt-stressed root tissues, which showed more pronounced up regulation of *cacyt* than that of *cacp*. This study suggests that abiotic stress conditions affecting the photosynthesis potential of plants also affect expression of different isoforms of β-CAs from C_3 plants differentially, which may contribute to plants' survival under stress conditions.

Keywords: β-carbonic anhydrase, *Leucaena leucocephala,* Abiotic stress, Carbon fixation, C_3 plants

1. Introduction

Carbonic anhydrase (CA) is a ubiquitous enzyme that is known to exist in all living forms including, plants, algae, animals, prokaryotes and viruses. CAs have been classified in five different families; α, β, γ, δ, and ζ (Zimmerman & Ferry, 2008; Moroney et al., 2011) and all these CA families catalyze the same biochemical reaction i.e. reversible hydration of carbon dioxide (CO_2) to form bicarbonate (HCO_3^-) and protons in a reaction represented as $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. Among all CA families, only β-CA and ζ-CA have small degree of sequence similarities at amino acid level; the sequences of other CA families do not exhibit any significant similarities with each other. First identified in spinach (Burnell et al., 1990; Fawcett et al., 1990), β-CAs have been reported to exist in eubacteria, algae, archaebacteria (Hewett-Emmett & Tashian, 1996; Smith et al., 2002) and in all the species of fungi and plants (Hewett-Emmett, 2000; Götz et al., 1999). The β-CAs exist in different oligomeric states and previous crystallographic studies have identified dimeric, tetrameric and octameric β-CAs (Strop et al., 2001; Kimber & Pai, 2000; Smith et al., 2000). CAs are metalloenzymes and most of the CAs require zinc ion as a prosthetic group. The zinc ion in the active site of CAs is coordinated by a zinc-binding hydrophobic pocket. The zinc-binding pocket of β-CAs is made of a histidine and two cysteine residues (Rowlett et al., 1994; Bracey et al., 1994). Additionally, a conserved aspartate residue in the active site of β-CAs has been suggested to be responsible for proton transport (Strop et al., 2001).

Based on different mechanisms for fixing inorganic carbon, plants can be categorized into Crassulacean acid metabolism (CAM), C_4, and C_3 plants and the role of β-CA differs in these plant groups (Burnell, 2000). In CAM and C4 plants, β-CAs act as the primary and first enzyme in CO_2 fixation (Badger & Price, 1994; Hatch & Burnell, 1990). A β-CA from a C_4 plant, *Pennisetum glaucum*, which is evolutionary very close to chloroplast β-CA isoform, has been shown to be differentially up-regulated under various abiotic stress conditions (Kaul et al., 2011), suggesting a possible new role of β-CAs in enhancing plants' capacity to withstand various abiotic stress conditions, in addition to the well-established role of carbon fixation. Such extensive study on β-CAs from C_3 plants is lacking and hence the role of β-CAs in C_3 plants is not well-defined.

β-CA is an abundant enzyme in the leaves of C_3 plants and represents ~2% of total protein (Okabeet al., 1984). In plants, the green tissues and roots both express β-CAs and they have been predicted to have diverse sub-cellular localization including chloroplasts, cytoplasm and mitochondria (Fabre et al., 2007; Tetu et al., 2007; Ludwig, 2012). However, only chloroplast-localized and cytoplasmic β-CAs have been suggested to have a role in carbon fixation (Syrjänen et al., 2010). Previously, two cDNAs, one each for chloroplastic and cytoplasmic β-CAs of *Arabidopisis thaliana* (*Arabidopsis*) were isolated and the transcript levels of these β-CAs were found to be regulated by light and dark conditions (Fett & Coleman, 1994). This report also suggested that cytoplasmic β-CA from *Arabidopsis* may have non-photosynthetic roles. Considering (i) limited study on β-CAs from C_3 plants, and (ii) possible role of β-CAs under abiotic stress conditions, it is important to study the role of chloroplastic and cytoplasmic β-CAs from a C_3 plant that is highly tolerant to abiotic stress conditions. We chose to study β-CAs of a C_3 plant *Leucaena leucocephala* (leucaena), which

can withstand a variety of abiotic stress conditions including drought. In this study, we have isolated two leucaena cDNAs encoding chloroplastic and cytoplasmic β-CA isoforms and investigated their expression under various stress conditions.

2. Materials and Methods

2.1 Plant Growth and Imitation of Stress Conditions

Leucaena leucocephala cv. K-636 (leucaena) seeds were collected from University of Hawaii research station, Waimanalo, Honolulu. The mature seeds were scarified and surface sterilized as described previously (Pal et al., 2012). The surface sterilized seeds were germinated on half-strength Murashige and Skoog (MS) media for 2-3 days under sterile conditions at 28 °C in dark. Germinated seedlings were then grown in vermiculite for four weeks using Hoagland solution containing 5 mM $Ca(NO_3)_2.H_2O$, 2 mM $MgSO_4$, 5 mM KNO_3, 1 mM KH_2PO_4, 0.02 mM $FeSO_4.7H_2O$, 0.02 mM Na-EDTA, 0.045 mM H_2BO_3, 0.01 mM $MnCl_2.H_2O$, 0.8 µM $ZnSO_4$, 0.1 µM $NaMoO_4.2H_2O$ and 0.3 µM $CuSO_4.5H_2O$. The plants were maintained at 25±2 °C with 16/8 h dark photoperiod with an irradiance of 30 µmol $s^{-1}m^{-2}$, unless otherwise stated. The plants were watered with Hoagland solution twice a week and water lost by evapotranspiration was compensated for by adding deionized water, daily. A few eight weeks old seedlings were used for RNA extraction, which later was used for isolating full-length cDNAs.

Eight weeks old leucaena plants were exposed to drought stress conditions by providing the plants with Hoagland solution supplemented with 15% polyethylene glycol (PEG 10000), the osmotic pressure of which is ~ -3.02 bars. The leaf, stem, and root samples were collected from the plants after 2, 6, 12, 24, 48, 72, and 96 h time points of treatment. For salt stress treatment, eight weeks old leucaena plants were watered with Hoagland solution containing 150 mM NaCl as used by (Kamal et al., 2012). The leaf, stem and root samples were collected after 1, 4, 6, 12, 24, and 48 h time points. Leaf, stem and root tissue samples isolated at same time points as those of treatments from leucaena plants that were watered only with Hoagland solution, without any addition of PEG or NaCl, served as controls for drought and salt stress conditions. To study the effect of light and dark on the transcript level of *cacp* and *cacyt*, the eight weeks old plants were kept under light or dark conditions for 1, 3, 8, 16, 24, 36 and 48 h. Plants grown under 16 h light and 8 h dark photoperiod served as control plants. The tissues collected from control plants just before the beginning of light photoperiod and dark photoperiod served as the controls for plants exposed to dark and light conditions, respectively. For bright light stress conditions eight weeks old plants were exposed to bright light 150 µmol $s^{-1}m^{-2}$ for 1, 3 and 8 h. Controls were collected at the same time points as stress-treated plant from the plants grown under normal light condition. The collected samples from control and treatments were immediately frozen in liquid nitrogen and stored at -80 °C until used for RNA extraction.

2.2 RNA Extraction and cDNA Synthesis

Frozen samples of whole seedlings or their different tissues were finely grounded in liquid nitrogen and total RNA was extracted using Qiagen RNeasy plant mini kit (Qiagen, CA,

USA) following the manufacturer's instruction. Total RNA was then treated with TURBO DNase (Ambion, TX, USA) for elimination of genomic DNA. The quality of RNA was verified by running 1 µg of total RNA on 1% non-denaturing Agarose gel. First-strand cDNA was synthesized from 2 µg of total RNA using MMLV reverse transcriptase (Promega, WI, USA).

2.3 Isolation of Chloroplastic and Cytoplasmic β-Carbonic Anhydrase cDNA

A 515 bp partial cDNA fragment, homologous to β-CA, was obtained from interspecies suppression subtractive hybridization (iSSH) clones of leucaena (Negiet al., 2011). To extend the partial cDNA fragment at both 5'- and 3'-ends, RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was performed using First Choice® RLM-RACE Kit (Ambion) according to the manufacturer's instruction. For 5'-RACE of the cDNA fragment, 5'-ROP-*cacp* and 5'-RIP-*cacp* gene-specific primers (Table 1) were used with the 5'-RACE outer and 5'-RACE inner primers (Ambion) and for 3'-RACE of the cDNA fragment, 3'-ROP-*cacp* and 3'-RIP-*cacp* (Table 1) were used with 3-RACE outer and 3'RACE inner primer (Ambion). The 5'- and 3'-extended sequences were cloned in pGEMT-easy vector (Promega) and sequenced. The obtained sequences were then assembled to obtain the full-length cDNA for chloroplastic β-CA from leucaena.

Table 1. Primers used in this study and their description

Primer name	Sequence (5' to 3')	Description
5'-ROP-*cacp*	TGGGTGAAGATCGGTTTACCTGCA	These primers were used in the 5'-RLM-RACE of *cacp*
5'-RIP-*cacp*	TGCTTGTGGTGGTATCAAGGGTCT	
3'-ROP-*cacp*	ACTTGGTCCCACCATATTGCCAGA	These primers were used in the 3'-RLM-RACE of *cacp*
3'-RIP-*cacp*	TCTCAAGGTGTCGGAAATCGTGGT	
F-*cacyt*	ATCTTGAATTTCCAACCTGGTGAGGCTTT	These primers were used to amplify 151bp*cacyt*
R-*cacyt*	CCTATCACCAAAATGTTCTCCACCTT	
3'-RP-*cacyt*	TGAGGCTTTTGTGGTTCGCAACATCGC	These primers were used in the 5'- and 3'-RLM-RACE of *cacyt*
5'-ROP-*cacyt*	CCACCTTTAAGTGCAACACTGC	

To isolate leucaena cDNA for cytoplasmic carbonic anhydrase (*cacyt*), three nucleotide sequences for *cacyt* one each from *Arabidopsis thaliana* (gi|145362379), *Ricinus communis* (gi|255568811) and *Populus trichocarpa* (gi|224107828) were obtained from NCBI. Nucleotide sequences of these *cacyt*were compared using ClustalW and F-*cacyt* and R-*cacyt*primers were designed from the conserved regions (Table 1) and PCR was performed to amplify a partial cDNA fragment of cytoplasmic β-CA. The 5'- and 3'-ends of partial cDNA sequences for *cacyt* was extended by RLM-RACE using First Choice® RLM-RACE Kit (Ambion) according to the manufacturer's instruction. The gene-specific primers,

5'-RP-*cacyt* was used in the 5'-RACE of the *cacyt* cDNA fragment whereas, 3'-RP-*cacyt* was used in the 3'-RACE (Table 1). The PCR products obtained as a result of 5'- and 3'-RACE were cloned in pGEMT-easy vector (Promega) and sequenced. The resultant sequences were then assembled to obtain the full-length cDNA for cytoplasmic β-CA from leucaena.

2.4 Sequence Analyses and Prediction of Sub-Cellular Localization

The homology searches of the partial and full-length cDNA sequences for the leucaena β-CAs were carried out using BLASTx program of NCBI against its reference protein database. The homology searches for the deduced amino acid sequences were performed using BLASTp program of NCBI against its reference protein database. The open reading frame (ORF) for each of the full-length *cacp* and *cacyt* were identified using 'ORF finder' tool of sequence manipulation suite. The deduced amino acid sequence for *cacp* and *cacyt* were then studied using TargetP 1.1 server (Emanuelsson et al., 2000) to predict their sub-cellular location. The TargetP 1.1 server predicts the sub-cellular location of eukaryotic proteins based on the presence of N-terminal pre-sequences such as chloroplast transit peptide, mitochondrial signal peptide and secretory pathway signal peptide. In this analysis 'Plant' was selected as the 'organism group' considering that the *cacp* and *cacyt* are of plant origin. The cleavage site prediction was also performed in this analysis.

2.5 In Silico Structural Analysis of Leucaena β-CAs

In order to study the secondary structure and active site architecture of leucaena β-CAs, the β-CA from the C_3 plant *Pisum sativum* (gi|8569250) was used as a reference sequence as its X-Ray crystallographic structure has already been resolved (Kimber & Pai, 2000). The β-CA amino acid sequences of leucaena and *Pisum sativum* were studied for their secondary structure using the PSIPRED server (McGuffinet al., 2000). The prediction method selected for analysis was PSIPRED v3.0. The signal peptide region of the chloroplastic β-CA was eliminated for analysis. The conserved regions in the chloroplastic β-CA of leucaena was determined by comparing its sequence with other chloroplastic β-CAs from C_3 plants including *Glycine max* (gi|356511666), *Medicago truncatula*(gi|357495985), *Populus trichocarpa* (gi|224055529), *Vitis vinifera* (gi|225452452, *Ricinus communis* (gi|255567325) and *Arabidopsis thaliana* (gi|30678353), whereas the conserved region of cytoplasmic β-CA of leucaena was identified by its comparison with cytoplasmic β-CAs of *A. thaliana* (accession NM_202390.2), *R. communis* (accession XM_002525331.1), and *P. trichocarpa* (accession XM_002314581.1). The homology-based modeling of three-dimensional (3D) structures of CAcp and CAcyt proteins were performed using SWISS-MODEL server (Arnold et al., 2006; Schwede et al., 2003; Guex & Peitsch, 1997).

2.6 Expression Analyses of cacp and cacyt

The designing of specific primers for *cacp* and *cacyt* and optimization of RT-PCR condition are described in Supplementary material (SM1, Table S1, and Figure S1). The semi-quantitative tissue specific expression of *cacp* and *cacyt* is described in Supplementary materials (SM2, and Figure S2). The suitable internal reference genes were

identified using the method as described previously by Negiet al., 2011. The results for identification of suitable internal reference gene are shown in Supplementary material (SM3, and Figure S8). For expression analysis of *cacp* and *cacyt*, the quantitative real-time PCR (qPCR) was performed using the 1µl of 10-fold diluted cDNA from control and treatments in a 15µl of reaction consisting of 0.3µl each of 10µM forward and 10µM reverse primer, and 1X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The reactions were carried out in Mini Opticon system (BioRad) and the qPCR parameter consisted of initial denaturation of 95 °C for 5 min and then 32 cycles of 95 °C for 15 s, 52 °C (for *cacp*) and 62 °C (for *cacyt*) for 20 s, and 72 °C for 25 s, followed by a final annealing for 5min at 72°C. In each qPCR reaction the specificity of primers were confirmed by performing melting curve analysis of the amplified products. The Ct values of control and treatments were recorded and were used for calculating the transcript expression levels under each condition. For each qPCR assay two biological and three PCR replicates were used. The Livak method (Livak & Schmittgen, 2001) was used to quantify the relative expression of the target gene normalized to that of the selected internal reference gene.

3. Results

3.1 Isolation of Chloroplastic and Cytoplasmic β-CA From Leucaena

The 515 bp partial sequence that was obtained from the iSSH clones of leucaena showed high homology with the carbonic anhydrase of *M. truncatula* in BLASTx analysis against reference protein database. The homology between the two sequences exhibited 82% identical and 90% positive amino acid residues (Supplementary materials, Figure S3). The sub-cellular localization of this partial sequence was uncertain at this point, therefore, we used the term ‘β-CA1’ for this leucaena β-CA cDNA fragment until its localization was predicted. The 5’- and 3’-RACE of β-CA1 resulted in the addition of 569 bp at the 5’-end and 65 bp at the 3’-end, respectively. This resulted in a total of 1149 bp long β-CA1 sequence with 34 bp 5’-UTR and 134 bp 3’-UTR. The ORF of the β-CA1 was found to be 981 bp with the deduced amino acid sequence of 326 residues. The BLASTp analysis of the deduced amino acid sequence of β-CA1 exhibited strong homology with the chloroplastic β-CA from *Glycine max*. Both the sequences share 76% identical and 85% positive amino acid residues (Supplementary materials, Figure S4). The high homology of β-CA1 with the chloroplastic carbonic anhydrase of *G. max* indicates that the β-CA1 may be a chloroplast-localized enzyme. However, to further verify its location, the amino acid sequence of β-CA1 was subjected to the TargetP1.1 server using the plant networks. The β-CA1 was predicted to have an N-terminal chloroplast signal peptide with the reliability class value of 1, which is the strongest confidence value of prediction (Table 2). The identified chloroplast transit peptide consists of 36 amino acids with the sequence ‘MSTASINGCCLSSFSSSKTSLPSKFSVSARLATPPP’. This suggests that the β-CA1 encodes a chloroplast-localized protein and hence we termed the cDNA as ‘chloroplastic β-CA’ (*cacp*).

After identifying and isolating the leucaena cDNA for chloroplastic β-CA, the next goal was to isolate the leucaena cDNA for cytoplasmic β-CA (*cacyt*). The PCR using leucaena cDNA as the template and F-*cacyt* and R-*cacyt* as the primers resulted in the amplification of a 151 bp sequence. Despite the small sequence coverage (50 amino acid), the BLASTx analysis of the resulted sequence showed high homology with the β-CA of *A. thaliana* with 86% identities and 94% positives residues (Supplementary materials, Figure S5). The 5'- and 3'-RACE resulted in the addition of 416 bp at the 5'-end and 443 bp additions at the 3'-end of the 151 bp partial sequence of leucaena β-CA. The final sequence assembled after 5'- and 3'-RACE consists of 1010 bp with 129 bp long 5'-UTR and 119 bp long 3'-UTR. The 1010-bp long cDNA has a 762-bp long ORF, which encodes for a β-CA with 253 amino acid residues. The BLASTp analysis of the full-length deduced amino acid sequence of leucaena β-CA exhibited strong homology with the carbonic anhydrase from *M. truncatula* with 79% identities and 90% positives (Supplementary materials, Figure S6). The sub-cellular localization of this sequence was predicted using TargetP1.1 server. The sequence was predicted to have no signal peptide with high reliability class value of 2 (Table 2), suggesting it to be a cytoplasmic β-CA. The GenBank accession numbers for chloroplastic and cytoplasmic β-CA from *L. leucocephala* are KC924756 and KC924757, respectively.

Table 2. Prediction of sub-cellular localization of the CAcp and CAcyt protein of leucaena using TargetP 1.1 server

Protein (amino acid residues)	Signal peptide neural network score on which final prediction is based			Other	Location	Reliability Class*	Signal peptide length
	Chloroplast transit peptide	Mitochondrial transit peptide	Secretory protein				
CAcp (323)	0.974	0.38	0.012	0.023	Chloroplast	1	36
CAcyt (253)	0.041	0.162	0.143	0.876	-	2	-

* Reliability class (RC), from 1 to 5, where 1 indicates the strongest prediction.

3.2 In Silico Structural Analysis of Leucaena CAcp and CAcyt

β-CAs in general has high homology and conserved secondary structure irrespective of their different sub-cellular localization. Therefore, to compare the sequence similarity between *cacp* and *cacyt* encoded proteins (CAcp and CAcyt), the deduced amino acid sequences of the two β-CAs from leucaena were aligned using ClustalW. The signal peptide region of the CAcp was eliminated from the alignment.

The two sequences showed high homology with 64% identical and 79% similar amino acid residues (Supplementary materials, Figure S7). For secondary structure analysis, the well

resolved and studied β-CA from the C_3 plant *P. sativum* (gi|8569250) was used as the reference. The secondary structure of β-CA from *P. sativum* follows a pattern of α-helix and β-strand in a sequence of (α1, α2, β1, β2, α3, β3, α4, α5, α6, α7, β4, β5). We identified the same sequential pattern of α-helix and β-strand in the predicted secondary structure of CAcp and CAcyt (Figure 1).

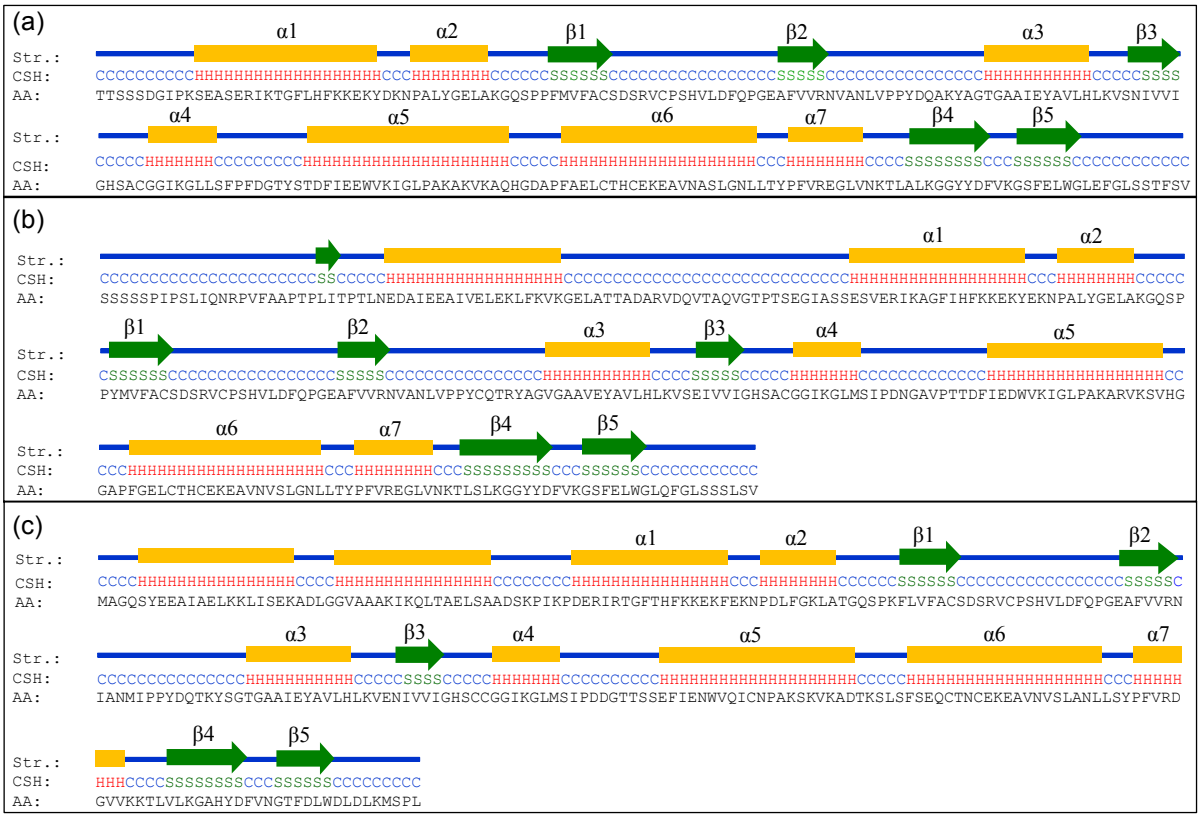

Figure 1. Prediction of secondary structure of chloroplastic β-CA (b) and cytoplasmic β-CA (c) from *L. leucocephala*. The β-CA from *P. sativum* (a) was used as a reference

There are some carbonic anhydrase-related proteins such as CARP VIII, CARP X and CARP XI, which, despite high homology with carbonic anhydrases, lack the functional activity because of substitution in one or more zinc-binding ligands (Sly & Hu, 1995). Therefore, it was important to identify the presence of zinc ligands in leucaena carbonic anhydrase. To study the active site architecture of leucaena β-CAs, the amino acid sequence of CAcp and CAcyt were compared with other chloroplastic and cytoplasmic β-CAs, respectively. Since the zinc ligands in functional β-CAs consist of one histidine and two cysteine residues, the sequences were first screened for the conserved histidine and cysteine residues. Leucaena CAcp, when compared with chloroplastic β-CAs of *G. max*, *M. truncatula*, *P. trichocarpa*, *V. vinifera*, *R. communis* and *A. thaliana*, showed three conserved histidine (His-162, His-202, and His-213) and three conserved cysteine residues (Cys-153, Cys-159, and Cys-216), whereas leucaena CAcyt, which was compared with the

cytoplasmic β-CAs of *A. thaliana*, *R. communis* and *P. trichocarpa* exhibited 4 conserved histidine (His-96, His-136, His-147 and His-233) and 6 conserved cysteine (Cys-87, Cys-93, Cys-149, Cys-150, Cys-197 and Cys-200) residues. To identify the zinc ligands among these conserved histidine and cysteine residues, the CAcp and CAcyt of leucaena were compared with the well-studied structure of C_3 β-CAs. It appears that the Cys-153 at the end of β-1 strand, Cys-216 before the beginning of α-4 helix and His-213 after β-3 strand are the zinc ligands in leucaena CAcp and Asp-155 is the conserved residue for the proton transport required in the β-CA-catalyzed reactions (Figure 2).

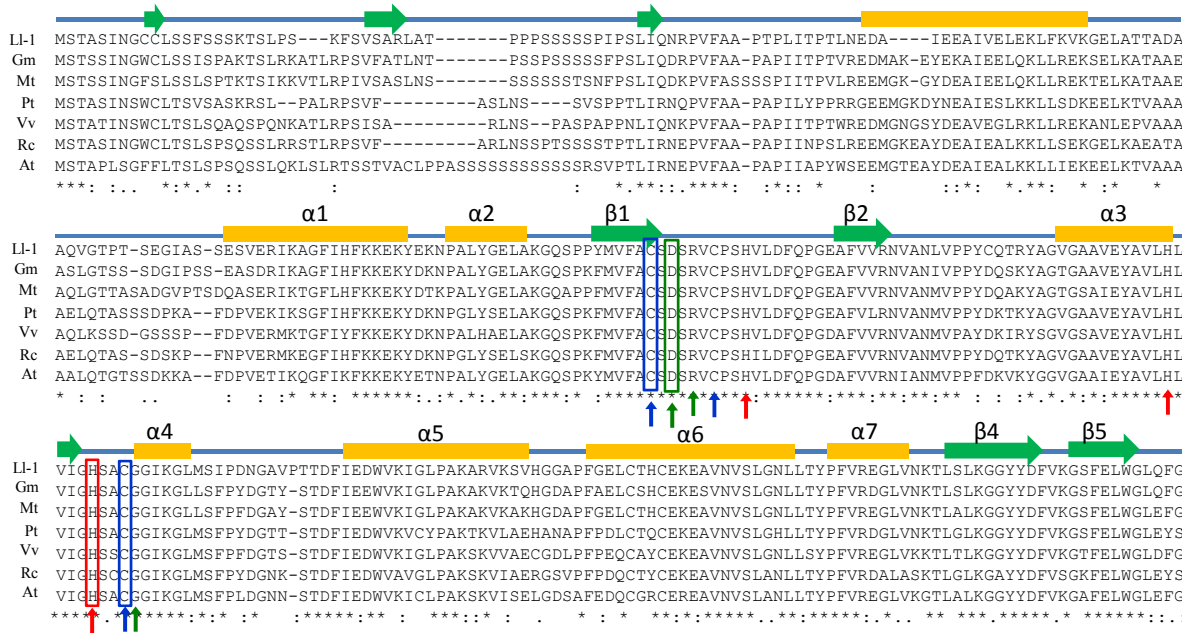

Figure 2. Chloroplastic β-CA of leucaena (Ll-1) was compared with the chloroplastic β-CAs of *G. max* (Gm), *M. truncatula* (Mt), *P. trichocarpa* (Pt), *V. vinifera* (Vv), *R. communis* (Rc) and *A. thaliana* (At). The conserved histidine and cysteine residues are represented by red and blue arrows, respectively. The green arrows represent some other conserved residues important in the active site of β-CAs. The cysteine and histidine residues in the blue and red rectangular boxes represent the zinc ligand and the aspartate shown in the green rectangular box is the residue involved in the proton transport step in β-CA catalyzed reactions

In CAcyt, it is likely that the Cys-87, Cys-139 and His-147 are the zinc ligand in CAcyt of leucaena and the Asp-89 is the residue involved in the proton transport (Figure 3).

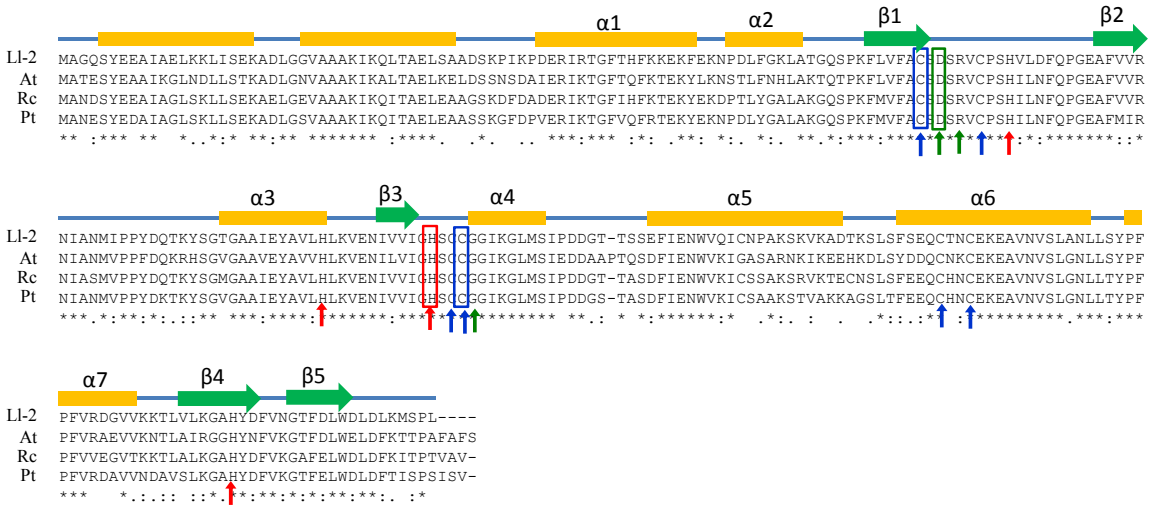

Figure 3. Cytoplasmic β-CA of leucaena (Ll-2) was compared with the cytoplasmic β-CAs of *A. thaliana* (At), *R. communis* (Rc) and *P. trichocarpa* (Pt). The conserved histidine and cysteine residues are represented by red and blue arrows, respectively. The green arrows represent some other conserved residues important in the active site of β-CAs. The cysteine and histidine residues in the blue and red rectangular boxes represent the zinc ligand and the aspartate shown in the green rectangular box is the residue involved in the proton transport step in β-CA catalyzed reactions

Although the β-CAs can be found in various oligomeric states, the fundamental structural unit of β-CA is a dimer (Rowlett, 2010). Therefore, to predict the oligomeric state of the two β-CA isoforms we performed the three-dimensional (3D) quaternary structure prediction of CAcp and CAcyt using the protein structure homology-modeling server; SWISS-MODEL (Arnold et al., 2006; Schwede et al., 2003). Based on the highest homology the SWISS-MODEL server selected the X-ray crystallographic structure of β-CA from the C_3 dicot *P. sativum*as the template structure and build the quaternary structure of both CAcp and CAcyt as octameric proteins (Figure 4). All the amino acid residues that interact with Zn ligand were found to be completely conserved between models and template. The predicted secondary structure, conserved active site residues, and 3D-model structure of CAcp and CAcyt suggest that these two leucaena β-CAs are octameric proteins and have all the essential elements that are required for the activity of functional β-CAs.

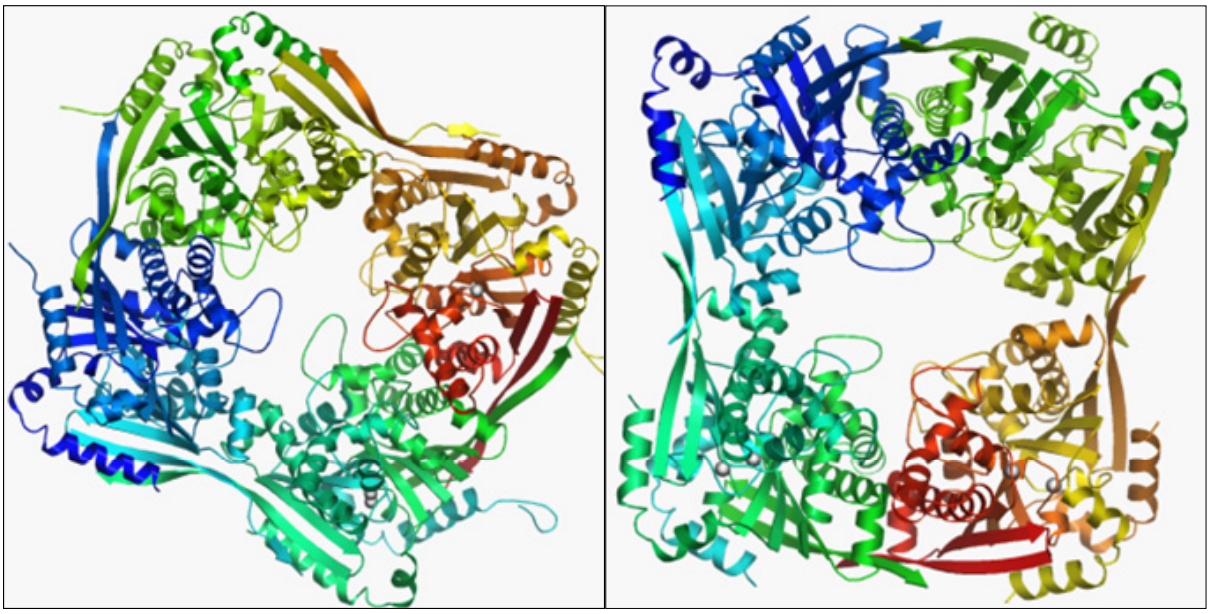

Figure 4. The 3D model of CAcp (a) and CAcyt (b) were build using SWISS-MODEL server. Both the models were predicted to be octameric proteins and each monomeric unit (polypeptide chain) of the proteins is represented in different color

3.3 Effects of Drought and Salt Stress Conditions on the Expression of cacp and cacyt

To determine the effects of drought and salt stress conditions on the expression of two β-CAs, the leucaena samples from different tissues exposed to drought stress conditions were collected at 2, 6, 12, 24, 48, 72, and 96 h of treatment. In the drought-exposed leaf tissues, the transcript of *cacp* increased up to ~6-fold in first 24 h; however, its expression sharply increased to ~17-fold in 48 h, which then remained unchanged at further time points. The stem tissues exhibited ~3-fold increase in the *cacp* transcript level in 24 h, which then increased to ~8-fold in 48 h and remained unchanged thereafter. The expression of *cacyt* in drought-stressed leaf and stem samples did not show much increase in the expression. In leaf tissues, the expression of *cacyt* increased to ~2-fold in 24 h, which further increased to ~4-fold in 48 h, and remained unchanged in further time points. The stem tissues showed maximum increase of ~2-fold in the *cacyt* expression in 24 h and got stabilized at this level in subsequent time points. No significant change in the transcript level of *cacp* and *cacyt* was observed in the root tissues from the drought-exposed plants (Figure 5a-b).

Different tissue samples of leucaena from salt stress-treated plants were collected at 1, 4, 6, 12, 24, and 48 h time points. The leaf tissues showed increase in the transcript levels of both *cacp* and *cacyt*. As compared with the transcript levels of *cacyt*, the overall increases in *cacp* expression level were more pronounced in leaf and stem tissues, however, the root tissues showed more up regulation of *cacyt*, than that of *cacp*. The *cacp* transcript level in leaf tissues first increased to ~6-fold in 1 h followed by nearly 15-fold increases in 4 and 6 h of treatment. In further time points of salt treatment, including 12, 24, and 48 h, the

increases in *cacp* transcript level compared to the expression level of control tissues were only ~10-, 3- and 2-fold, respectively. The stem tissues exhibited ~2- to 7-fold increases in the levels of *cacp* transcripts in first 12 h of treatment. The *cacp* transcript level in the subsequent time points was almost similar to those of controls. The *cacp* transcript levels in salt-stressed root tissues were found to be ~2 to 3-fold higher in 1 to 24 h time points, and at 48 h there was no significant change observed. The *cacyt* transcript level in salt-stressed leaf tissues increased to ~5- to 6-fold in 1 to 24 h time points and it decreased to 2-fold in 48 h. In stem tissues, the *cacyt* transcript level increased to ~2- to 6-fold in 1 to 6 h time points. At 12 h of treatment, there was only a 3-fold increase in the transcript level of *cacyt*, which then became similar to the control in 24 h and remained unchanged until 48 h. The transcript level of *cacyt* in root tissues first increased to ~5- to 10-fold in 1 to 6 h and then became stabilized at ~4- to 5-fold in 12 h (Figure 5c-d).

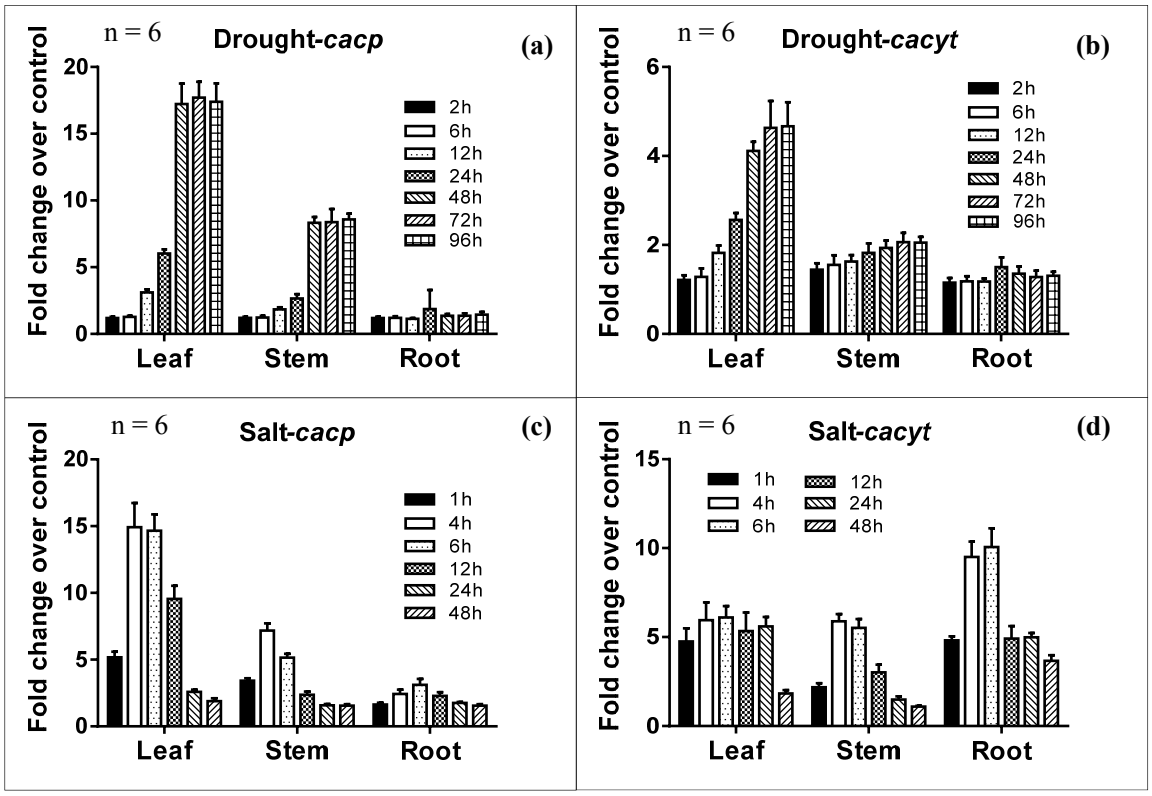

Figure 5. Tissue-specific differential expression of cacp and cacyt from *L. leucocephala* exposed to drought (a-b) and salt stress (c-d) conditions. The bars represent mean fold change in expression of gene compared to that of control, whereas the error bar represents the standard deviation. The statistical significance was calculated using a two-way ANOVA

3.4 Effects of Light, Bright Light, and Dark on the cacp and cacyt Transcripts

Under light conditions the *cacp* and *cacyt* transcripts showed increased expressions in leaf and stem tissues. The root tissues did not show any significant changes in the levels of *cacp* and *cacyt* transcripts. The *cacp* and *cacyt* transcript levels in leaf and stem tissues first exhibited a gradual increase until 16 h of treatment and then became stabilized in further time points. The *cacp* transcript levels in leaf tissues first increased from ~9- to 34-fold in 1 to 16 h time and remained at the same level in further time points. The effect of light on the *cacp* transcript levels in leaf tissues was less pronounced than that of *cacp*. The *cacp* transcript levels first increased from ~3- to 11-fold and then remained at the same level in further time points. In stems, the *cacp* transcript levels increased to ~6- to 24-fold in 1 to 24 h of treatments after which no further increase in *cacyt* transcript levels was observed. The *cacyt* transcript levels, on the other hand, increased only from ~6- to 10-fold in 1 to 48 h time points (Figure 6a-b).

Bright light conditions, as compared with moderate light that served as the control, induced only small changes in the level of *cacp* and *cacyt* transcripts and we did not observe fluctuation in changes in different time points. The level of *cacp* transcripts exhibited small increases in the leaf and stem tissues. The increase in the *cacp* transcripts was found to be ~2- to 4-fold in leaf tissues and ~2-fold in stem tissues. The *cacyt* transcript level showed 2-fold increase in leaf tissues. The transcript levels of *cacp* in root tissues and the transcript levels of *cacyt* in stem and root tissues did not show any change compared to those of controls.

Other than root tissues, the leaf and stem exhibited significant decrease in the transcript levels of both *cacp* and *cacyt* under dark conditions. In leaf tissues, the decrease in *cacp* level was found to be ~4- to 22-fold in 3 to 24 h of treatment and no further decrease was observed in the subsequent time points. The *cacyt* transcript level showed decrease of ~9-fold in 8 h and then remained at the same level in further time points. In stems, the decreases in the *cacp* and *cacyt* transcript levels were found to be ~3- to 10-fold and ~2- to 5-fold, respectively. The root tissues, however, did not exhibit any significant decrease in the transcript levels of *cacp* and *cacyt*.

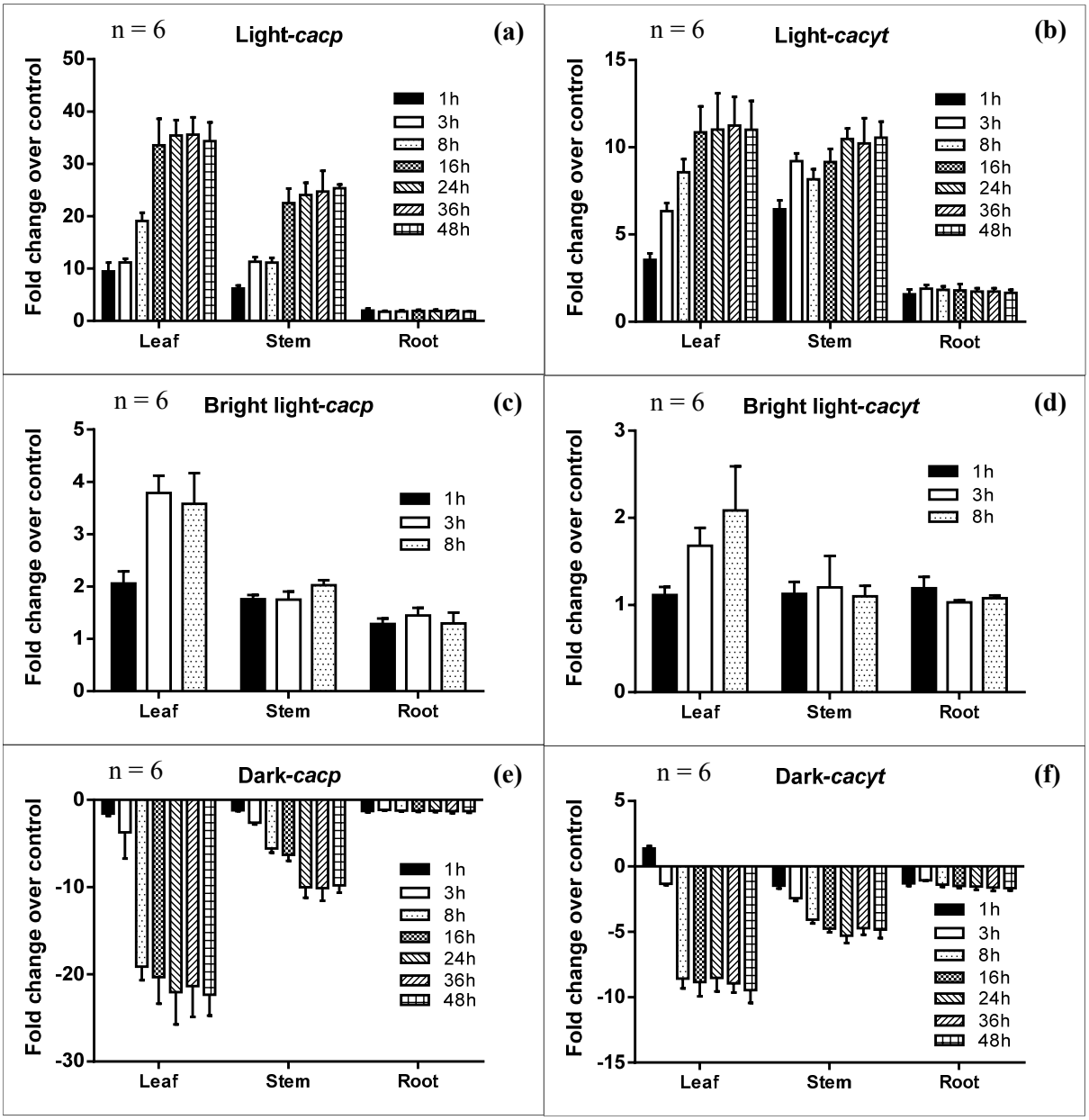

Figure 6. Effect of light (a-b), bright light (c-d), and dark (e-f) conditions on the tissue-specific expressions of *cacp* and *cacyt* from *L. leucocephala*. The bars represent mean fold change in expression of *cacp* or *cacyt* compared to that of control, whereas the error bar represents the standard deviation. The statistical significance was calculated using a two-way ANOVA.

4. Discussion and Conclusion

β-CAs have been well-studied in CAM and C_4 plants in which most of the CO_2 that is fixed comes through β-CAs (Badger & Price, 1994). In these plants, β-CAs act as a primary enzyme in the CO_2 fixation. However, the role of β-CAs in C_3 plants is inexplicit because of limited study. Therefore, the goal of this research was to isolate cDNA sequence for

chloroplastic and cytoplasmic β-CAs fromleucaena, a C_3 plant and to study the effects of various stress conditions on the expression of leucaena β-CAs. In this report, we isolated two full-length cDNAs for β-CAs from leucaena and studied their expression in various stress conditions. Although the sub-cellular localization of one of these β-CAs was predicted to be chloroplastic (*cacp*) and the other as cytoplasmic (*cacyt*), their amino acid sequences were 79% similar and both have similar secondary structures.

Carbonic anhydrase-related proteins (CARPs) such as CARP VIII, CARP X and CARP XI, are homologous to carbonic anhydrases but lack the functional activity because of substitution in one or more amino acid residues involved in zinc binding. Therefore, for a functionally active CA it is important to have all the zinc-binding residues at the active site of the enzyme. Moreover, the enzyme should have all the conserved folds as found in functionally active forms of CAs. Therefore, for analyzing structural folds and active site residues, we used structurally resolved β-CA from *P. sativum* (Kimber & Pai, 2000) as the reference. The secondary structure of both the β-CAs from leucaena was found conserved and followed the same sequential pattern of α-helixes and β-strands as found in the β-CA from *P. sativum*. A previous study on spinach β-CA identified that one histidine and two cysteine residues make the hydrophobic pocket that serves as the zinc-ligand in the active site of the enzyme (Rowlett et al., 1994; Bracey et al., 1994; Kimber & Pai, 2000). In our active site analysis, we have predicted that Cys-153, Cys-216 and His-213 may constitute the zinc ligand of CAcp, whereas the zinc ligand of CAcyt may be made of Cys-87, Cys-139 and His-147. Crystallographic studies on β-CA from *Cryptococcus neoformans* show that an aspartate residue in the active site of β-CA activates water molecule for nucleophilic attack of CO_2 by making a hydrogen bond with the Zn(II)- coordinated water molecule (Schlicker et al., 2009). This aspartate residue is also conserved in plant β-CAs and based on the analysis of conserved residues at the active site of chloroplastic and cytoplasmic β-CAs from C_3 plants, we predicted Asp-155 of CAcp and Asp-89 of CAcyt as the respective residues of CAcp and CAcyt involved in proton transport; a feature necessary for the catalytic function of the enzyme. The 3D structures of CAcp and CAcyt, which were built using homology-based modeling, suggest that both the β-CAs of leucaena are octameric proteins with all the active site residues and zinc-binding residues conserved among CAcp and CAcyt.

β-CA is abundant in C_3 plants and represent ~2% of total protein in leaf tissues (Okabe et al., 1984). However, the information on individual abundance of chloroplastic and cytoplasmic β-CA in C_3 plants is lacking. We designed and used specific primer sets for *cacp* and *cacyt*in RT-PCR to identify tissue-specific transcript abundance of *cacp* and *cacyt* in leucaena. Our results demonstrate that transcript abundance of *cacp* was higher than that of *cacyt* in leaf and stem tissues while in root tissues the abundance of *cacyt* transcripts and *cacp* transcripts was similar. Transcript level of *cacp* was found maximum in leaf followed by stem and root tissu es whereas the maximum *cacyt* transcript level was observed in roots followed by leaf and stem tissues. Although the overall high β-CA content in C_3 plants indicates their important physiological role, the varying abundance of chloroplastic and cytoplasmic β-CAs of leucaena in different tissues and also within the same tissue suggests that they have either different or complementary physiological role. Considering the alkaline nature of chloroplast

stroma of C_3 plants, their chloroplastic β-CAs have been proposed to be involved in the diffusion of CO_2 into the chloroplast stroma, where CO_2 and ribulosebis phosphate (RuBP) serve as the substrate for rubisco, which fixes the inorganic carbon into 3-phosphoglycerate (Badger & Price, 1994). On the other hand, the cytoplasm of C_3 plants has acidic environment in which the HCO_3^- levels are usually low. Additionally, the CO_2 that diffuses through the cell wall may directly enter the chloroplast because the diffusion distance between the chloroplast envelope and the cell wall is typically small (Cowan, 1986; Badger & Price, 1994). This suggests that the cytoplasmic β-CAs may have little role, if any, in carbon fixation. However, considering that leucaena is well-adapted to alkaline soil condition, the abundance of *cacyt* in leucaena root tissues may be an adaptive measure of leucaena to improve buffering capacity of root cells in alkaline soils.

Photosynthesis potential of plants has been reported to be affected by some stress conditions such as drought and salt stress (Lawlor, 1995; Munns, 2002). Further study on correlation of photosynthesis with salt and drought stress demonstrated that these stress conditions predominantly limit the influx of CO_2 in the leaves by decreasing the conductance of stomata and mesophyll (Flexas et al., 2004; Sudhir & Murthy, 2004). Considering these studies and also the high abundance of β-CAs along with its biochemical role in carbon fixation, we hypothesized that the β-CAs in C_3 plants might be involved in dealing with the physiological stress conditions that could limit the photosynthetic potential of C_3 plants and therefore, the levels of β-CAs should be significantly increased under drought and salt stress conditions. To test this hypothesis, we studied the effects of drought and salt stress conditions on the transcript abundance of leucaena *cacp* and *cacyt*. As expected, we observed an enhanced expression of *cacp* and *cacyt* under both drought and salt stresses. In fact, the expression of *cacyt*was found to be higher in salt-stressed root tissues as compared to salt-stressed leaf and stem tissues. The overall expression of *cacp* was higher than *cacyt* in leaf and stem tissues under drought and salt stress conditions, however in root tissues *cacyt* expressed at higher levels compared to *cacp*. This may be because root is the first tissue to encounter these stresses and also may be because the root tissues lack chloroplasts and thereby it has a low level of *cacp.* Therefore, overexpression of *cacyt* in root tissues may be an adaptive measure of leucaena to withstand salinity stress. Drought and salt stress conditions lead to reduced CO_2 solubility or decreased CO_2 supply as a result of stomata closure. Previously, a similar increase in the expression of β-CA under drought and salt stress conditions was reported in a C_4 plant, *Pennise tumglaucum* (Kaulet al., 2011).

Like drought and salt stress conditions, photosynthesis potential of plants is also affected by light and dark conditions. Also, the level of nucleus-encoded mRNAs for photosynthesis-related proteins have been shown to decrease under dark conditions (Giuliano et al., 1988; Chory et al., 1989). Therefore, we also tested the effects of light, dark, and bright light conditions on the expression levels of *cacp* and *cacyt*. The transcript levels of *cacp* and *cacyt* increased in light conditions in leaf and stem tissues only and the increase in the transcript levels of *cacp* was much higher than that of *cacyt*. Additionally, the increase in the expression of these transcripts got stabilized after 24 h of the treatment. The bright-light exposed plants, as compared to the control plants that were grown under moderate light

conditions, exhibited only ~2- to 4-fold increase in the transcript levels of *cacp* in the leaves, whereas transcript levels of *cacyt* exhibited only ~2-fold increase in the leaves. In the dark condition, the transcript levels of *cacp* and *cacyt* decreased in leaf and stem tissues and the overall decrease was observed more in the *cacp* levels than that of *cacyt* levels. The decrease in the levels of *cacp* transcripts and *cacyt* transcripts got stabilized after 24 h and 8 h time points, respectively and no further decrease in their expression levels was observed after these time points. A similar decrease in the level of β-CA was previously observed in *A. thaliana* in the dark condition (Fett & Coleman, 1994). The increase or decrease in the expression of β-CAs under light or dark condition was found to change with time intervals. This indicates that β-CAs level may also be responsive to the normal circadian rhythm. The relatively higher expression of *cacp* as compared to *cacyt* under light condition may be because of its location and possibly crucial role in carbon fixation. Considering the alkaline microenvironment in chloroplast stroma, *cacp* is the major β-CA involved in inorganic carbon fixation into 3-phosphoglycerate (Badger & Price, 1994). In contrast, the microenvironment of cytoplasm is acidic where the HCO_3^- levels is usually low, therefore, *cacyt* may have little or no role in carbon fixation under light condition (Cowan, 1986; Badger & Price, 1994).

In summary, we have isolated cDNAs for chloroplastic and cytoplasmic β-CA from the C_3 plant leucaena and we have shown that the transcript levels of *cacp* and *cacyt* increase under drought, salt and light conditions and decrease under dark condition. The differential expression of leucaena β-CAs under these conditions suggest that β-CAs may have a possible role in enhancing leucaena's ability to withstand abiotic stress conditions that affect the photosynthesis potential of the plant. However, it does not rule out another possibility in which β-CAs are only responsive to these stresses and do not play any active role in enhancing abiotic stress tolerance.

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