

**FUNCTIONAL CHARACTERIZATION OF
CHLOROPLASTIC AND CYTOPLASMIC β -
CARBONIC ANHYDRASE ISOFORMS FROM
*LEUCAENA LEUCOCEPHALA***

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF
THE UNIVERSITY OF HAWAI'I AT MĀNOA IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MOLECULAR BIOSCIENCES AND BIOENGINEERING

DECEMBER 2013

By

Archana Pal

Dissertation Committee:

Dulal Borthakur, Chairperson
Jon-Paul Bingham
John Hu
Qing X. Li
Richard Manshardt

DEDICATION

To the loving memory of my father,

Sri Haridas Pal.

*His words of inspiration and encouragement
in pursuit of excellence, still linger on.*

ACKNOWLEDGMENTS

I would never have been able to finish my dissertation without the guidance of my Ph.D. supervisor, committee members, help from friends, and support from my family and husband.

I would like to express my deepest gratitude to my Ph.D. advisor, Dr. Dulal Borthakur for his guidance, support and all his help that made this dissertation possible. I offer my sincere appreciation for the learning opportunities provided by him. His recommendations and instructions have enabled me to assemble and finish the dissertation effectively.

I cannot express enough thanks to my committee, Drs. John Hu, Jon-Paul Bingham, Qing Li, and Richard Manshardt for their encouraging words, thoughtful criticism, and time and attention during busy semesters.

I would like to thank all my lab mates, who as good friend were always willing to help and give their best suggestions. It would have been a lonely lab without them. My research would not have been possible without their helps.

I would like to extend my gratitude to the Department of Molecular Biosciences and Bioengineering staff, Joanne, Shan, Ardi, and Karen for assisting me with the administrative tasks necessary for completing my doctoral program.

I would also like to thank my parents, two elder sisters, and elder brother. They were always supporting me and encouraging me with their best wishes.

Finally, I would like to thank my husband, Vishal. He was always there cheering me up and stood by me through the good times and bad. Your encouragement when the times got rough are much appreciated and duly noted. It was a great comfort and relief to know that you were willing to provide management of our household activities while I completed my work.

This research was supported by the National Science Foundation Award No. CBET 08-27057 and partially by a HATCH grant (CRIS0216234).

ABSTRACT

Carbonic anhydrases (CAs) catalyze rapid interconversion of CO₂ and water to bicarbonate and protons. Carbonic anhydrases (CAs) have been extensively studied and are known to be involved in various physiological processes such as respiration, cell growth and acid/base regulation in animals, and carbon fixation and photosynthesis in plants, algae and cyanobacteria. Although the beta-type carbonic anhydrases (β -CAs) are crucial for carbon fixation in C₄ plants, algae, and cyanobacteria, the role of β -CA in C₃ plants is not well-studied. Therefore, the specific objective of this research is to isolate and characterize the genes encoding chloroplastic and cytoplasmic β -CAs from the C₃ plant, *Leucaena leucocephala* (leucaena) and investigate their possible role in the plant by overexpressing the leucaena β -CA in *Nicotiana tabacum* and also to study the expression pattern of leucaena β -CAs under various physiological stress conditions that affect photosynthesis potential in plants. In this study, two cDNA clones, *ca_{cp}* and *ca_{cyt}*, encoding chloroplastic and cytoplasmic forms of β -CA, respectively, were isolated from a leucaena cDNA library. These clones were further extended to full-length by 5'- and 3'-rapid amplification of cDNA ends (RACE). Deduced amino acid sequences of *ca_{cp}* and *ca_{cyt}* exhibited 79 % homology with each other. To determine the possible role of these genes in leucaena under physiological stresses, quantitative PCR (qPCR) was performed from different tissues of leucaena grown under normal, drought, saline, light, bright light, and dark conditions. The expression of *ca_{cp}* and *ca_{cyt}* was found to be up

regulated under drought, salt, and light conditions and down regulated under dark conditions. Bright light conditions, compared with normal light condition, did not exhibit much change in the transcript levels of ca_{cp} and ca_{cyt} . Additionally, tobacco plants were transformed with leucaena's ca_{cp} and ca_{cyt} separately and also together resulting in three types of transgenic tobacco plants (i) with ca_{cp} only (ii) with ca_{cyt} only and (iii) with both ca_{cp} and ca_{cyt} . These transgenic tobaccos were also studied under stress conditions. This study will give us a deeper insight to understand the role of chloroplastic and cytoplasmic β -CAs in leucaena and also in C3 plants in general.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	iii
CHAPTER 1.....	1
<i>Introduction and Review of Literature</i>	1
1.1 Introduction.....	1
1.2 CA gene families	2
1.2.1 α -carbonic anhydrase gene family.....	2
1.2.2 β -carbonic anhydrase gene family.....	4
1.2.3 γ -carbonic anhydrase gene family	7
1.2.4 δ -carbonic anhydrase gene family.....	8
1.2.4 ζ -carbonic anhydrase gene family	9
1.3 Mechanism of action.....	11
1.4 Role of β -CAs in higher plants	13
1.4.1 Role of β -CAs in CAM plants.....	13
1.4.2 Role of β -CAs in C4 plants	15
1.4.2 Role of β -CAs in C3 plants	19
1.5 Hypotheses	22
1.6 Objectives.....	23
CHAPTER 2.....	27
<i>Isolation of Leucaena genes for chloroplastic and cytoplasmic β-CAs and their comparative studies.....</i>	27
2.1 Introduction.....	27
2.2 Materials and methods	28
2.2.1 Plant growth.....	28
2.2.3 Isolation of chloroplastic β -carbonic anhydrase cDNA	30
2.2.4 Isolation of cytoplasmic β -carbonic anhydrase cDNA	31

2.2.5	Sequence analyses and prediction of sub-cellular localization	32
2.2.6	In silico structural analysis of leucaena β -CAs	33
2.3	Results	34
2.3.1	Isolation of chloroplastic β -CA from leucaena.....	34
2.3.2	Isolation of cytoplasmic β -CA from leucaena.....	37
2.3.3	In silico structural analysis of leucaena CAcp and CAcyt.	41
2.4	Discussion	51
CHAPTER 3	53
	<i>Tissue-specific expression of two β-CA isoforms from Leucaena under various physiological stress conditions</i>	53
3.1	Introduction.....	53
3.2	Materials and methods	55
3.2.1	Plant growth and imitation of stress conditions	55
3.2.2	RNA extraction and cDNA synthesis	57
3.2.3	Identification of suitable internal reference gene.....	57
3.2.4	ca_{cp} and ca_{cyt} specific primer design and optimization of RT-PCR condition	57
3.2.5	qPCR analysis.....	58
3.2.6	Tissue-specific expression of ca_{cp} and ca_{cyt}	59
3.3	Results	59
3.3.1	Confirmation of qRT-PCR primer sets specific for ca_{cp} and ca_{cyt}	59
3.3.2	Expression stability of HKGs in control and treatments	62
3.3.3	Tissue-specific expression of ca_{cp} and ca_{cyt}	67
3.3.4	Effects of drought and salt stress conditions on the expression of ca_{cp} and ca_{cyt}	68
3.3.5	Effects of light, bright light, and dark on the ca_{cp} and ca_{cyt} transcripts	70
3.4	Discussion	74
CHAPTER 4	77
	<i>Transgenic tobacco expressing leucaena β-CA isoforms</i>	77

4.1 Introduction.....	77
4.2 Materials and methods	79
4.2.1 Construction of entry clones and binary constructs	79
4.2.2 Plant material and transformation.....	80
4.2.3 Verification of transgenic plants.....	81
4.2.4 CA activity assay	84
4.2.5 Chlorophyll measurements.....	85
4.2.6 Plant dry weight measurement	85
4.3 Results	85
4.3.1 Binary constructs preparation.....	86
4.3.2 Tobacco transformation.....	87
4.3.3 Screening of putative transgenic <i>Tobacco</i> plants	88
4.3.4 CA activity in wild type and transgenic tobacco plants.....	91
4.3.4 Chlorophyll content and dry weight of wild type and transgenic tobacco plants	92
4.4 Discussion	96
SUMMARY AND FUTURE WORK.....	98

LIST OF FIGURES

Figure 1: Step-wise mechanism of CA-catalyzed reaction.....	12
Figure 2: Schematic representation of the role of β -CA in the carbon fixation in CAM plants.....	14
Figure 3: Schematic representation of the role of β -CA in the carbon fixation in C4 plants.	18
Figure 4: Representation of possible role of β -CA in the carbon fixation in C3 plants	20
Figure 5: The blastx analysis of 515bp partial iSSH clone of <i>Leucaena</i> . The query sequence exhibited homology with the carbonic anhydrase with <i>M. truncatula</i> (Gene ID: 11428425 MTR_6g006990).....	34
Figure 6: The blastp analysis of full-length deduced amino acid sequence of β -CA-1. The query sequence exhibited homology with the chloroplastic carbonic anhydrase with <i>G. max</i> (Gene ID: 100500448 LOC100500448).....	36
Figure 7: The multiple sequence alignment of the cytoplasmic β -CAs nucleotide sequences of C3 dicots from NCBI database. The Rc, Pt and At represents the ORF of the cytoplasmic β -CA sequences from <i>Ricinus communis</i> (gi 255568811), <i>Populus trichocarpa</i> (gi 224107828), and <i>Arabidopsis thaliana</i> (gi 145362379), respectively. The bold, underlined, and green highlighted text represents the sequences used for designing forward and reverse primers. ...	39

- Figure 8:** The tblastx analysis of the 151 bp *Leucaena* β -CA obtained as a result of PCR using primers designed from the conserved region of cytoplasmic β -CAs of C3 dicots.40
- Figure 9:** The blastp analysis of full-length deduced amino acid sequence of *Leucaena* β -CA. The query sequence exhibited homology with the carbonic anhydrase with *M. truncatula* (GENE ID: 11407419 MTR_5g034250).....41
- Figure 10:** The sequence alignment of the deduced amino acid sequence of CA_{cyt} and CA_{cp} using ClustalW.....42
- Figure 11:** 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.43
- Figure 12:** Chloroplastic β -CA of *Leucaena* (LI1) 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.46
- Figure 13:** Cytoplasmic β -CA of *Leucaena* (LI) 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.....47

Figure 14: The 3D model of CA_{cp}. The quaternary structure was build using SWISS-MODEL server. The model for CA_{cp} was predicted to be octameric proteins. Each monomeric unit (polypeptide chain) of the proteins is represented in different color.....49

Figure 15: The 3D model of CA_{cyt}. The quaternary structure was build using SWISS-MODEL server. The model for CA_{cyt} was predicted to be octameric proteins. Each monomeric unit (polypeptide chain) of the proteins is represented in different color.....50

Figure 16: The sequence alignment of the ORF of *ca_{cp}* and *ca_{cyt}* using ClustalW. The primers for *ca_{cp}* and *ca_{cyt}* were designed from the yellow and green highlighted regions, respectively.61

Figure 17: Expression stabilities of HKGs in leaf tissues under control and treatments conditions in four experimental groups that include control and drought (a), control and salt (b), light and dark (c), and light and bright light (d). The bars represent inter-group variance whereas the error bars represent the average of intra-group variances. The most stably expressed HKG in each group is represented as the bar shaded in grey color.64

Figure 18: Expression stabilities of HKGs in stem tissues under control and treatments conditions in four experimental groups that include control and drought (a), control and salt (b), light and dark (c), and light and bright light (d).

The bars represent inter-group variance whereas the error bars represent the average of intra-group variances. The most stably expressed HKG in each group is represented as the bar shaded in grey color.65

Figure 19: Expression stabilities of HKGs in stem tissues under control and treatments conditions in four experimental groups that include control and drought (a), control and salt (b), light and dark (c), and light and bright light (d). The bars represent inter-group variance whereas the error bars represent the average of intra-group variances. The most stably expressed HKG in each group is represented as the bar shaded in grey color.66

Figure 20: Comparison of transcript abundance of ca_{cp} and ca_{cyt} in leaf, stem, and root tissues of *L. leucocephala*. (a) Bands of ca_{cp} and ca_{cyt} amplified by RT-PCR from leaf, stem, and root tissues. β -actin was used as loading and PCR control. (b) Quantitative representation of transcript abundance from the same bands in agarose gel. The band intensities were digitized using ImageJ software. The band intensities of β -actin was used to normalized the transcript abundance.67

Figure 21: Tissue-specific differential expression of ca_{cp} and ca_{cyt} from *L. leucocephala* exposed to drought (a-b) and salt stress (c-d) conditions.70

Figure 22: Effect of light (a-b), bright light (c-d), and dark (e-f) conditions on the tissue-specific expression of ca_{cp} and ca_{cyt} from *L. leucocephala*73

Figure 23: pMDC binary constructs containing transgene. The transgene ca_{cyt} was cloned in pMDC140 and pMDC100 to make the recombinant binary constructs pMDC140- ca_{cyt} (a) and pMDC100- ca_{cyt} (b), respectively. The ca_{cyt} transgene was cloned in pMDC140 to make the binary construct namely pMDC140- ca_{cp} 86

Figure 24: Steps of tobacco transformation. Transformed leaf discs were cultured in full-strength MS (PH-5.7) supplemented with 1µg/mL BAP and 0.1µg/mL NAA (a). The leaf discs cultured on the same MS media and plant growth regulator composition supplemented with 250µg/mL cefotaxime and 40 µg/mL hygromycin started to swell up followed by callus formation by 3-4 weeks (b) and shoot regeneration from the callus mass in 5-6 weeks (c). The plants were induced for root formation and the rooted plants were then transferred to pots containing garden soil (d).....88

Figure 25: verification of presence of transgene and its expression in transgenic plants using PCR from genomic DNA (a) and RT-PCR from cDNA (b).....90

Figure 26: The GUS assay on the wild type (a) and transgenic plants containing pMDC140-*ca_{cyt}* construct (b), pMDC140-*ca_{cyt}* (c), and constructs pMDC100-*ca_{cyt}* and pMDC140*ca_{cp}* (c).....91

Figure 27: CA enzyme activity in Wilbur and Anderson unit per mg of total leaf protein from wild type and transgenic tobacco plants.....92

Figure 28: Phenotypes of wild type (Wt) and transgenic tobacco plants expressing leucaena *ca_{cp}* (a-c), *ca_{cyt}* (d-f), and *ca_{cp}* and *ca_{cyt}* together (g-i).93

Figure 29: SPAD value representing chlorophyll contents from wild type and transgenic plants containing *ca_{cp}* (a), *ca_{cyt}* (b), and *ca_{cp}* and *ca_{cyt}* together (c).94

Figure 30: Dry weight of leaves, stem and root tissue samples in wild type and transgenic tobacco plants expressing leucaena *ca_{cp}* (a), *ca_{cyt}* (b), and *ca_{cp}* and *ca_{cyt}* together (c).95

LIST OF TABLES

Table 1: Classification and characteristics of CA families	10
Table 2: Primers used and their description	31
Table 3: Prediction of sub-cellular localization of the seq3-encoded protein of leucaena using TargetP 1.1 server	37
Table 4: The qPCR primers used in the study and their specificity for the ca_{cp} and ca_{cp} templates	62
Table 5: Primer used for cloning of ca_{cp} and ca_{cyt} gene sequences in binary constructs.....	83

LIST OF ABBREVIATIONS

Acronym	Definition
3-PGA	3-phosphoglycerate
3D	three-dimensional
Asp	aspartate
BAP	6-Benzylaminopurine
BLAST	basic local alignment search tool
BLASTp	protein blast
bp	base pair
CA	carbonic anhydrase
<i>ca_{cp}</i>	chloroplastic carbonic anhydrase
<i>ca_{cyt}</i>	cytoplasmic carbonic anhydrase
CAM	crassulacean acid metabolism
CA-RPs	carbonic anhydrase-related proteins
Ct	Cycle threshold
Cys	cysteine
EXAFS	X-ray absorption spectroscopy
GUS	β -glucuronidase
His	histidine
HKGs	housekeeping genes
mRNAs	messenger RNAs
MS media	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid
NaCl	Sodium chloride
NCBI	national center for biotechnology information
ORF	open reading frame
PEP	phosphoenolpyruvate
PEPCase	Phosphoenolpyruvate carboxylase
PMSF	phenylmethylsulfonyl fluoride
PSIPRED	protein structure prediction server
qPCR	quantitative PCR
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RT	reverse transcriptase
RTPCR	reverse transcription polymerase chain reaction
Rubisco	ribulose-1,5-bisphosphate carboxylase oxygenase
RuBP	ribulose bisphosphate

SA	salicylic acid
Acronym	Definition
UTR	untranslated region
WAU	Wilbur and Anderson unit
X-Gluc	5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid, cyclohexylammonium

CHAPTER 1

Introduction and Review of Literature

1.1 Introduction

Carbonic anhydrase (CA) represents a family of metalloenzymes that catalyze reversible hydration of carbon dioxide (CO_2) to form bicarbonate (HCO_3^-) and protons in a reaction represented as $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$. CA was first discovered and purified from red blood cells (Meldrum and Roughton, 1933) and has been reported to exist in almost all living organisms including prokaryotes, algae, plants, and animals (Hewett-Emmett and Tashian, 1996).

Based on (i) the use of HCO_3^- as a substrate in various biological processes and production of CO_2 as a waste product in animals and (ii) its use in carbon fixation in plants, CAs are believed to play a crucial role in a variety of physiological processes by regulating the interconversion of both the molecules. In animals, CAs are involved in respiration and acid/base regulation, insulin secretion, bone resorption, cell growth, calcification, and transport of carbon dioxide out of tissues, whereas in plants, algae and cyanobacteria CAs play an important role in photosynthesis. In plants CAs has been suggested to be involved in stress tolerance (Fett and Coleman, 1994; Kaul et al., 2011). In bacteria, CAs are also involved in the transport of carbon dioxide and bicarbonate.

1.2 CA gene families

CAs are ubiquitous metalloenzymes that can be classified in five different families namely, α , β , γ , δ , and ζ (Zimmerman and Ferry, 2008). Despite catalyzing the same biochemical reaction, these CA families have no significant sequence similarity at amino acid level and structures, except some degree of similarity between β -CA and ζ -CA. Therefore, these CA families appear to have evolved independent of each other through convergent evolution (Hewett-Emmett and Tashian, 1996). However, β - and ζ -CA have nearly identical geometry of active site and similar fold indicating that ζ -CA may be a diverged subtype of β -CA (Xu et al., 2008). The summary of features of each CA families is represented in Table 1.

1.2.1 α -carbonic anhydrase gene family

The CAs in α -CA family are monomeric zinc metalloenzymes that exist as various isoforms in animals and plants and also have been found in fungi, algae, bacteria and viruses (Moroney et al., 2001; Tripp et al., 2001). In mammals, α -CAs are classified into four subgroups consisting of several isoforms; (i) the cytosolic α -CAs (CA-I, CA-II, CA-III, CA-VII and CA XIII), (ii) mitochondrial α -CAs (CA-VA and CA-VB), (iii) secreted α -CAs (CA-VI), (iv) membrane-associated α -CAs (CA-IV, CA-IX, CA-XII, CA-XIV and CA-XV). These α -CAs exhibit some degree of antigenic difference, based on which the α -CA family of vertebrates can be divided into two groups: (i) soluble isoform such as cytoplasmic and mitochondrial, and (ii) membrane-bound and secreted α -CAs; antibodies raised against one isoform do

not cross-react with the other α -CA isoforms, for example polyclonal antibodies raised against CA VI (secreted α -CA) did not show any cross-reactivity with CA I or CA II (cytoplasmic α -CAs) (Parkkila et al., 1990).

The X-ray crystallographic studies of α -CA identified that the side chains of the three histidine residues and a hydroxyl ion bind the zinc atom (Christianson and Cox, 1999), which is obligatory for CO₂ hydration. A hydrophobic cluster adjacent to zinc-binding domain is important for the specific binding of zinc metal as hydrophobic core orients histidine residues in a geometry that stabilizes zinc binding and discourages binding of other metals (Hunt et al., 1999). This hydrophobic core is highly conserved among all the α -CA isoforms; a minimum width and depth of hydrophobic pocket and the volume of the amino acid moiety present at the base of the pocket are important for efficient catalytic activity (Alexander et al., 1991; Fierke et al., 1991; Nair et al., 1991; Nair and Christianson, 1993).

Apart from the three histidine residues, which serve as zinc ligand, the active site contains another histidine residue that transfers protons between the zinc-bound water and solvent water at the active site cavity resulting in the regeneration of zinc-bound hydroxyl ion (Duda et al., 2001). Some proteins namely, CA-related proteins (CA-RPs) have high homology with α -CAs but lacks the catalytic activity (Sly and Hu, 1995) because of substitution in one or more histidine residues required to bind the zinc ion and therefore, their biological function of these proteins is not clear.

The α -CAs have been extensively studied in animals, whereas a few α -CAs have been also identified in photosynthetic organisms including cyanobacteria (Soltes-Rak et al., 1997), the green algae *Chlamydomonas reinhardtii* (Karlsson et al., 1998) and *Dunaliella salina* (Fisher et al., 1996), and plants. Eight α -CA isoenzymes were identified from the genome sequences of *Arabidopsis thaliana* (Fabre et al., 2007). The α -CAs identified in plants so far appear to be soluble protein despite different sub-cellular localization (Moroney et al., 2001).

In general, α -CAs are catalytically functional as ~30 kDa monomeric enzyme with one active site. However, a periplasmic α -CA from *C. reinhardtii* and a CA from *D. salina* are exceptions; the periplasmic α -CA from *C. reinhardtii*, ~75 kDa in size, is a heterotetramer composed of a two large subunits made of 37 kDa homodimer and a two small subunits made of 4 kDa homodimer held together with disulfide bonds (KAMO et al., 1990), whereas the CA from *D. salina* is ~60 kDa protein, which is internally duplicated with two active sites (Fisher et al., 1996). Both of the exceptional α -CAs from green algae have two active sites and the ~60 kDa single polypeptide of *D. salina* CA resembles the four subunits of ~75 kDa α -CA from *C. reinhardtii*.

1.2.2 β -carbonic anhydrase gene family

The β -CA gene family was first identified in spinach (Burnell et al., 1990; Fawcett et al., 1990), and its occurrence was initially thought to be confined to the plant kingdom only. However, the β -CA gene family is widely distributed in prokaryotes

and eukaryotes its presence has been reported in algae (Yagawa and Miyachi, 1987; Hiltonen et al., 1995), eubacteria and archaeobacteria (Hewett-Emmett and Tashian, 1996; Smith et al., 1999; Smith et al., 2002). It exists in almost all the species of fungi and plants (Götz et al., 1999; Hewett-Emmett, 2000). The presence of β -CA in animal remained controversial for long time, however recently its widespread presence was demonstrated among invertebrates (Syrjänen et al., 2010).

Unlike monomeric form of α -CAs, β -CAs exists in different oligomeric states. Crystallographic studies have identified dimeric, tetrameric, hexameric and octameric β -CAs (Kimber and Pai, 2000; Smith et al., 2000; Strop et al., 2001). The oligomeric form in the multiple of 2 indicates that the basic building block of β -CAs is dimeric (Tripp et al., 2001). The studies involving X-ray absorption spectroscopy (EXAFS) of β -CA from spinach identified that the zinc ligands at the active site of the enzyme include a histidine and two cysteine residues (Bracey et al., 1994; Rowlett et al., 1994; Strop et al., 2001). Since both histidine and cysteine are hydrophobic amino acids, it appears that the amino acid residues at the active site of the β -CA are similar to those of α -CAs hydrophobic pocket (Kimber and Pai, 2000).

An aspartate residue is conserved at the active site of β -CAs and the crystal structure of β -CA predicted the role of aspartate residue in the proton transport (Strop et al., 2001). The structure of β -CA from pathogenic fungus *Cryptococcus neoformans* shows that the aspartate residue in the active site of β -CA activates

water molecule for nucleophilic attack of the CO₂ by making a hydrogen bond with the Zn(II) coordinated water molecule (Schlicker et al., 2009). Although the structures and fold of α -CAs and β -CAs are strikingly different, the active site residues are highly conserved and the active site architecture of the two families appears to be the mirror image of each other (Kimber and Pai, 2000).

β -CA gene family represents the most diverse CA gene family (Smith et al., 1999). In phylogenetic analysis, the β -CAs from archaea, monocots and dicots form distinct clades (Smith et al., 1999; Smith et al., 2000). The β -CAs from the archaea is known as 'Cab type' β -CA (Strop et al., 2001) and the β -CAs from monocots and dicots are classified as 'plant type' β -CA (Kimber and Pai, 2000). Both these subtypes of β -CAs are structurally different and respond differently to CA inhibitors (Smith and Ferry, 2000).

Within the 'plant type' β -CAs, the dicot and monocot β -CAs exhibit differences in structure and size. Antibodies developed against purified β -CA from maize, a monocot plant, showed cross-reactivity against leaf extracts from a variety of monocot as well as dicots. However, these antibodies could quantitatively titrate CA activity only in leaf extracts of maize and sorghum, but not in dicots (Burnell, 1990). Similarly, the antibodies developed against spinach, a dicot, exhibited cross-reactivity with leaf extracts of several C3, C4 dicot, and Crassulacean acid metabolism (CAM) dicot plants but not with C4 monocot plants, and green *C. reinhardtii* (Okabe et al., 1984), indicating that the β -CAs from monocots and dicots are antigenically different from each other.

The expression of β -CAs in plants has been reported in both green tissues and roots. Although the sub-cellular localization of plant β -CAs has been predicted in chloroplasts, cytoplasm and mitochondria (Fabre et al., 2007; Tetu et al., 2007), the β -CAs with sub-cellular localization of chloroplasts and cytoplasm are known to be involved in carbon fixation (Syrjänen et al., 2010).

1.2.3 γ -carbonic anhydrase gene family

The γ -CA gene family was first discovered in the archaeon *Methanosarcina thermophila* (Alber and Ferry, 1994), and has been estimated to evolve around 3.0 and 4.5 billion years ago (Smith et al., 1999). Besides archaeon, two and five γ -CAs have been identified in *Escherichia coli* (*E. coli*) (Tripp et al., 2004) and in *A. thaliana* (Parisi et al., 2004), respectively.

The crystal structure of γ -CAs is significantly different from that of α -CAs or β -CAs (Kisker et al., 1996). It function as as a homotrimer where the structure of each subunit is dominated by a left-handed β -helix (Kisker et al., 1996). Each subunit of the homotrimer contains a zinc atom. Like the active site of α -CAs, the active site of γ -CAs also consists of three histidine residues as the zinc ligand (Cox et al., 2000), however, the histidines for one zinc atom are from two different subunits (Kisker et al., 1996). Unlike α -CAs, the γ -CA from *M. thermophila* does not have esterase activity and also it exhibits relatively lower inhibition in response to sulfonamide (Alber and Ferry, 1996).

The polyclonal antibodies developed against arabidopsis γ -CA indicated its presence in mitochondrial protein complex I. In the homozygous knockout mutant of γ -CA in *A. thaliana*, a reduction in the level of mitochondrial protein complex I was observed (Perales et al., 2005). The mitochondrial protein complex I consists of five structurally related subunits representing γ -CA (Sunderhaus et al., 2006) and when grown in suspension culture, arabidopsis cells from γ -CA homozygous knockout mutant exhibited reduced growth rates and respiration suggesting role of γ -CAs in mitochondria and respiration in arabidopsis (Perales et al., 2005).

1.2.4 δ -carbonic anhydrase gene family

A 27-kDa monomeric carbonic anhydrase, namely TWCA1 was purified from marine diatom *Thalassiosira weissflogii* and has been suggested to play a critical role in inorganic carbon acquisition in marine diatoms (Roberts et al., 1997). The active site of TWCA1 is similar to that of α -CAs and γ -CA; the zinc atom at the active site was shown to be coordinated by three histidine residues and a water molecule (Roberts et al., 1997). However, the deduced amino acid sequence of TWCA1 does not show any significant sequence similarity with other CA gene families and may represent an example of convergent evolution at the molecular level. Therefore, TWCA1 was proposed as the fourth class of CA gene family designated as the δ -CA (Tripp et al., 2001). Similar δ -CAs has also been reported in marine coccolithophorid algae *Emiliana huxleyi* (Soto et al., 2006) and in free-living marine dinoflagellate *Lingulodinium polyedrum*. The δ -CAs in these organisms appears to play a role in carbon acquisition.

1.2.4 ζ -carbonic anhydrase gene family

All the CA gene families discussed so far are zinc containing metalloenzymes, however, a novel cadmium (Cd)-dependent carbonic anhydrase was purified and characterized from marine diatom *Thalassiosira weissflogii* (Lane et al., 2005). The Cd-dependent CA is distinct from all other CAs and hence has been proposed as the fifth type of CA gene family designated as ζ -carbonic anhydrase.

In general, Cd is not believed to have a biological function, but in marine environment which usually has low Zn, the marine diatom *T. weissflogii* has been shown to utilize Cd reflecting its biological role in such environment (Lane and Morel, 2000). With the addition of Cd in Zn-limited cultures of *T. weissflogii*, an increase in the growth rate of the diatom and the levels of CA activity was observed. This increased CA activity was because of increased activity of ζ -CA, not because of the TWCA1, which is the major Zn-requiring isoform of CA in *T. weissflogii*. The diatom *T. weissflogii* expresses the Cd-requiring CA particularly in Zn-deficient conditions, in which ζ -CA replace Zn containing enzyme TWCA1 in its role as carbon-concentrating mechanism (Lane and Morel, 2000).

The fold in ζ -CAs have some similarity with that of β -CAs. Additionally, the geometry of the active site of the ζ -CAs is nearly identical to that of β -CAs suggesting that ζ -CAs might be a diverged subtype of β -CAs structure (Xu et al., 2008).

Table 1: Classification and characteristics of CA families

Features	α -carbonic anhydrase	β -carbonic anhydrase	γ -carbonic anhydrase	δ -carbonic anhydrase	ζ -carbonic anhydrase
Existence	200-300 million years	NA	3-4.5 billion years	NA	NA
Distribution	Animals, plants, fungi, algae, bacteria and viruses	Plants, Fungi, (Götz et al., 1999; Hewett-Emmett, 2000)(Götz et al., 1999; Hewett-Emmett, 2000) invertebrates eubacteria and archaeobacteria	Archaea, bacteria and plants	Marine diatoms, marine dinoflagellate and marine coccolithophorid algae	Marine diatom
Metal required for activity	Zinc	Zinc	Zinc or Cobalt	Zinc	Cadmium
Active site	Three histidine	Two cysteine and one histidine	Three histidine	Three histidine	Two cysteine and one histidine
Zinc ligand	Three Histidine	One Histidine, Two cysteine	Three Histidine	Similar to α and γ	Similar to β
Proton transport	Histidine	Aspartate	Water molecule	Similar to α and γ	Similar to β
Polypeptide	Monomer	Dimer, tetramer, hexamer and octamer (basic building block is dimer)	Homotrimer	Monomer	Monomer
Polypeptide arrangement	Antiparallel β - sheet	α helices with parallel β sheet core	Left handed β - helix	NA	NA
Size	~30 kDa [exceptions: <i>D. Salina</i> (60 kDa), <i>C. reinhardtii</i> (82KDa)]	Monomeric unit is ~ 25 kDa	19 kDa	27 kDa	NA

1.3 Mechanism of action

The mechanism of CA-catalyzed interconversion of CO_2 and HCO_3^- is same in all the CA families; the only difference is the amino acid residues at the active site of each family. In the active site of CAs three amino acid residues serves as zinc-ligands that holds the zinc atom in proper position. The water molecule then binds with zinc atom, which serves as a Lewis acid to decrease of pka of water from 15.7 to 7. The resulting neutral pH causes removal of a proton from the bound water molecule forming a zinc-bound hydroxide ion (Step 1, Fig. 1).

Carbonic anhydrases have high turnover number and performs very fast catalytic reaction; therefore to keep up with the speed of the reaction a specific amino acid at the active site serves as proton shuttle to diffuse away the proton removed from zinc-bound water molecule. This proton shuttling is the rate-limiting step for carbonic anhydrase-catalyzed reactions.

The zinc-bound hydroxide ion produced as a result of proton removal acts as a strong nucleophile, which attacks on CO_2 and forms bicarbonate (Step 2 and 3, Fig. 1). The water molecule then replaces the bicarbonate resulting in the formation of zinc bound H_2O to begin the cycle again (Step 4, Fig. 1).

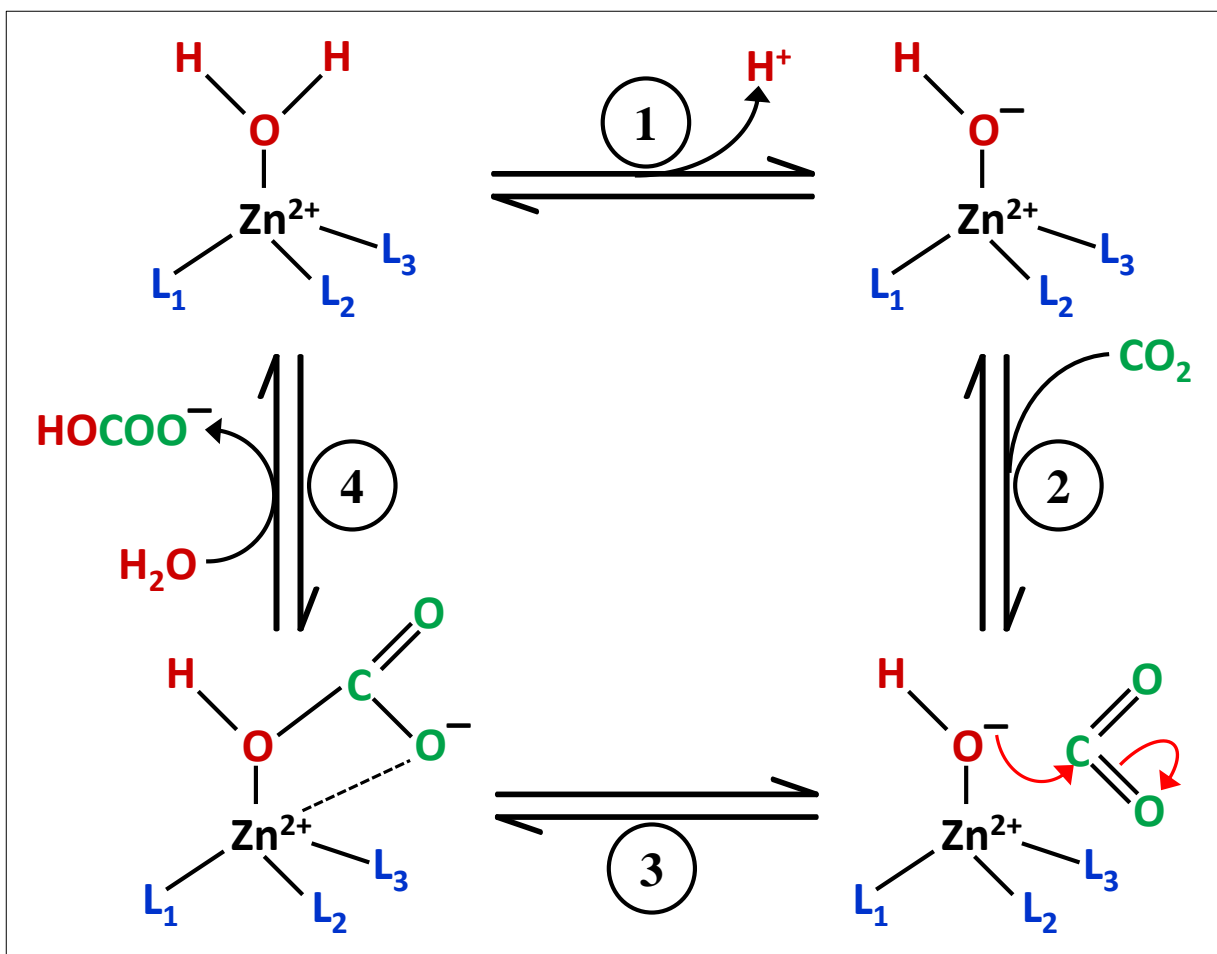


Figure 1: Step-wise mechanism of CA-catalyzed reaction. (1) In the active site of CAs three amino acid residues serves as zinc-ligands that holds the zinc atom in proper position. The water molecule then binds with zinc atom, which serves as a Lewis acid to decrease of pka of water from 15.7 to 7. The resulting neutral pH causes removal of a proton from the bound water molecule forming a zinc-bound hydroxide ion. (2-3) The zinc-bound hydroxide ion produced as a result of proton removal acts as a strong nucleophile, which attacks on CO₂ and forms bicarbonate. (4) The water molecule then replaces the bicarbonate resulting in the formation of zinc bound H₂O to begin the cycle again

1.4 Role of β -CAs in higher plants

In animals the major role of carbonic anhydrase is to facilitate the removal of CO_2 from the body, however, CAs have quite different roles in plants. Among all the CA gene families, β -CAs are the most abundant CAs in plants and their major role is to facilitate the diffusion of CO_2 to the site of inorganic carbon fixation.

Based on the mechanism of inorganic carbon fixation the plants may be classified in three distinct groups including crassulacean acid metabolism (CAM), C4 and C3 plants. Although the primary role of β -CA in plants is CO_2 accumulation and carbon fixation, the role of β -CA differs among different plant groups with different mechanisms for fixing inorganic carbon into sugar (Burnell, 2000).

1.4.1 Role of β -CAs in CAM plants

CAM plants fix CO_2 via crassulacean acid metabolism, which evolved in CAM plants as an adaptation in dry climates. CAM plants have separated the mechanism for inorganic carbon fixation in two time zones, night and day. At night, the CAM plants have their stomata open which let the CO_2 diffuse into the leaves where β -CA catalyzes the conversion of CO_2 into bicarbonate, which is then used as a substrate by PEP carboxylase. PEP carboxylase catalyzes the formation of oxaloacetate from PEP and bicarbonate. The resulted oxaloacetate is then converted into malate, which is then stored in vacuoles during night (Fig. 2).

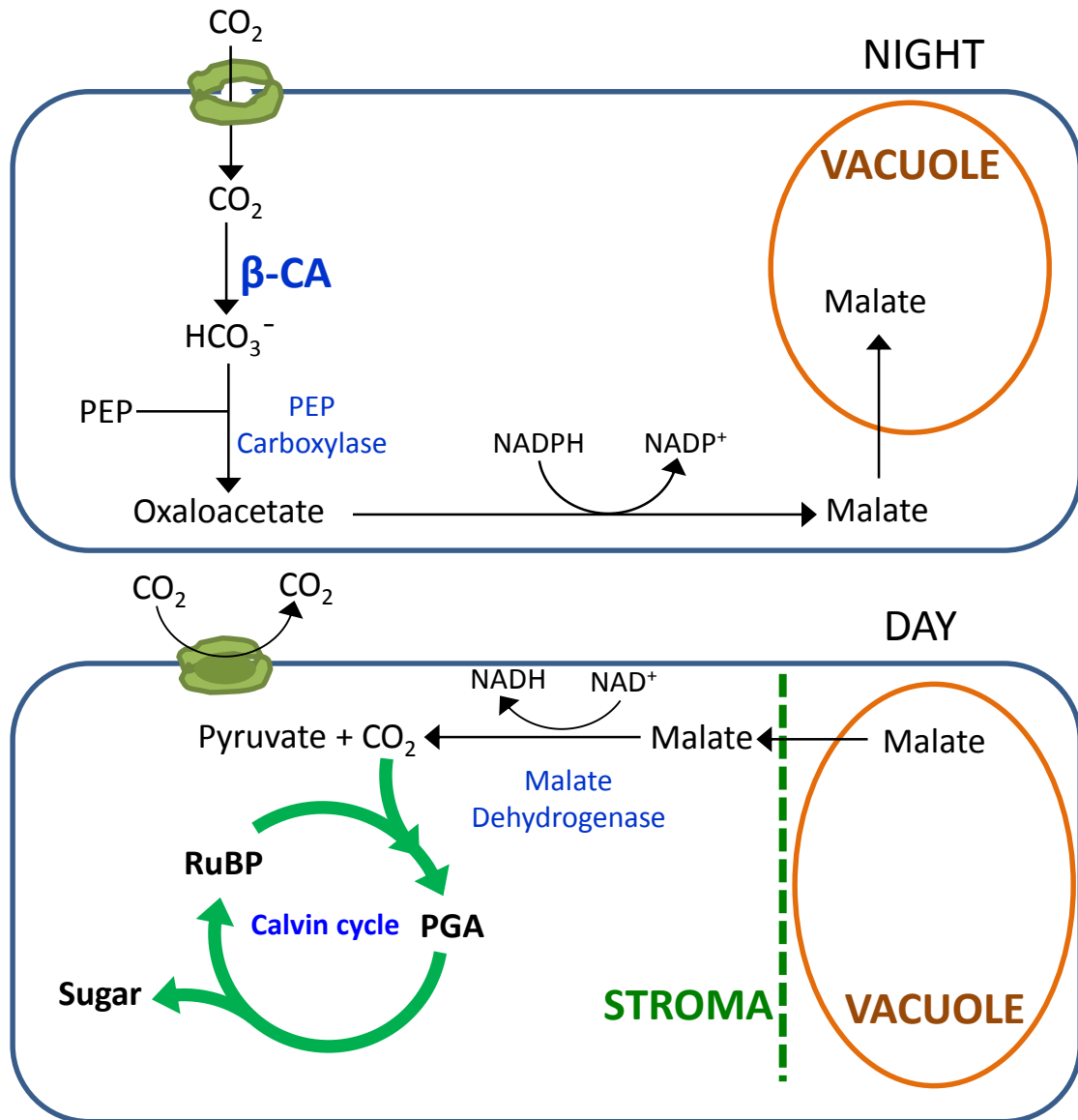


Figure 2: Schematic representation of the role of β -CA in the carbon fixation in CAM plants. At night, the CAM plants have their stomata open which let the CO_2 diffuse into the leaves where β -CA catalyzes the conversion of CO_2 into bicarbonate, which is then used as a substrate by PEP carboxylase. PEP carboxylase catalyzes the formation of oxaloacetate from PEP and bicarbonate. The oxaloacetate is then converted into malate, which is then stored in vacuoles during night.

However, during the day, stored malate is released from vacuole of mesophyll cells and enters the stroma of chloroplasts where it is decarboxylated to

release CO_2 . The released CO_2 is then refixed by the Calvin cycle. In CAM plants, β -CA is an important enzyme, which maintains the equilibria of CO_2 and HCO_3^- for carbon fixation.

1.4.2 Role of β -CAs in C4 plants

The C4 plants have specialized anatomy, called Kranz anatomy, in which the photosynthetic tissue is anatomically and biochemically divided into two distinct cell types. Anatomically, the vascular bundles of leaves have two rings of cells, an outer ring that is made of mesophyll cells and an inner ring, which is made of bundle-sheath cells that contains starch-rich chloroplasts lacking grana. Biochemically, the two different cell types have two separate mechanisms for fixing inorganic carbon.

The first inorganic carbon fixation is catalyzed by phosphoenolpyruvate (PEP) carboxylase in the cytoplasm of mesophyll cells, whereas the second is catalyzed by Rubisco in the chloroplast of bundle sheath cells. Rubisco can utilize the diffused CO_2 as a substrate, however, PEP carboxylase in mesophyll cells cannot use CO_2 directly as the substrate. Instead, the inorganic carbon substrate for PEP carboxylase is HCO_3^- . Therefore, the conversion of diffused CO_2 into HCO_3^- in the mesophyll cells is essential to fix inorganic carbon and this critical requirement makes the β -CA crucial for carbon fixation in C4 plants. The β -CA in the outer ring of mesophyll cells catalyzes rapid conversion of diffused CO_2 into HCO_3^- and therefore, serves as the first enzyme in carbon fixation in C4 plants

(Burnell and Hatch, 1988). The resultant HCO_3^- then combines with PEP to form organic compounds predominantly oxaloacetate and malate by the action of PEP carboxylase (Fig. 3), which unlike rubisco, can operate at much lower CO_2 level, without any inhibition by O_2 . Additionally, C4 plants can maintain sufficient CO_2 influx even when the stomata are closed at the leaf surface to prevent water loss in hot arid environment.

The C4 products (oxaloacetate and malate) formed in the mesophyll cells of C4 plants are transported through plasmodesmata into thick-walled bundle-sheath cells that are sealed off from atmospheric gases. In the bundle-sheath cells, the splitting of C4 products to produce CO_2 increases its level suitable for carbon fixation by rubisco. As the inorganic carbon substrate for PEP carboxylase is HCO_3^- instead of CO_2 , the β -CA acts as the first enzyme for carbon fixation in C4 plants (Hatch and Burnell, 1990).

In a study on various C4 plants, including *Zea mays*, *Spinacia oleracea*, *Setaria viridis* and *Triticum aestivum* cv. Omase, the significant β -CA activity was detected in the inner side of the plasma membrane of mesophyll cells (Utsunomiya and Muto, 1993). Moreover, the bundle sheath cells from leaves of a variety of C4 species were found to have little or no β -CA activity (Burnell and Hatch, 1988) and this deficiency of CA activity in the bundle sheath cells helps in maintaining high CO_2 level for proper functioning of rubisco. Using antibodies developed against β -CA, the isolated bundle sheath cells of *Flaveria bidentis*, a C4 plant, were shown to have small but significant amount of CA activity (Ludwig and Burnell, 1995).

Overexpression of β -CA from *Nicotiana tabacum* in bundle-sheath cells of *F. bidentis* resulted in ~10-50% increase in the CA activity compared to non-transformed control plants. However, this increase in CA activity in the cytoplasm of bundle sheath cells caused rapid conversion of CO_2 into HCO_3^- and its leakage from bundle sheath cells to mesophyll cells through plasmodesmata. The increased permeability of the bundle sheath cells to inorganic carbon then resulted in the decrease in the CO_2 availability for rubisco (Jenkins et al., 1989; Ludwig et al., 1998). Therefore, high β -CA activity in mesophyll cells and low or no β -CA activity in bundle sheath cells is vital for the efficient carbon fixation in C4 plants.

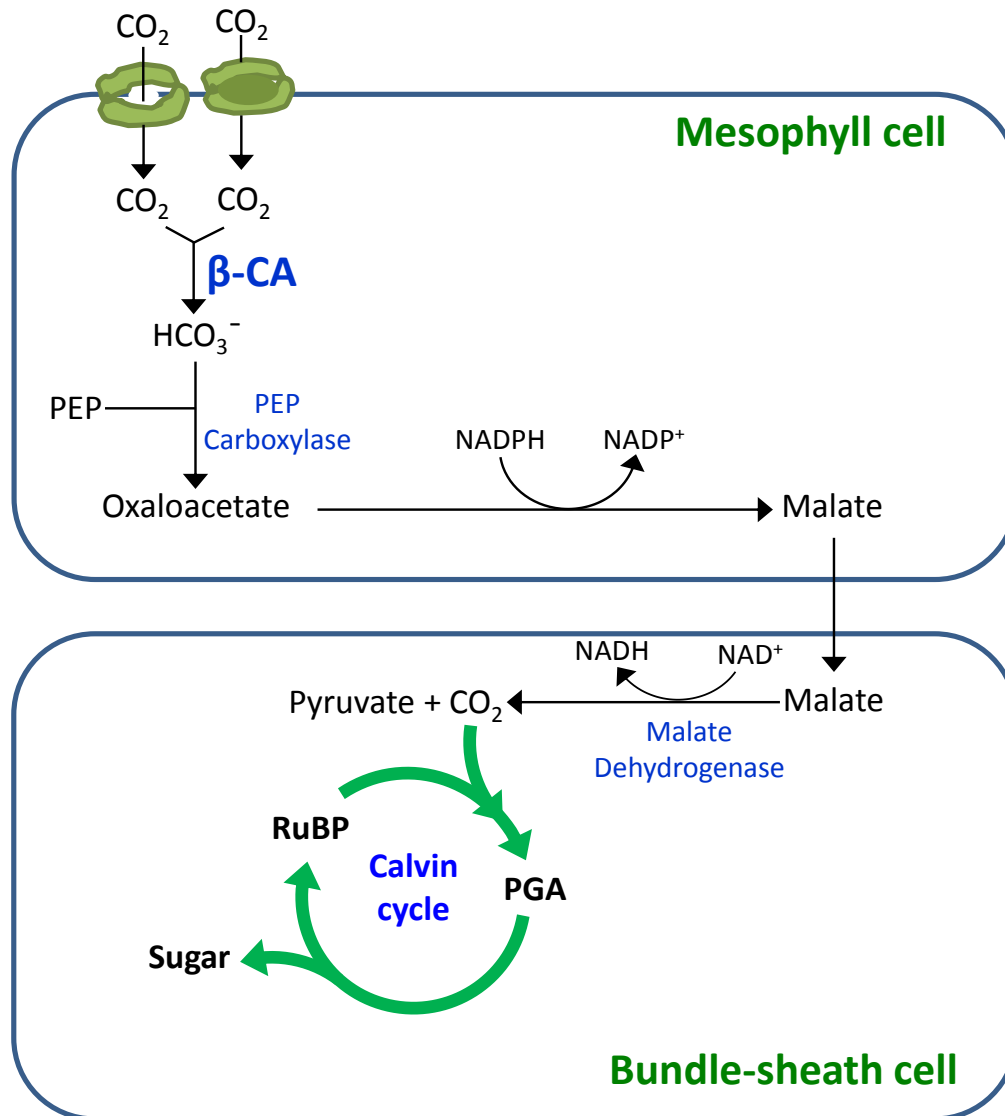


Figure 3: Schematic representation of the role of β-CA in the carbon fixation in C4 plants. The photosynthetic tissue in C4 plants is anatomically and biochemically divided into two distinct cell types; an outer ring that is made of mesophyll cells and an inner ring, which is made of bundle-sheath cells. The first inorganic carbon fixation is catalyzed by phosphoenolpyruvate (PEP) carboxylase in the cytoplasm of mesophyll cells, whereas the second is catalyzed by Rubisco in the chloroplast of bundle sheath cells. The β-CA in the outer ring of mesophyll cells catalyzes rapid conversion of diffused CO₂ into HCO₃⁻. The resultant HCO₃⁻ then combines with PEP to form organic compounds predominantly oxaloacetate and malate by the action of PEP carboxylase. The C4 products (oxaloacetate and malate) formed in the mesophyll cells are transported through plasmodesmata into thick-walled bundle-sheath cells that are sealed off from atmospheric gases. In the

bundle-sheath cells, the splitting of C₄ products to produce CO₂ increases its level suitable for carbon fixation by rubisco.

1.4.2 Role of β -CAs in C₃ plants

In CAM and C₄ plants β -CAs act as a primary enzyme in the CO₂ fixation and most of the CO₂ that is fixed comes through the β -CAs (Badger and Price, 1994). However, the role of β -CAs in C₃ plants is uncertain because of limited study. The C₃ plants have significantly different anatomy than that of C₄ and CAM plants; unlike C₄ and CAM plants, the carbon fixation machinery in C₃ plants is neither spatially nor timely separated.

The β -CAs are highly abundant proteins in C₃ plants and represent ~0.5 to 2% of total soluble protein from leaf tissues (Reed and Graham, 1981; Okabe et al., 1984). Two β -CA isoenzymes including a chloroplastic and a cytoplasmic isoform have been identified in many C₃ plants (Kachru and Anderson, 1974; Fett and Coleman, 1994). It has been suggested that the predominant isoform of C₃ plant β -CAs is the chloroplast localized β -CA, which account for most of the CA activity in the leaves of C₃ plants (Burnell, 2000).

The chloroplast stroma of C₃ plants is alkaline and hence the chloroplastic β -CAs has been proposed to be involved in the diffusion of CO₂ across the chloroplast stroma where diffused CO₂ and ribulose bisphosphate (RuBP) serves as the substrate for Rubisco, which fixes the inorganic carbon into 3-phosphoglycerate (Badger and Price, 1994) (Fig. 4). In contrast to the

chloroplastic β -CAs, the cytoplasmic β -CAs may have little role in carbon fixation because the cytoplasm is the acidic environment and hence the HCO_3^- levels are usually low in the acidic environment. Additionally, the CO_2 that diffused 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 and Price, 1994).

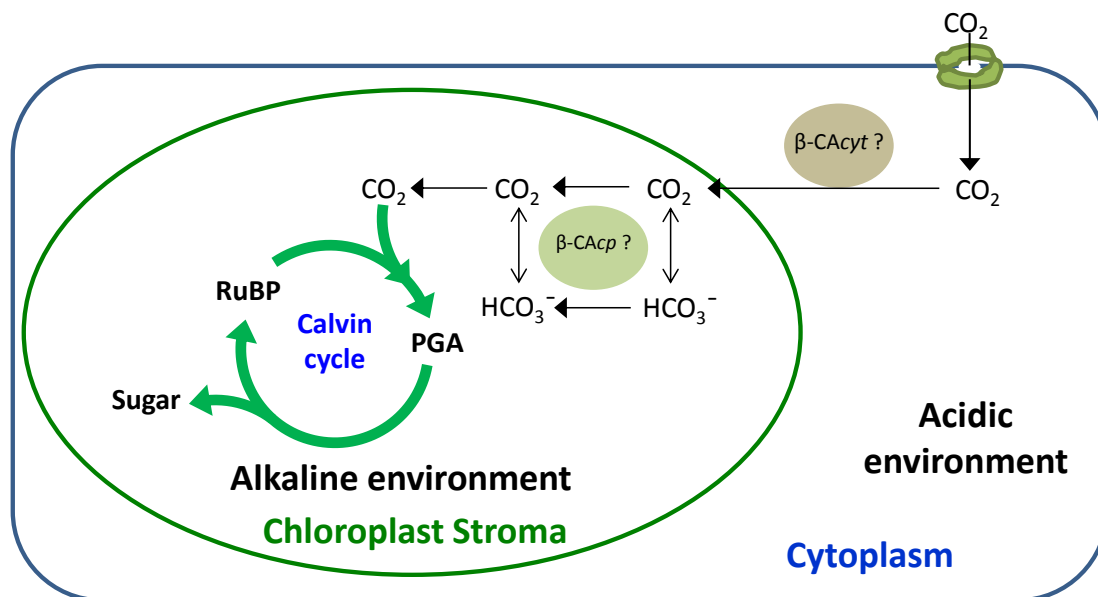


Figure 4: Representation of possible role of β -CA in the carbon fixation in C3 plants. The chloroplast stroma of C3 plants is alkaline and hence the chloroplastic β -CAs has been proposed to be involved in the diffusion of CO_2 across the chloroplast stroma where diffused CO_2 and ribulose bisphosphate (RuBP) serves as the substrate for Rubisco, which fixes the inorganic carbon into 3-phosphoglycerate. In contrast to the chloroplastic β -CAs, the cytoplasmic β -CAs may have little role in carbon fixation because the cytoplasm is the acidic environment and hence the HCO_3^- levels are usually low in the acidic environment. Additionally, the CO_2 that diffused through the cell wall may directly enter the chloroplast because the diffusion distance between the chloroplast envelope and the cell wall is typically small.

Although β -CA represents ~2% of the total protein in leaf tissue of C3 plants, significant reduction in β -CA activity by limiting its cofactor, zinc, has insignificant impact on the photosynthesis in C3 plants (Edwards and Mohamed, 1973; Randall and Bouma, 1973). This finding contradicts the proposed importance of β -CAs in CO₂ influx and photosynthesis in the C3 plants, however it does not rule out the possibility of very small level of β -CAs being adequate in maximum diffusion of CO₂ in plants. Previous studies in which expression of β -CA in C3 plants were suppressed by antisense RNA techniques resulted in conflicting results. In *Tobacco*, decrease in β -CA expression did not show any difference in CO₂ assimilation and phenotype (Majeau et al., 1994), however a recent report shows that reduced activity of plastid-localized β -CA has negative impact on the survival of *Arabidopsis* seedling at ambient level of CO₂ but not at elevated CO₂ level or at high sucrose concentration (Ferreira et al., 2008). This report suggests that the β -CA is involved in photosynthetic performance of cotyledons by facilitating the diffusion of CO₂ in plants during phototropic growth before the development of true leaves.

Large content of β -CA in leaves of C3 plants suggests their importance in the physiology of the C3 plants. Besides their proposed role in the carbon fixation and photosynthesis, they may also be involved in dealing with the physiological stress conditions that may limit the photosynthetic potential of C3 plants. 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 limits the influx of CO₂ in the leaves by decreasing the conductance of stomata and mesophyll (Flexas et al., 2004; Sudhir and Murthy, 2004). Similarly, light and dark conditions are also related with photosynthetic potential of plants; therefore, it would be interesting to test any possible role of β-CA under physiological stress conditions. The possible role of a β-CA isoform in such physiological stress conditions was shown recently in a C₄ plant, *Pennisetum glaucum* (Kaul et al., 2011). The *Pennisetum glaucum* β-CA, which is evolutionary close to chloroplast β-CA isoform displayed differential up-regulation under various physiological stress conditions indicating its involvement in improving plants tolerance against physiological stresses. Such studies on C₃ plant β-CAs are lacking and therefore, the role of β-CAs isoforms in C₃ plants is not well-studied. Previously, cDNAs corresponding to chloroplastic and cytoplasmic β-CAs were isolated from a C₃ plant *Arabidopsis thaliana* (arabidopsis) and the expression of the two transcripts were shown to be up- and down-regulated by light and dark conditions, respectively (Fett and Coleman, 1994). These finding strengthens the possible role of C₃ β-CA isoforms in dealing with physiological stress conditions.

1.5 Hypotheses

- β-CAs are highly abundant in C₃ plants and besides fixation of inorganic carbon they may also be involved in conferring tolerance to various physiological stress conditions.

- *Leucaena leucocephala*, a tropical tree-legume, is highly tolerant to various physiological stress conditions and therefore, it may be an ideal plant to study the possible role of chloroplastic and cytoplasmic isoforms of β -CAs under abiotic stress conditions.
- If C3 plant β -CAs are involved in conferring tolerance against various stress conditions, their expression level should be differentially regulated under physiological stresses. The change in expression may be studied by using real-time PCR.
- Individual role of chloroplastic and cytoplasmic β -CA isoforms of *Leucaena leucocephala* in inorganic carbon fixation and in improving plants ability to withstand physiological stress conditions can be studied by overexpressing genes for these β -CA isoforms, individually as well as together, in model plant tobacco and comparing transgenic tobaccos with wild-type tobaccos for their ability to tolerate stress conditions.

1.6 Objectives

The overall objective of this research is to study chloroplastic and cytoplasmic isoform of β -CAs and their potential role in inorganic carbon fixation.

The specific objectives include:

I. The isolation of *Leucaena* genes encoding chloroplastic β -CA (ca_{cp}) and cytoplasmic β -CA (ca_{cyt}) and their comparative studies.

- a. Isolation of cDNA fragments for chloroplastic and cytoplasmic β -CA isoforms from *Leucaena*
- b. Extension of the 5'- and 3'-end of the partial cDNA fragments for β -CAs
- c. Prediction of sub-cellular localization of ca_{cp} and ca_{cyt}
- d. Prediction of secondary structure, active site residues, and three-dimensional structures of the two β -CAs

II. Determining the expression of ca_{cp} and ca_{cyt} in *Leucaena leucocephala* under various stress conditions.

- a. Design of qPCR primer sets specific for ca_{cp} and ca_{cyt} .
- b. High quality RNA preparation and cDNA synthesis from leaf, root, and stem tissues of *Leucaena leucocephala* grown under normal conditions.
- c. RT-PCR analysis of ca_{cp} and ca_{cyt} for quantitative estimation of their expression in different tissue types of *Leucaena leucocephala*.

- d. Imitation of various stress conditions and exposing *Leucaena* plants to these stress conditions.
- e. High quality RNA preparation and cDNA synthesis from leaf, root, and stem tissues of *Leucaena leucocephala* exposed to various stress conditions.
- f. Identification of suitable internal reference gene with highest stability of expression and least variation in expression in each stress condition and tissue type of *Leucaena leucocephala*.
- g. Real-time PCR analysis of ca_{cp} and ca_{cyt} for quantitative estimation of their expression in different tissue types of *Leucaena leucocephala* exposed to various stress conditions.

III. Determining the role of ca_{cp} and ca_{cyt} from *Leucaena leucocephala* in stress tolerance and carbon assimilation in transgenic tobacco plants containing these genes.

- a. Gateway cloning of ca_{cp} and ca_{cyt} to prepare binary constructs
- b. *Agrobacterium*-mediated transformation of *N. tabacum* with the binary construct containing ca_{cyt}

- c. *Agrobacterium*-mediated transformation of *N. tabacum* with the binary construct containing ca_{cp}
- d. *Agrobacterium*-mediated transformation of *N. tabacum* with the binary constructs for ca_{cyt} and ca_{cp} together
- e. Screening of transgenic plants for heterologous expression of transgene
- f. Comparative study of β -CA activity
- g. Measurement of carbon assimilation
- h. Comparative study of wild-type and transgenic tobacco plants under stress conditions

CHAPTER 2

Isolation of Leucaena genes for chloroplastic and cytoplasmic β -CAs and their comparative studies

2.1 Introduction

The β -CAs are zinc metalloenzymes, which are highly conserved among plants. In C3 plants β -CAs are one of the most abundant proteins representing ~0.5 to 2% of total leaf protein (Reed and Graham, 1981; Okabe et al., 1984). They are expressed in both green tissues as well as root tissues.

The β -CA isoforms are nuclear encoded proteins but have been predicted to have different sub-cellular localizations such as mitochondria, chloroplast, and cytoplasm (Fabre et al., 2007; Tetu et al., 2007). The β -CA isoforms, chloroplast-localized and cytoplasmic in particular, have been suggested to have a role in carbon fixation (Syrjänen et al., 2010). In C3 plants, chloroplast-localized and cytoplasmic β -CA isoforms have been reported (Kachru and Anderson, 1974; Fett and Coleman, 1994) of which the chloroplast-localized β -CA has been proposed to be the most predominant isoform (Burnell, 2000). Fett and Coleman (1994) isolated cDNA for chloroplastic and cytoplasmic β -CA isoforms from *Arabidopsis thaliana* λ YES library and demonstrated that the expression level of the two β -CA isoforms is regulated by light and dark conditions.

Previous crystallographic studies have identified different oligomeric forms of β -CA including dimeric, tetrameric, hexameric and octameric forms (Kimber and Pai, 2000; Smith et al., 2000; Strop et al., 2001). The monomeric subunit of β -CA has a molecular weight of ~25 kDa (Johansson and Forsman, 1992). Mutagenesis and extended X-ray absorption fine structure (EXAFS) studies on β -CAs identified that pea and spinach β -CAs utilizes one cysteine and two histidine residues to bind the zinc ion at the active site of the enzyme (Provar et al., 1993; Bracey et al., 1994; Rowlett et al., 1994). Each monomeric subunit in the multimeric enzyme binds with one zinc atom, which is essential for catalysis and serves as the prosthetic group. The functionally active β -CAs exhibit similar folds, secondary structure, and display conserved active site residues. However, the three-dimensional structure of β -CAs varies depending on the oligomeric states.

The β -CAs has been well-studied in C4 plants but not in C3 plants, therefore the role of C3 plant β -CA isoforms is still elusive. In this chapter, the chloroplastic and cytoplasmic β -CAs from *Leucaena leucocephala*, a tropical C3 plant, has been discussed.

2.2 Materials and methods

2.2.1 Plant growth

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 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 5mM $\text{Ca}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$, 2mM MgSO_4 , 5mM KNO_3 , 1mM KH_2PO_4 , 0.02mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02mM Na-EDTA, 0.045mM H_2BO_3 , 0.01mM $\text{MnCl}_2 \cdot \text{H}_2\text{O}$, 0.8 μM ZnSO_4 , 0.1 μM $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ and 0.3 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The plants were watered with Hoagland solution twice a week and water lost by evapotranspiration was compensated for by adding deionized water, daily.

Eight-week old seedlings were immediately frozen in liquid nitrogen and stored at -80 °C until used for RNA extraction.

2.2.2 RNA extraction and cDNA synthesis

Frozen samples of whole seedlings were finely ground 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).

2.2.3 Isolation of chloroplastic 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 (Negi et 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 FirstChoice[®] RLM-RACE Kit (Ambion) according to the manufacturer's instruction. For 5'-RACE of the cDNA fragment, 5'-ROP-*ca_{cp}* and 5'-RIP-*ca_{cp}* gene-specific primers (Table 2) were used with the 5'-RACE outer and 5'-RACE inner primers (Ambion) and for 3'-RACE of the cDNA fragment, 3'-ROP-*ca_{cp}* and 3'-RIP-*ca_{cp}* (Table 2) 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 2: Primers used and their description

Primer name	Sequence (5' to 3')	Description
5'-ROP- <i>ca</i> _{cp}	TGGGTGAAGATCGGTTTACCTGCA	These primers were used in the 5'-RLM-RACE of <i>ca</i> _{cp}
5'-RIP- <i>ca</i> _{cp}	TGCTTGTGGTGGTATCAAGGGTCT	
3'-ROP- <i>ca</i> _{cp}	ACTTGGTCCCACCATATTGCCAGA	These primers were used in the 3'-RLM-RACE of <i>ca</i> _{cp}
3'-RIP- <i>ca</i> _{cp}	TCTCAAGGTGTCGGAAATCGTGGT	
F- <i>ca</i> _{cyt}	ATCTTGAATTTCCAACCTGGTGAGGCTTT	These primers were used to amplify 151bp <i>ca</i> _{cyt}
R- <i>ca</i> _{cyt}	CCTATCACCAAAAATGTTCTCCACCTT	
3'-RP- <i>ca</i> _{cyt}	TGAGGCTTTTGTGGTTCGCAACATCGC	These primers were used in the 5'- and 3'-RLM-RACE of
5'-ROP- <i>ca</i> _{cyt}	CCACCTTTAAGTGCAACACTGC	<i>ca</i> _{cyt}

2.2.4 Isolation of cytoplasmic β -carbonic anhydrase cDNA

To isolate leucaena cDNA for cytoplasmic carbonic anhydrase (*ca*_{cyt}), three nucleotide sequences for *ca*_{cyt} one each from *Arabidopsis thaliana* (gi|145362379), *Ricinus communis* (gi|255568811) and *Populus trichocarpa* (gi|224107828) were obtained from NCBI. Nucleotide sequences of these *ca*_{cyt} were compared using

ClustalW and F-*ca_{cyt}* and R-*ca_{cyt}* primers were designed from the conserved regions (Table 2) and PCR was performed to amplify a partial cDNA fragment of cytoplasmic β -CA. The 5'- and 3'-ends of partial cDNA sequences for *ca_{cyt}* was extended by RLM-RACE using FirstChoice[®] RLM-RACE Kit (Ambion) according to the manufacturer's instruction. The gene-specific primers, 5'-RP-*ca_{cyt}* was used in the 5'-RACE of the *ca_{cyt}* cDNA fragment whereas, 3'-RP-*ca_{cyt}* was used in the 3'-RACE (Table 2). 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.

2.2.5 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 *ca_{cp}* and *ca_{cyt}* were identified using 'ORF finder' tool of sequence manipulation suite. The deduced amino acid sequence for *ca_{cp}* and *ca_{cyt}* 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 ca_{cp} and ca_{cyt} are of plant origin. The cleavage site prediction was also performed in this analysis.

2.2.6 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 C3 plant *Pisum sativum* (gi|8569250) was used as a reference sequence as its X-Ray crystallographic structure has already been resolved (Kimber and Pai, 2000). The β -CA amino acid sequences of leucaena and *Pisum sativum* were studied for their secondary structure using the PSIPRED server (McGuffin et 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 C3 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 CA_{cp} and CA_{cyt} proteins were performed using SWISS-MODEL server (Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006).

2.3 Results

2.3.1 Isolation of chloroplastic 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 (Fig. 5).

GENE ID: 11428425 MTR_6g006990 Carbonic anhydrase [Medicago truncatula]				
Score = 247 bits (631), Expect = 1e-79, Method: Compositional matrix adjust.				
Identities = 119/146 (82%), Positives = 132/146 (90%), Gaps = 1/146 (1%)				
Frame = +3				
Query	3	VANLVPPYCQTRCAGVGAAVEYAVLHLKVSEIVVIGHVSACGGIKGLMSIPDNGAVPTTDF	182	
		VAN+VPPY Q + AG G+A+EYAVLHLKVS IVVIGHVSACGGIKGL+S P +GA +TDF		
Sbjct	115	VANMVPPYDQAKYAGTGS AIEYAVLHLKVSNI VVIGHVSACGGIKGLLSFPFDGAY-STDF	173	
Query	183	IXDWVKIGLPAKARVKS VHGAPFGELCTHCEKEVVNVS LGNLLTYPFVREGLVNKTLSL	362	
		I +WVKIGLPAKA+VK+ HG APFGELCTHCEKE VNVSLGNLLTYPFVREGLVNKTL+L		
Sbjct	174	IEEWVKIGLPAKAKVKAKHGDAPFGELCTHCEKEAVNVS LGNLLTYPFVREGLVNKTLAL	233	
Query	363	KGGYYDFVKGSFELWGLKFGLSSSLS	440	
		KGGYYDFVKGSFELWGL+FGLSS+ S		
Sbjct	234	KGGYYDFVKGSFELWGLEFGLSSTFS	259	

Figure 5: The blastx analysis of 515bp partial iSSH clone of *Leucaena*. The query sequence exhibited homology with the carbonic anhydrase with *M. truncatula* (Gene ID: 11428425 MTR_6g006990).

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 (Fig. 6).

GENE ID: 100500448 LOC100500448 carbonic anhydrase, chloroplastic [Glycine max] Length=328 amino acids				
Score = 485 bits (1248), Expect = 1e-169, Method: Compositional matrix adjust. Identities = 249/329 (76%), Positives = 281/329 (85%), Gaps = 7/329 (2%)				
Query	1	MSTASINGCCLSSFSSSKTSLPS---KFSVSARLATPPPPSSSSSIPSLIQNRPVFAAPT	57	
		MST+SING CLSS S +KTSL + SV A L TP SSSS PSLIQ+RPVFAAP		
Sbjct	1	MSTSSINGWCLSSISPAKTSLRKATLRPSVFATLNTFPSSPSSSSSFPSLIQDRPVFAAPA	60	
Query	58	PLITPTLNEDA---IEEAIVELEKLFKVKGELATTADARVDQVTAQVGTPTSEGIASSES	114	
		P+ITPT+ ED E+AI EL+KL + K EL TA +V+Q+TA +GT +S+GI SSE+		
Sbjct	61	PIITPTVREDMAKEYEKAIEELQKLLREKSELKATAAEKVEQITASLGTSSSDGIPSEA	120	
Query	115	VERIKAGFIHFKEKYEKNPALYGELAKGQSPPYMVFACSDSRVCPSHVLDFQPGEAFVV	174	
		+RIKAGFIHFKEKY+KNPALYGELAKGQSP +MVFACSDSRVCPSHVLDFQPGEAFVV		
Sbjct	121	SDRIKAGFIHFKEKYDKNPALYGELAKGQSPKFMVFACSDSRVCPSHVLDFQPGEAFVV	180	
Query	175	RNVANLVPPYCQTRYAGVGAAVEYAVLHLKVSEIVVIGHSACGGIKGLMSIPDNGAVPTT	234	
		RNVAN+VPPY Q++YAG GAAVEYAVLHLKVSEIVVIGHSACGGIKGL+S P +G +T		
Sbjct	181	RNVANIVPPYDQSKYAGTGAAVEYAVLHLKVSEIVVIGHSACGGIKGLSFPYDGTY-ST	239	
Query	235	DFIEDWVKIGLPAKARVKSVMGGAPFGELCTHCEKEAVNVSLGNLLTYPFVREGLVNKTL	294	
		DFIE+WVKIGLPAKA+VK+ HG APF ELC+HCEKE+VNVSLGNLLTYPFVR+GLVNKTL		
Sbjct	240	DFIEEWVKIGLPAKAKVKTQHGDAFPAELCSHCEKESVNVSLGNLLTYPFVRDGLVNKTL	299	
Query	295	SLKGGYYDFVKGSFELWGLQFGLSSSLSV	323	
		SLKGGYYDFVKGSFELWGLQFGL+SS SV		
Sbjct	300	SLKGGYYDFVKGSFELWGLQFGLASSFSV	328	

Figure 6: The blastp analysis of full-length deduced amino acid sequence of β -CA-1. The query sequence exhibited homology with the chloroplastic carbonic anhydrase with *G. max* (Gene ID: 100500448 LOC100500448).

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 3). 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' (ca_{cp}).

Table 3: Prediction of sub-cellular localization of the seq3-encoded protein of leucaena using TargetP 1.1 server

Protein	Length (amino acid)	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				
ca_{cp}	323	0.974	0.38	0.012	0.023	Chloroplast	1	36
ca_{cyt}	253	0.041	0.162	0.143	0.876	-	2	-

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

2.3.2 Isolation of cytoplasmic β -CA from leucaena

After identifying and isolating the leucaena cDNA for chloroplastic β -CA, the next goal was to isolate the leucaena cDNA for cytoplasmic β -CA (ca_{cyt}). Considering that leucaena is a C3 dicot plant, we obtained three cytoplasmic β -CA nucleotide sequences of C3 dicots from the NCBI database. The obtained β -CA sequences were from *Arabidopsis thaliana* (gi|145362379), *Ricinus communis* (gi|255568811) and *Populus trichocarpa* (gi|224107828). These nucleotide sequences were then aligned using ClustalW to identify the highly conserved regions in the cytoplasmic β -CAs of C3 dicots (Fig. 7). Two primers namely F- ca_{cyt} and R- ca_{cyt} were designed from the conserved regions of the cytoplasmic β -CAs as forward and reverse primers, respectively (Table 2). The PCR using leucaena cDNA as the template and F- ca_{cyt} and R- ca_{cyt} 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 (Fig. 8). The gene specific primers for performing 5'- and 3'-RLM-RACE were designed from the 151 bp sequence (Table 2).


```

GENE ID: 821134 CA1 | carbonic anhydrase 1 [Arabidopsis thaliana]
Score = 96.3 bits (238), Expect = 3e-23, Method: Composition-based
stats.
Identities = 43/50 (86%), Positives = 47/50 (94%), Gaps = 0/50 (0%)
Frame = +2

Query 2    VLDFQPGEAFVVRNIANMIPPYDQTKYSGTGAAIEYAVLHLKVENIVVIG 151
          VLDFQPG+AFVVRNIANM+PP+D+ KY G GAAIEYAVLHLKVENIVVIG
Sbjct 100  VLDFQPGDAFVVRNIANMVPPFDKVKYGGVGAAIEYAVLHLKVENIVVIG 149

```

Figure 8: The tblastx analysis of the 151 bp *Leucaena* β -CA obtained as a result of PCR using primers designed from the conserved region of cytoplasmic β -CAs of C3 dicots.

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 (Fig. 9). 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 3), suggesting it to be a cytoplasmic β -CA.

GENE ID: 11407419 MTR_5g034250 Carbonic anhydrase [Medicago truncatula]				
Score = 434 bits (1115), Expect = 2e-151, Method: Compositional matrix adjust.				
Identities = 198/252 (79%), Positives = 228/252 (90%), Gaps = 0/252 (0%)				
Query	1	MAGQSYEEAIAELKKLISEKADLGGVAAAKIKQLTAELSAADSKPIKPDERTGTFTHFK	60	
		MAG+++E++IA L +L+ EKA+LG +AA KIK+LTAE L A SKP PDERIR+GF FK		
Sbjct	23	MAGETFEDSIATLTRLKKEKAE LGDIAAVKIKELTAELEANGSKPFPDERIRSGFVSFK	82	
Query	61	KEKFEKNPDLFGKLATGQSPKFLVFACSDSRVCP SHVLD FQPGEAFVVRNIANMIPPYDQ	120	
		EKF KNP+L+G+LA GQSPKF+VFACSDSRVCP SH+LDFQPGEAFVVRNIANM+PP+D+		
Sbjct	83	TEKFLKNPELYGELAKGQSPKFMVFACSDSRVCP SHILDFQPGEAFVVRNIANMVPPFDK	142	
Query	121	TKYSGTGAAIEYAVLH LKVENIVVIGHSCCGGIKGLMSIPDDGTT SSEFIENWVQICNPA	180	
		TKYSG GAAIEYAVLH LKVENIVVIGHSCCGGIKGLMSIPDDGTT+S+FIE WVQICNPA		
Sbjct	143	TKYSGAGAAIEYAVLH LKVENIVVIGHSCCGGIKGLMSIPDDGTTASDFIEQWVQICNPA	202	
Query	181	KSKVKADTKSLSFSEQCTNCEKEAVNVSLANLLSYPFVRDGVVKKTLVLKGAHYDFVNGT	240	
		+SKVK +T SLSF+EQCTNCEKEAVNVSL NLL+YPFVRDGVVKK+L LKGAHY+FVNGT		
Sbjct	203	RSKVKLETSSLSFAEQCTNCEKEAVNVSLGNLLTYPFVRDGVVKKSLALKGAHYNFVNGT	262	
Query	241	FDLWDLDLKMSP 252		
		F+LWDL+ + P		
Sbjct	263	FELWDLNFNLLP 274		

Figure 9: The blastp analysis of full-length deduced amino acid sequence of *Leucaena* β -CA. The query sequence exhibited homology with the carbonic anhydrase with *M. truncatula* (GENE ID: 11407419 MTR_5g034250).

2.3.3 In silico structural analysis of leucaena CA_{cp} and CA_{cyt}.

β -CAs in general have high homology and conserved secondary structure irrespective of their different sub-cellular localization. Therefore, to compare the sequence similarity between ca_{cp} and ca_{cyt} encoded proteins (CA_{cp} and CA_{cyt}), the deduced amino acid sequences of the two β -CAs from leucaena were aligned using ClustalW. The signal peptide region of the CA_{cp} was eliminated from the alignment. The two sequences showed high homology with 64% identical and 79% similar amino acid residues (Fig. 10).



Figure 10: The sequence alignment of the deduced amino acid sequence of CA_{cyt} and CA_{cp} using ClustalW.

For secondary structure analysis, the well resolved and studied β -CA from the C3 plant *Pisum 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 (α 1, α 2, β 1, β 2, α 3, β 3, α 4, α 5, α 6, α 7, β 4, β 5). We identified the same sequential pattern of α -helix and β -strand in the predicted secondary structure of CA_{cp} and CA_{cyt} (Fig. 11).

There are some carbonic anhydrase-related proteins, which, despite high homology with carbonic anhydrases, lack the functional activity because of substitution in one or more zinc-binding ligands. 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 CA_{cp} and CA_{cyt} 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 CA_{cp}, 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 CA_{cyt}, 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 CA_{cp} and CA_{cyt} of leucaena were compared with the well-studied structure of C3 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 CA_{cp} and Asp-155 is the conserved residue for the proton transport required in the β -CA-catalyzed reactions (Fig. 12). In CA_{cyt}, it is likely that the Cys-87, Cys-139 and His-147 are the zinc ligand in CA_{cyt} of

leucaena and the Asp-89 is the residue involved in the proton transport (Fig. 13).

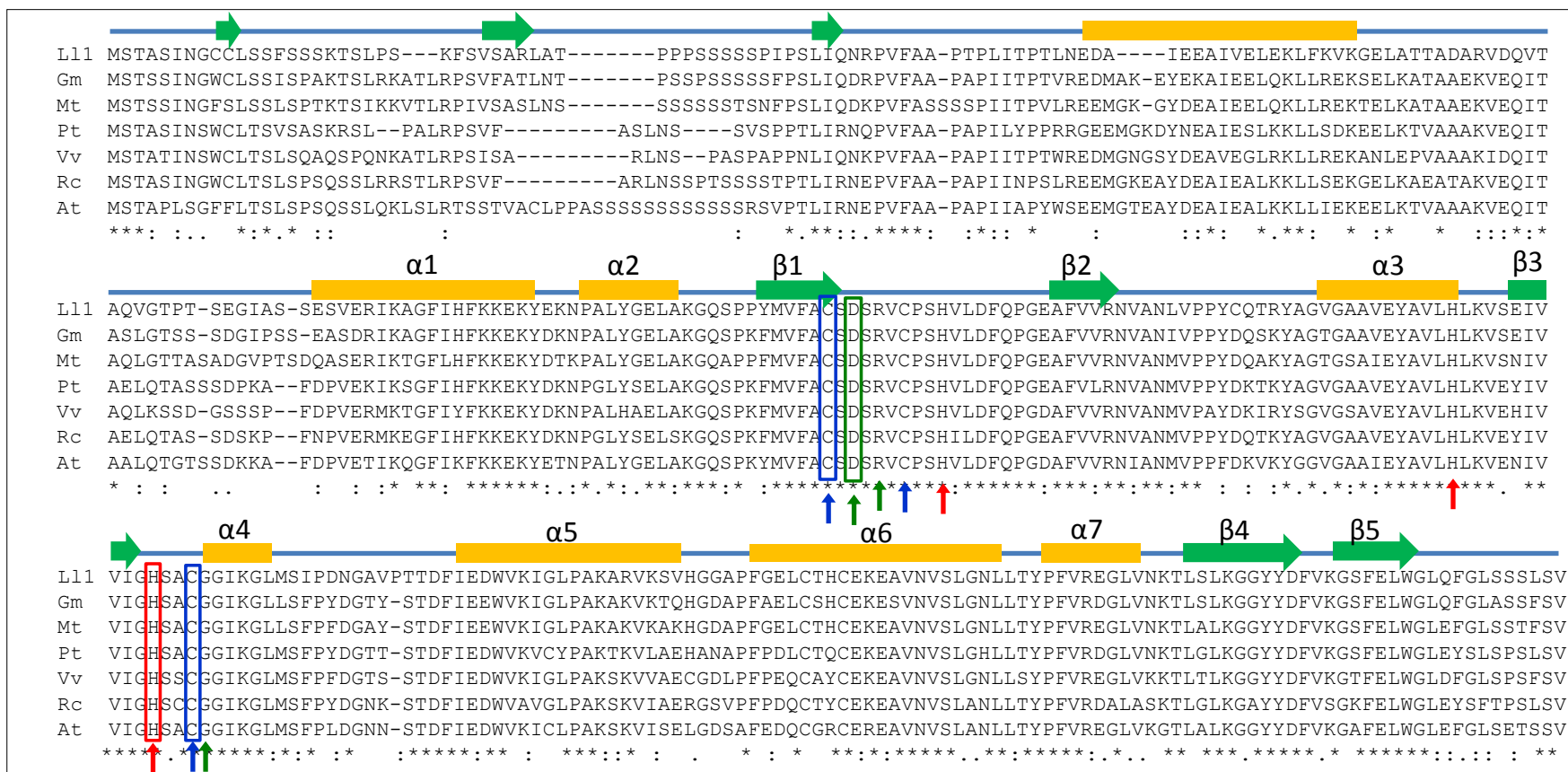


Figure 12: Chloroplastic β -CA of *Leucaena* (Ll1) was compared with the chloroplastic β -CAs of *G. max* (Gm), *M. truncatula* (Mt), *P. trichocarpa* (Pt), *V. vinifera* (Vv), *R. communis* (Rc) and *A. thaliana* (At). The conserved histidine and cysteine residues are represented by red and blue arrows, 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.

The three-dimensional (3D) quaternary structure of CA_{cp} and CA_{cyt} was predicted using the protein structure homology-modeling server; SWISS-MODEL (Schwede et al., 2003; Arnold et al., 2006). Based on the highest homology, the SWISS-MODEL server selected the X-ray crystallographic structure of β -CA from the C3 dicot *Pisum sativum* as the template structure and built the quaternary structure of both CA_{cp} and CA_{cyt} as octameric proteins (Figs. 14 and 15). 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 CA_{cp} and CA_{cyt} 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.

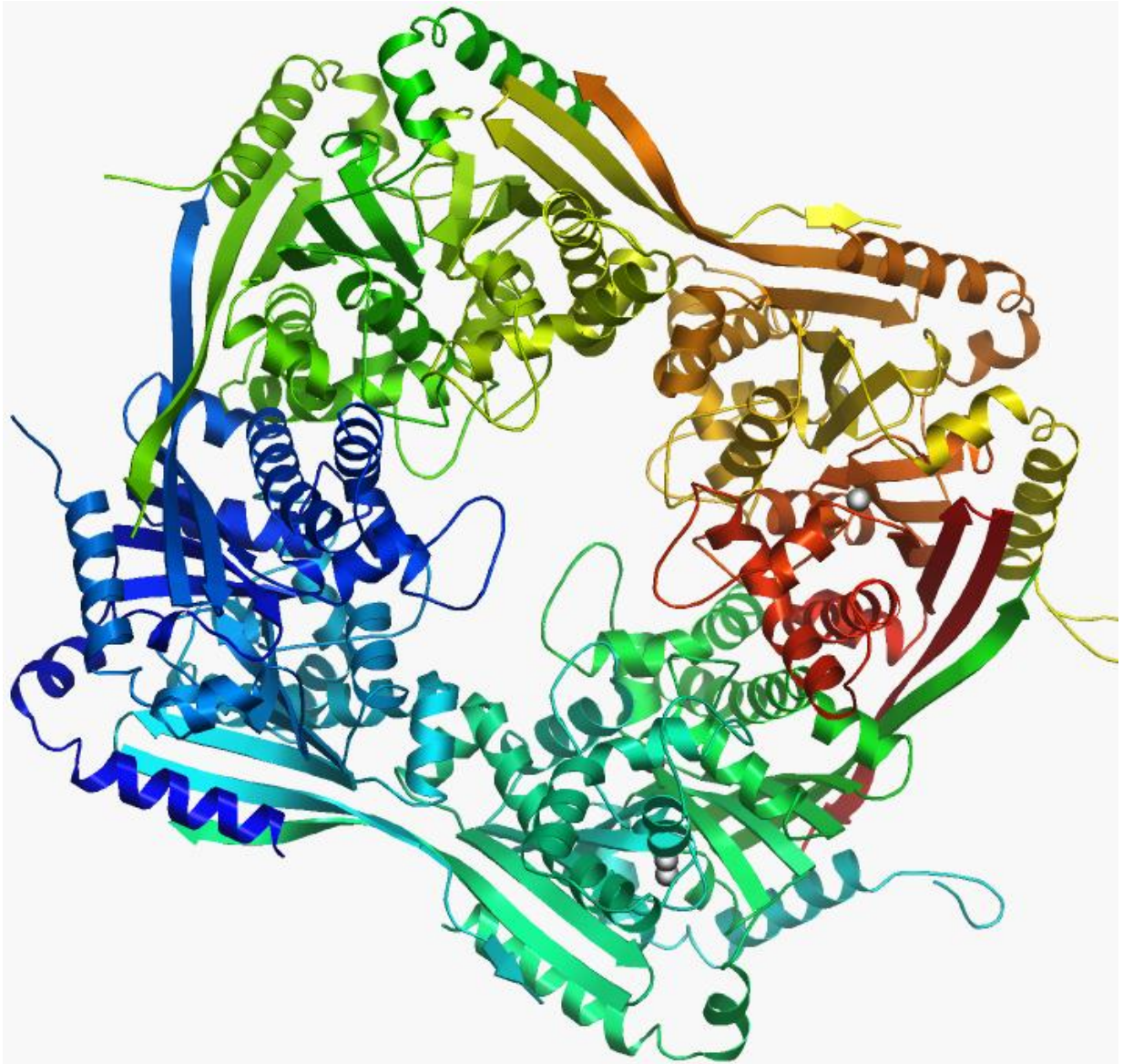


Figure 14: The 3D model of CA_{cp}. The quaternary structure was built using SWISS-MODEL server. The model for CA_{cp} was predicted to be octameric proteins. Each monomeric unit (polypeptide chain) of the proteins is represented in different color.

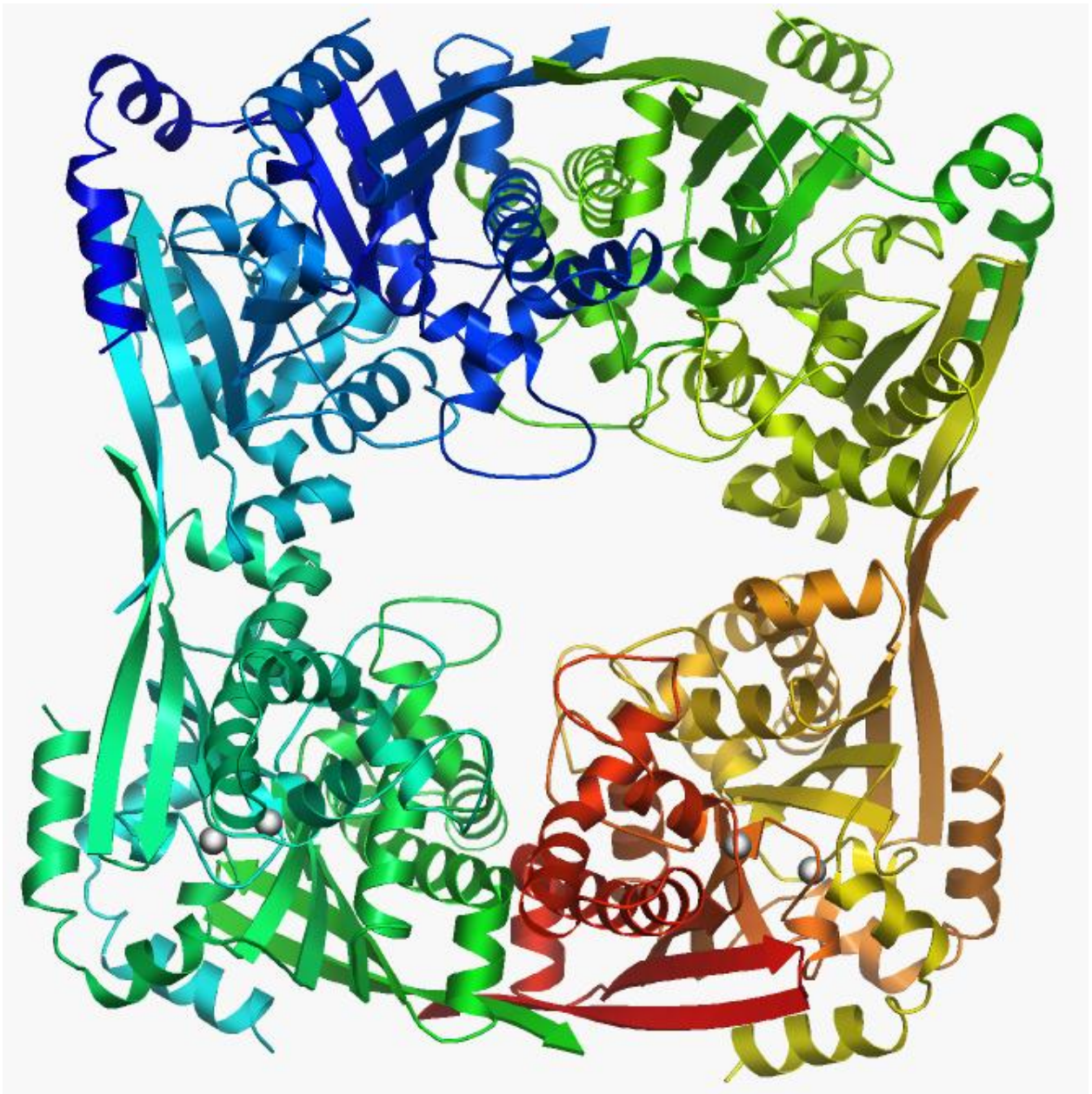


Figure 15: The 3D model of CA_{cyt}. The quaternary structure was built using SWISS-MODEL server. The model for CA_{cyt} was predicted to be octameric proteins. Each monomeric unit (polypeptide chain) of the proteins is represented in different color.

2.4 Discussion

This chapter demonstrates the identification and isolation of leucaena cDNAs for two β -CA isoforms and the structural studies of the encoded enzymes through in silico methods. Although the sub-cellular localization of one of these β -CAs was predicted to be chloroplastic (ca_{cp}) and the other as cytoplasmic (ca_{cyt}), their amino acid sequences were 79% similar and both have similar secondary structures.

There are some proteins namely carbonic anhydrase-related proteins (CARPs), which despite being homologous to carbonic anhydrases 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 fold as found in functionally active forms of CAs. Therefore, for analyzing structural fold and active site residues, we used structurally resolved β -CA from *Pisum sativum* (Kimber and 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 (Bracey et al., 1994; Rowlett et al., 1994; Kimber and Pai, 2000). In our active site analysis, we predicted that Cys-153, Cys-216 and His-213 constitute the zinc ligand of CA_{cp} ,

whereas the zinc ligand of CA_{cyt} is made of Cys-87, Cys-139 and His-147. Crystallographic studies on β -CA from *Cryptococcus neoformans* shows that an aspartate residue in the active site of β -CA activates water molecule for nucleophilic attack of CO₂ 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 C3 plants, we predicted Asp-155 of CA_{cp} and Asp-89 of CA_{cyt} as the respective residues of CA_{cp} and CA_{cyt} involved in proton transport; a feature necessary for the catalytic function of the enzyme. The 3D structures of CA_{cp} and CA_{cyt}, which were built using homology-based modeling, indicate that both the β -CAs of leucaena are octameric proteins with all the active site residues and zinc-binding residues conserved among CA_{cp} and CA_{cyt}.

CHAPTER 3

*Tissue-specific expression of two β -CA isoforms from *Leucaena* under various physiological stress conditions*

3.1 Introduction

The β -CA superfamily was first identified in spinach (Burnell et al., 1990; Fawcett et al., 1990) and exists in prokaryotes including eubacteria and archaeobacteria, as well as in eukaryotes including algae, fungi and plants (Hewett-Emmett and Tashian, 1996; Götz et al., 1999; Hewett-Emmett, 2000; Smith et al., 2002).

The primary role of β -CA in plants is CO_2 accumulation and carbon fixation. However, based on different mechanisms for fixing inorganic carbon into sugar, the role of β -CA differs in different plant groups, including crassulacean acid metabolism (CAM), C4, and C3 plants (Burnell, 2000).

Most of the CO_2 that is fixed in CAM and C4 plants comes through the β -CAs, which acts as the primary enzyme in CO_2 fixation (Badger and Price, 1994). In these plants, β -CAs convert CO_2 to HCO_3^- , which along with phosphoenolpyruvate (PEP) is used to form organic compounds predominantly oxaloacetate and malate by the action of PEP carboxylase. As the inorganic carbon substrate for PEP carboxylase is HCO_3^- instead of CO_2 , the β -CA acts as

the first enzyme for carbon fixation in CAM and C4 plants (Hatch and Burnell, 1990).

Besides a primary role in fixation of inorganic carbon, β -CA from C4 plants appears to have some role in conferring tolerance against various physiological stresses. A β -CA from a C4 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 plant's capacity to withstand various abiotic stress conditions, in addition to the well-established role of carbon fixation. Such extensive study on β -CAs from C3 plants is lacking, and hence the role of β -CAs in C3 plants is not well-defined.

β -CA is an abundant enzyme in the leaves of C3 plants and represents ~2% of total protein (Okabe et 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). 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 *Arabidopsis thaliana* (arabidopsis) were isolated and the transcript level of these β -CAs were found to be regulated by light and dark conditions (Fett and 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 C3 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 C3 plant that is highly tolerant to abiotic stress conditions. We chose to study β -CAs of a C3 plant *Leucaena leucocephala* (leucaena), which can withstand a variety of abiotic stress conditions including drought. The isolation of cDNAs for chloroplastic and cytoplasmic β -CA isoforms, namely ca_{cp} and ca_{cyt} , and their active site, and secondary and quaternary structure has been described in Chapter 2. The current chapter discusses the tissue-specific expression of ca_{cp} and ca_{cyt} under various stress conditions.

3.2 Materials and methods

3.2.1 Plant growth and imitation of stress conditions

Leucaena leucocephala cv. K-636 (leucaena) seeds were collected from University of Hawaii research station, Waimanalo, Honolulu. Leucaena seedlings were grown following the procedure as described in Section 2.2.1 of Chapter 2.

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 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. 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 ca_{cp} and ca_{cyt} , the eight weeks old plants were kept under light or dark conditions for 1, 3, 8, 16, 24, 36 and 48 h. The light condition was maintained by an irradiance of $30 \mu\text{mol s}^{-1}\text{m}^{-2}$ and dark was complete absence of light in the growth room. Plants grown under 16 h light and 8h 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 $150 \mu\text{mol s}^{-1}\text{m}^{-2}$ 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 $-80 \text{ }^{\circ}\text{C}$ until used for RNA extraction.

3.2.2 RNA extraction and cDNA synthesis

Frozen samples of different tissues from stress treated and control plants were finely ground in liquid nitrogen and RNA extraction and cDNA synthesis were exactly performed as described previously in Section 2.2.2 of Chapter 2.

3.2.3 Identification of suitable internal reference gene

The six housekeeping genes (HKGs) including β -actin, tubulin-1, ubiquitin-5, 18S rRNA, 5.8 S rRNA and ef1a were tested as possible internal reference for each of the treatment and control. The method and primers used for identification of suitable internal reference were the same as we discussed previously (Negi et al., 2011). The qPCR assay for these HKGs was performed in two biological replicates and three PCR replicates for each control and treatment group.

3.2.4 ca_{cp} and ca_{cyt} specific primer design and optimization of RT-PCR condition

To design specific primers for ca_{cp} and ca_{cyt} , the ORF of the two sequences were compared using ClustalW and two sets of real-time PCR primers were designed for each β -CA-subtype (Table 4). RT-PCR was performed using ca_{cp} and ca_{cyt} as templates with each primer sets to test their specificity. RT-PCR amplified

products were then analyzed in agarose gel electrophoresis and the gene-specific primer sets were selected.

3.2.5 qPCR analysis

For expression analysis of ca_{cp} and ca_{cyt} , the quantitative real-time PCR (qPCR) was performed using the 1 μ L of 10-fold diluted cDNA from control and treatments in a 15 μ L of reaction consisting of 0.3 μ L each of 10 μ M forward and 10 μ M reverse primer, and 1X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The reactions were carried out in MiniOpticon 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 ca_{cp}) and 62 °C (for ca_{cyt}) 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 (also called 'threshold cycle' is the intersection between an amplification curve and a threshold line) 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 and Schmittgen, 2001) was used to quantify the relative expression of the target gene normalized to that of the selected internal reference gene.

3.2.6 Tissue-specific expression of ca_{cp} and ca_{cyt}

For tissue specific expression of ca_{cp} and ca_{cyt} , we performed reverse transcription PCR (RT-PCR) using the 1 μ L of 10-fold diluted cDNA from leaf, stem and root tissues of leucaena in a 15 μ L reaction consisting of 0.3 μ L each of 10 μ M forward and 10 μ M reverse primer and analyzed the product in agarose gel electrophoresis. The transcript abundance in each tissue type was calculated by digitizing the band densities using ImageJ software from NCBI. The relative density of bands corresponding to ca_{cp} and ca_{cyt} in each tissue type were calculated and were normalized against the relative band intensities of β -actin bands, which were used as controls.

3.3 Results

3.3.1 Confirmation of qRT-PCR primer sets specific for ca_{cp} and ca_{cyt}

The sequence alignment of ca_{cp} and ca_{cyt} ORFs showed high homology (Fig. 16) between the two sequences. Two sets of qPCR primers were designed for each gene from the regions of low homology and the specificity of primers were tested by PCR using ca_{cp} and ca_{cyt} as the templates for each primer set in separate reactions. Both primer sets (set1 and set2) of ca_{cp} amplified only ca_{cp} template but not ca_{cyt} . However, the band intensity in agarose gel

electrophoresis was higher in case of set2 primers as compared to the set1 primers (Table 4). Similarly, the two primer sets of (set1 and set2) ca_{cyt} amplified only ca_{cyt} template but not ca_{cp} template and the band intensity was higher in case of set1 primers as compared to set2 primers (Table 4). The PCR products obtained using set2 primers for ca_{cp} and set1 primers of ca_{cyt} were sequenced and 100% homology to the 120 bp and 177 bp cDNA fragments of ca_{cp} and ca_{cyt} , respectively were confirmed. The set1 primers for ca_{cp} and set2 primers for ca_{cyt} were then selected for expression studies of leuaena ca_{cp} and ca_{cyt} , respectively.

```

cacp ATGTCGACCGCTTCCATCAACGGCTGCTGCCTCTCTTTCTCTTCTCCTCTAAGACTTCTCTTCCATCCAAGTTCTCTGTCTCTGCAAGGCTCGCCACTCCTCCTCTTCATCTTCTTCT 120
cacyt -----

cacp TCCCCAATCCCTTCTCTCATCCAAAACAGGCCCGTCTTCGCCGCCCTACTCCCCTTATCACGCCACCCTGAACGAGGATGCGATCGAGGAAGCTATTGTAGAACTCGAGAAACTGTTC 240
cacyt -----ATGGCAGGGCAGTC-----ATA----CGAGGAAGCCATTGCAGAGCTGAAGAGCTTATC 51
      *   **** *   ***                               **   *****   ****   *   *   *   *   *   *   *

cacp AAGGTGAAGGGTGAGC TGGCAACCACAGCAGAT GCAAGGGTGGATCAAGTAACAGCTCAGGTGGGAACACCGAC-ATCTGAAG GCATCGCATCATCTGAATCT GTCGAGAGGATCAAGGC 359
cacyt AGCGAGAAAGCTGACCTTG GGGCCGTGCGCCGC GCAAAGATCAAGCAGCTGACAGCCGAGTTG--AGCGCCGCCGATTCGAAG-----CCGATTAAACCGGACGAGAGGATCCGAAC 161
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

cacp TGGTTTTATTCACTTCAAGAAAGAGAAATATGAGAAGAACCCTGCTCTATACGGTGAACCTGCCAAGGGACAGAGCCCCCGTACATGGTATTTGCTTGCTCAGACTCTAGGGTCTGCC 479
cacyt CGGGTTCACTCACTTTAAGAAAGAGAAGTTCGAGAAGAATCCAGATTTGTTTGGGAAACTTGCCA CCGCCAAAGCCAAAGTTT TGGTATTTGCATGCTCAGACTCTAGAGTTTGCC 281
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

cacp ATCTCACGTGCTAGACTTCCAACCAGGGGAGGCTTTTGTGCTCAGAAATGTTGCTAACTTGGTCCCACCATATGGCAGACAAGGTATGCTGGAGTTGGAGCTGCCGTTGAGTACGCCGT 599
cacyt ATCGCATGTACTGGATTTTCAACCGGGTGAAGCTTTTGTGGTGCAGAACATCGCCAACATGATCCCGCCTTATGACCAGACGAAATATTCAGGAACGGGGCAGCCATTGAATATGCAGT 401
      *** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

cacp TCTGCATCTCAAGGTGTCGGAATCGTGGTGATTTGGTACAGTGTCTGTGGTGGTATCAAGGGTCTCATGTCTATCCAGACAATGGAGCCGTCCCACCCTGACTTCATAGAGGACTG 719
cacyt GTTGCATTTAAAGGTGAGAAATATAGTAGTATTGGACATAGCTGTTGTGGAGGTATAAAGGGCTCATGTCTATCCAGATGATGGGACCA-CTTCA--AGTGAATTCATAGAGAAGT 518
      ***** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

cacp GGTGAAGATCGGTTTACCTGCAAAGGCAAGAGTGAATCAGTACATGGAG-GCGCACCTTTC GGTGAGCTCTGCACACA CTGTGAGAAGGAAGCTGTGAACGTGTCACTTGGGAATCTGC 838
cacyt GGTGCAAATTTGTAATCCAGCAAATCCAAGGTTAAAGCAG-ACACAAAAGCTTGAGTTTC TCGGAGCAGTGTACTAA CTGCAGAGAAGGAAGCGTGAATGTGTCGCTTGCAAATCTGC 637
      ***** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

cacp TGACATATCCATTTGTGAGAGAGGGATTGGTGAACAAGACACT GTCACTGAAAGGAGGATAC TATGACTTTGTGAAGGATCATTGTAGCTGTGGGCCTTCAGTTTGGCCTGTCTTCT 958
cacyt TGAGCTATCCGTTTGTAGAGATGGAGTTGTGAAGAAAACACT AGTTTTAAAGGGTGTCT CATTACGATTTTGTAAATGGCACCTTTGACCTCTGGGATCTGGACCTCAAATGTCT---- 748
      ***   *****   *****   *****   * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

cacp CCCTCTCCGTATGA 972
cacyt -CCTCTT----TAA 762
      *****   * *

```

Figure 16: The sequence alignment of the ORF of *ca_{cp}* and *ca_{cyt}* using ClustalW. The primers for *ca_{cp}* and *ca_{cyt}* were designed from the yellow and green highlighted regions, respectively.

Table 4: The qPCR primers used in the study and their specificity for the ca_{cp} and ca_{cp} templates

Gene	Primer set	Primer ID	Sequence (5'→3')	Amplicon size	Template	
					ca_{cp}	ca_{cp}
ca_{cp}	Set1	CAcp-F1	TGGCAACCACAGCAGAT	87bp	+	-
		CAcp-R1	AGATTCAGATGATGCGATGC			
	Set2	CAcp-F2	GGTGAGCTCTGCACACA	120bp	+++	-
		CAcp-R2	GTATCCTCCTTTCAGTGAC			
ca_{cyt}	Set1	CAcyt-F1	GGGGCGTCGCCCGG	177bp	-	+++
		CAcyt-R1	AAAACCTTTGGGCTTTGGCCGG			
	Set2	CAcyt-F2	TCGGAGCAGTGTACTAA	118bp	-	++
		CAcyt-R2	GAGCACCTTTAAACT			

- sign represents no amplification of template

+ sign represents amplification of template but faint band

++ sign represents amplification of template with relatively higher intensity of band

+++ sign represents amplification of template with maximum intensity of band

3.3.2 Expression stability of HKGs in control and treatments

The six HKGs were tested for their expression stabilities under control and various stress conditions using qPCR. The primers used for these HKGs were found to be specific as the amplicons for each of the HKGs appeared as a single band in agarose gel electrophoresis and also exhibited single peak in melting curve analysis. The stability of HKGs were tested in three tissue types (leaf, stem and roots) and in four experimental groups that include control and

drought, control and salt, light and dark, and light and bright light. The inter-group and intra-group variance for each group was calculated using NormFinder applet (Andersen et al., 2004). The inter-group variance for each HKGs were plotted as a bar and the confidence intervals on the inter-group variance was obtained by plotting the average of intra-group variances as error bars in the inter-group variances. The expression stabilities in leaf tissues under various stress condition with respect to control is represented in (Fig. 17a-d). In leaf tissues, *ef1a* was found to be the most stably expressed HKG in the three experimental groups including 'control and drought', 'light and dark', and 'light and bright light'. In the experimental group of 'control and salt', the *5.8SrRNA* had the most stable expression with the least inter-group and intra-group variance. In case of stem tissues, *18SrRNA*, and *ubiquitin-5*, were found to be the most stably expressed HKGs in 'control and drought', and in 'control and salt' experimental groups, respectively, whereas the expression of *β -actin* was the most stable in 'light and dark' and in 'light and bright light' experimental groups (Fig. 18a-d). In root tissues of leucaena, *5.8SrRNA*, and *18SrRNA* had the most stable expression in 'control and drought', and in 'light and dark' groups, respectively, whereas *β -actin* showed the most stable expression in 'control and salt' and 'light and bright light' groups (Fig. 19a-d).

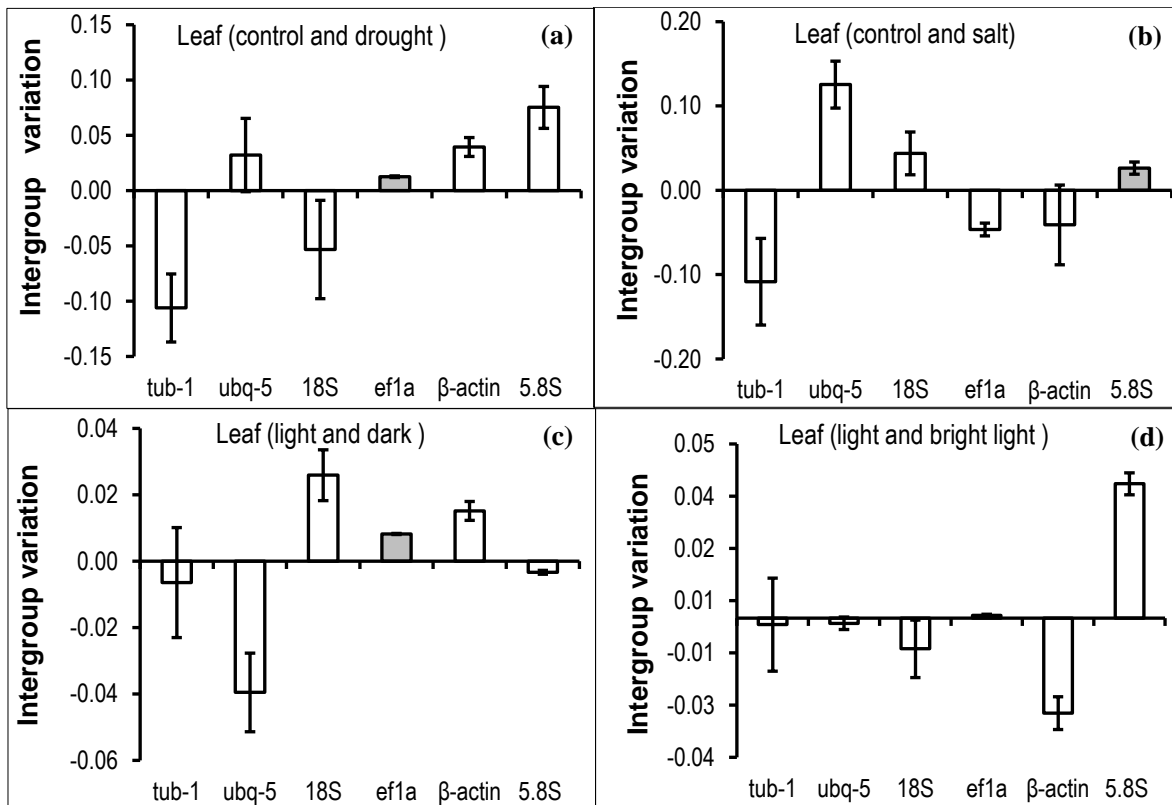


Figure 17: Expression stabilities of six housekeeping genes (HKGs) including β -actin, tubulin-1, ubiquitin-5, 18S rRNA, 5.8SrRNA and ef1a in leaf tissues under stress treatments (drought, salt, light, and dark). Control plants were grown in Hoagland solution under 16 h light and 8h dark photoperiod. The expression stabilities of HKGs were tested in four experimental groups that include control and drought (a), control and salt (b), light and dark (c), and light and bright light (d). Each experiment has six replicates (n=6). The bars represent inter-group variance whereas the error bars represent the average of intra-group variances. The most stably expressed HKG in each group is represented as the bar shaded in grey color

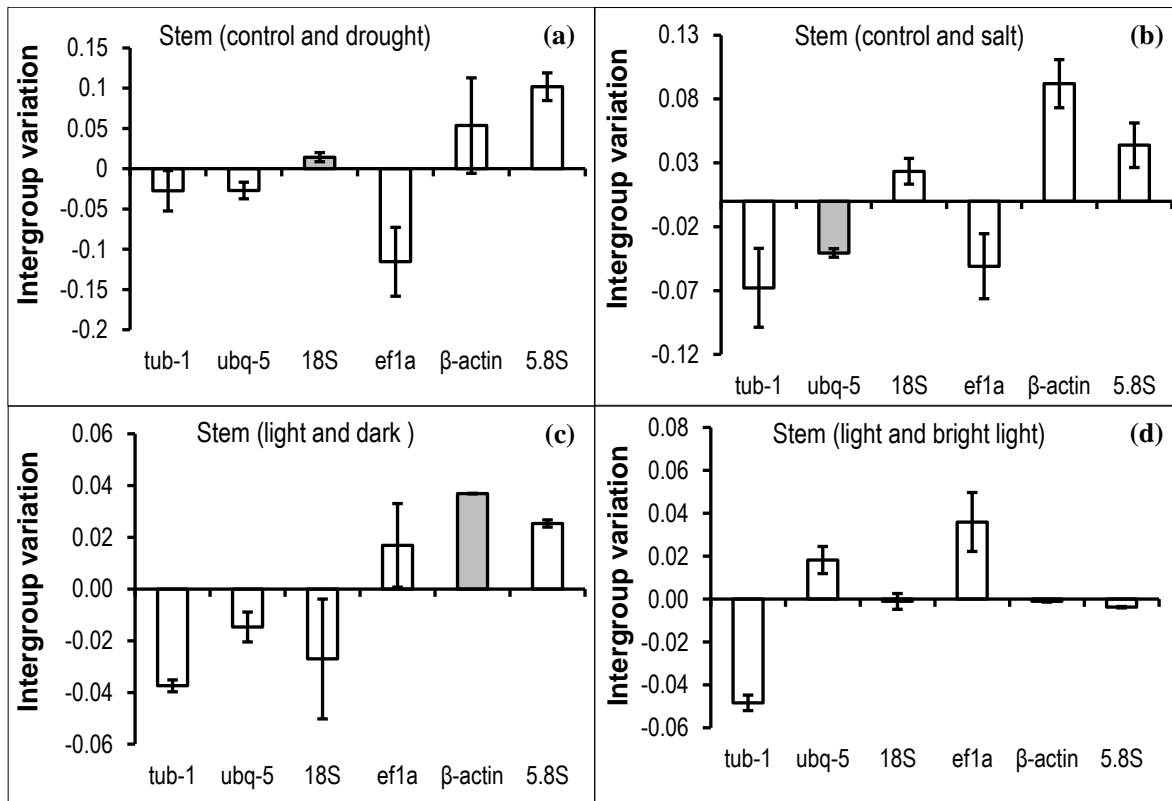


Figure 18: Expression stabilities of six housekeeping genes (HKGs) including β -actin, tubulin-1, ubiquitin-5, 18S rRNA, 5.8SrRNA and ef1a in stem tissues under stress treatments (drought, salt, light, and dark). Control plants were grown in Hoagland solution under 16 h light and 8h dark photoperiod. The expression stabilities of HKGs were tested in four experimental groups that include control and drought (a), control and salt (b), light and dark (c), and light and bright light (d). Each experiment has six replicates (n=6). The bars represent inter-group variance whereas the error bars represent the average of intra-group variances. The most stably expressed HKG in each group is represented as the bar shaded in grey color

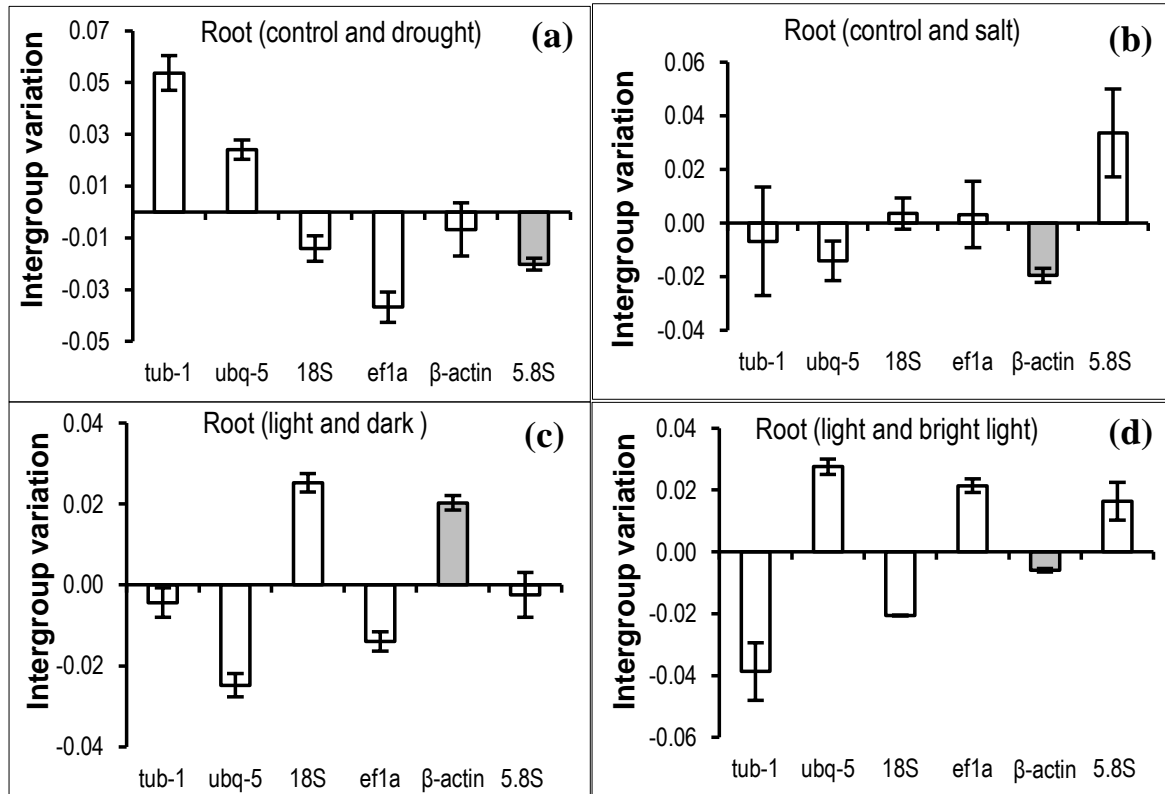


Figure 19: Expression stabilities of six housekeeping genes (HKGs) including β -actin, tubulin-1, ubiquitin-5, 18S rRNA, 5.8SrRNA and ef1a in root tissues under stress treatments (drought, salt, light, and dark). Control plants were grown in Hoagland solution under 16 h light and 8h dark photoperiod. The expression stabilities of HKGs were tested in four experimental groups that include control and drought (a), control and salt (b), light and dark (c), and light and bright light (d). Each experiment has six replicates (n=6). The bars represent inter-group variance whereas the error bars represent the average of intra-group variances. The most stably expressed HKG in each group is represented as the bar shaded in grey color

3.3.3 Tissue-specific expression of ca_{cp} and ca_{cyt}

The transcript abundance of ca_{cp} and ca_{cyt} in different tissues were quantified by digitizing the band densities of RT-PCR amplified products. The transcript abundance of ca_{cp} was found maximum in leaf tissues followed by stem and root tissues, whereas the transcript abundance of ca_{cyt} was found maximum in root tissues followed by leaf and stem tissues (Fig. 20 a-b). The overall expression of ca_{cp} was higher than the expression of ca_{cyt} in leaf and stem tissues but in root tissues the transcript abundance of both ca_{cp} and ca_{cyt} were almost similar.

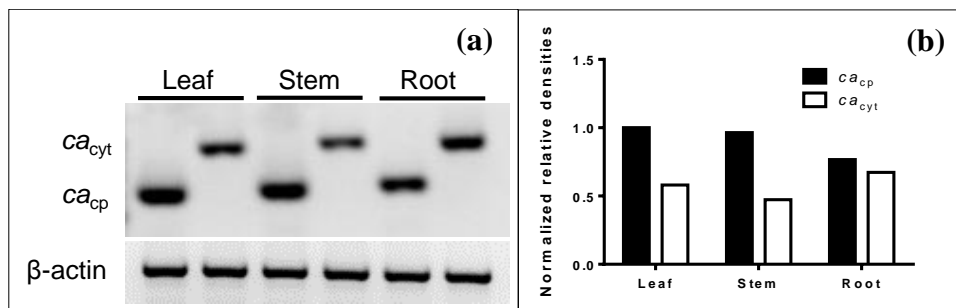


Figure 20: Comparison of transcript abundance of ca_{cp} and ca_{cyt} in leaf, stem, and root tissues of *L. leucocephala*. (a) Bands of ca_{cp} and ca_{cyt} amplified by RT-PCR from leaf, stem, and root tissues. β -actin was used as loading and PCR control. (b) Quantitative representation of transcript abundance from the same bands in agarose gel. The band intensities were digitized using ImageJ software. The band intensities of β -actin were used to normalized the transcript abundance.

3.3.4 Effects of drought and salt stress conditions on the expression of ca_{cp} and ca_{cyt}

To determine the effects of drought and salt stress on the expression of the two β -CAs, the leucaena samples from different tissues exposed to drought were collected at 2, 6, 12, 24, 48, 72, and 96 h of treatment. In the drought-exposed leaf tissues, the transcript of ca_{cp} 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 ca_{cp} transcript level in 24 h, which then increased to ~8-fold in 48 h and remained unchanged thereafter. The expression of ca_{cyt} in drought-stressed leaf and stem samples did not show much increase in the expression. In leaf tissues, the expression of ca_{cyt} increased ~2-fold in 24 h, which further increased ~4-fold in 48 h, and remained unchanged in further time points. The stem tissues showed maximum increase of ~2-fold in the ca_{cyt} expression in 24 h and gets stabilized at this level in subsequent time points. No significant change in the transcript level of ca_{cp} and ca_{cyt} was observed in the root tissues from the drought-exposed plants (Fig. 21a-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 ca_{cp} and ca_{cyt} . As compared with the

transcript levels of ca_{cyt} , the overall increases in ca_{cp} expression level were more pronounced in leaf and stem tissues, however, the root tissues showed more up regulation of ca_{cyt} , than that of ca_{cp} . The ca_{cp} 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 ca_{cp} 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 level of ca_{cp} transcripts in first 12 h of treatment. The ca_{cp} transcript level in the subsequent time points was almost similar to those of controls. The ca_{cp} 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 ca_{cyt} 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 ca_{cyt} 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 ca_{cyt} , which then became similar to the control in 24 h and remained unchanged until 48 h. The transcript level of ca_{cyt} 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 (Fig. 21c-d).

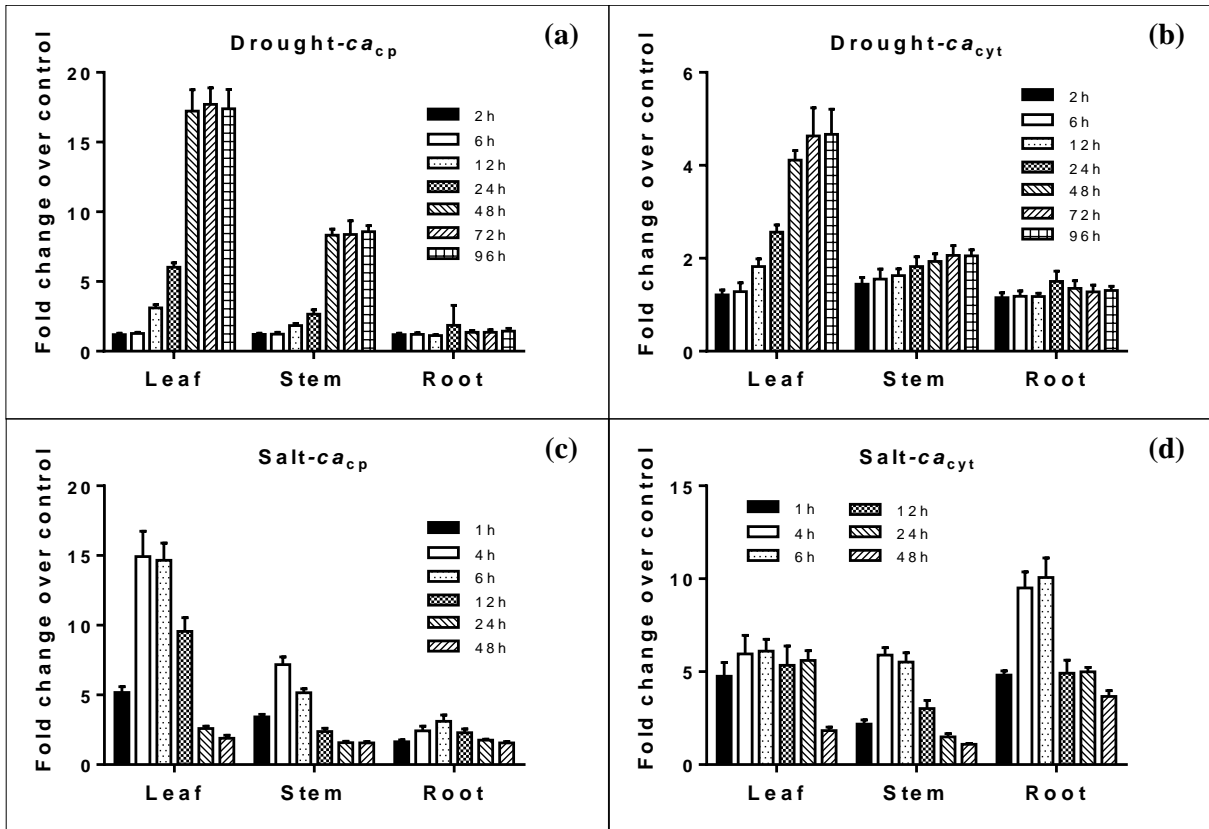


Figure 21: Tissue-specific differential expression of *cACP* and *cACYT* from *L. leucocephala* exposed to drought (a-b) and salt stress (c-d) conditions. Each experiment has six replicates (n=6). The bars represent mean fold change in expression of gene compared to that of control, whereas the error bar represents the standard deviation. The statistical significance was calculated using a two-way ANOVA

3.3.5 Effects of light, bright light, and dark on the *ca*_{cp} and *ca*_{cyt} transcripts

Under light conditions the *ca*_{cp} and *ca*_{cyt} transcripts showed increased expressions in leaf and stem tissues. The root tissues did not show any significant changes in the levels of *ca*_{cp} and *ca*_{cyt} transcripts. The *ca*_{cp} and *ca*_{cyt} 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 ca_{cp} 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 ca_{cyt} transcript levels in leaf tissues was less pronounced than that on ca_{cp} . The ca_{cp} transcript levels first increased from ~3 to 11 fold and then remained at the same level in further time points. In stems, the ca_{cp} transcript levels increased ~6 to 24 fold in 1 to 24 h of treatments after which no further increase in ca_{cp} transcript levels was observed. The ca_{cyt} transcript levels, on the other hand, increased only from ~6 to 10 fold in 1 to 48 h time points (Fig. 22a-b).

Bright light conditions, as compared with moderate light that served as the control, induced only small changes in the level of ca_{cp} and ca_{cyt} transcripts and we did not observe fluctuation in changes in different time points. The level of ca_{cp} transcripts exhibited small increases in the leaf and stem tissues. The increase in the ca_{cp} transcripts was found to be ~2 to 4 fold in leaf tissues and ~2 fold in stem tissues. The ca_{cyt} transcript level showed 2-fold increase in leaf tissues. The transcript levels of ca_{cp} in root tissues and the transcript levels of ca_{cyt} in stem and root tissues did not show any change compared to those of controls (Fig. 22c-d).

Other than root tissues, the leaf and stem exhibited significant decrease in the transcript levels of both ca_{cp} and ca_{cyt} under dark conditions. In leaf tissues, the decrease in ca_{cp} 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 ca_{cyt} 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 ca_{cp} and ca_{cyt} 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 ca_{cp} and ca_{cyt} (Fig. 22e-f).

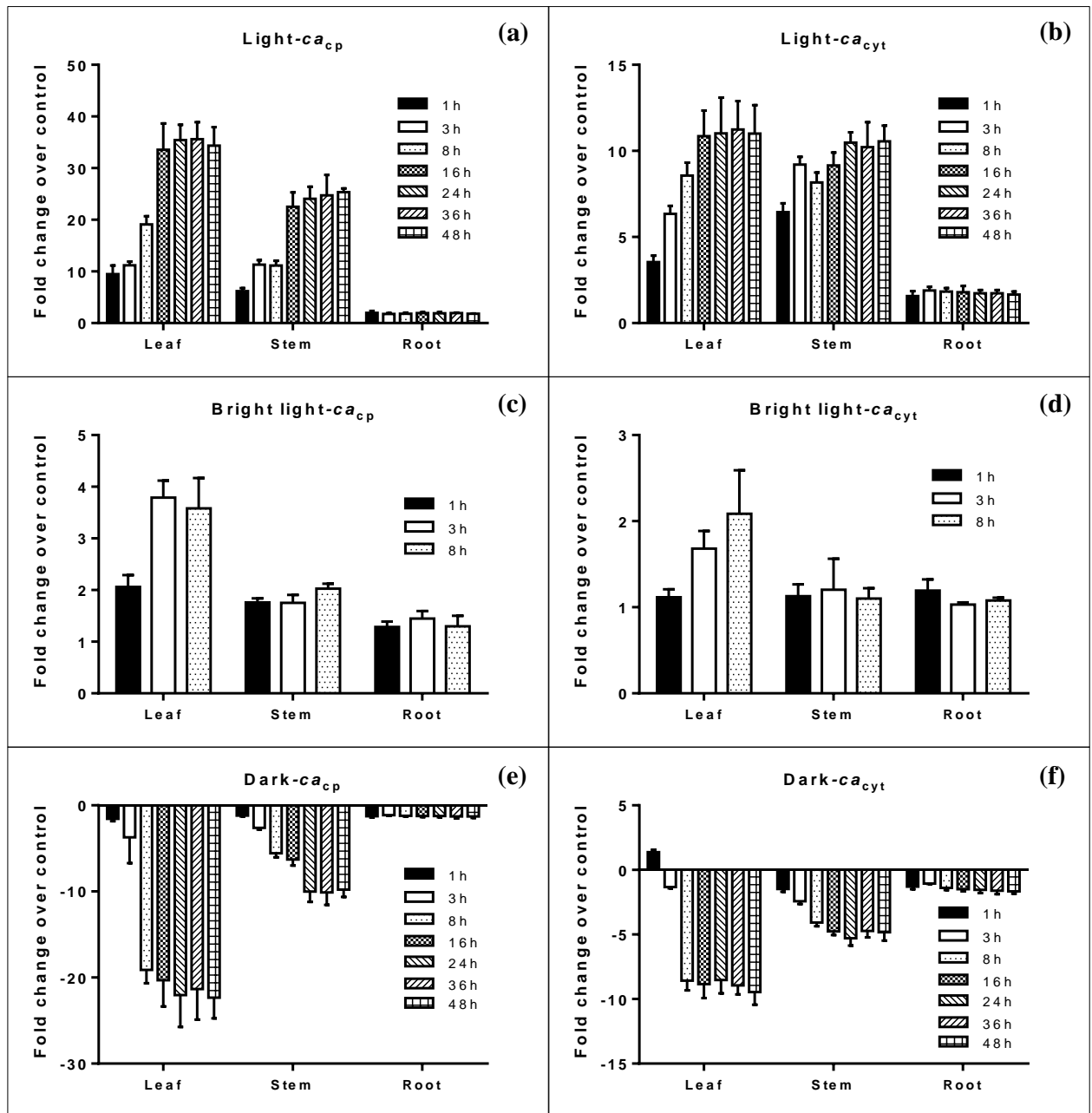


Figure 22: Effect of light (a-b), bright light (c-d), and dark (e-f) conditions on the tissue-specific expressions of ca_{cp} and ca_{cyt} from *L. leucocephala*. Each experiment has six replicates ($n=6$). The bars represent mean fold change in expression of ca_{cp} or ca_{cyt} compared to that of control, whereas the error bar represents the standard deviation. The statistical significance was calculated using a two-way ANOVA

3.4 Discussion

β -CA is abundant in C3 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 C3 plants is lacking. In the previous chapter the identification and isolation of chloroplastic and cytoplasmic β -CA isoforms and their structural features were discussed. In this chapter the tissue-specific abundance of the two isoforms and also their expression under various stress conditions has been described.

We designed and used specific primer sets for ca_{cp} and ca_{cyt} in RT-PCR to identify tissue-specific transcript abundance of ca_{cp} and ca_{cyt} in leucaena. Our results demonstrate that transcript abundance of ca_{cp} was higher than that of ca_{cyt} in leaf and stem tissues while in root tissues the abundance of ca_{cyt} transcripts and ca_{cp} transcripts was similar. Transcript level of ca_{cp} was found maximum in leaf followed by stem and root tissues whereas the maximum ca_{cyt} transcript level was observed in roots followed by leaf and stem tissues. Although the overall high β -CA content in C3 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.

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 CO₂ in the leaves by decreasing the conductance of stomata and mesophyll (Flexas et al., 2004; Sudhir and 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 C3 plants might be involved in dealing with the physiological stress conditions that could limit the photosynthetic potential of C3 plants and therefore, in such case the levels of β-CAs should be significantly altered compared to normal growth conditions. To test this hypothesis, we studied the effects of drought and salt stress conditions on the transcript abundance of leucaena *ca_{cp}* and *ca_{cyt}*. As expected, we observed an enhanced expression of *ca_{cp}* and *ca_{cyt}* under both drought and salt stresses. In fact, the expression of *ca_{cyt}* was found to be higher in salt-stressed root tissues as compared to salt-stressed leaf and stem tissues. Considering that root tissues are first to encounter salt stress conditions, overexpression of *ca_{cyt}* in root tissues may be an adaptive measure of leucaena to withstand salinity stress. Drought and salt stress conditions lead to reduced CO₂ solubility or decreased CO₂ 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 C4 plant, *Pennisetum glaucum* (Kaul et 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 ca_{cp} and ca_{cyt} . The transcript levels of ca_{cp} and ca_{cyt} increased in light conditions in leaf and stem tissues only and the increase in the transcript levels of ca_{cp} was much higher than that of ca_{cyt} . Additionally, the increase in the expression of these transcripts gets 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 ca_{cp} in the leaves, whereas transcript levels of ca_{cyt} exhibited only ~2-fold increase in the leaves. In the dark condition, the transcript levels of ca_{cp} and ca_{cyt} decreased in leaf and stem tissues and the overall decrease was observed more in the ca_{cp} levels than that of ca_{cyt} levels. The decrease in the levels of ca_{cp} transcripts and ca_{cyt} transcripts gets 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 and Coleman, 1994).

CHAPTER 4

Transgenic tobacco expressing leucaena β -CA isoforms

4.1 Introduction

β -CAs are crucial for inorganic carbon fixation in C4 plants, however the role of β -CAs in C3 photosynthesis remains indefinable because of lack of any conclusive and convincing study. Although β -CA in C3 plants was proposed to maintain sufficient level of CO₂ for Rubisco, no significant impact on photosynthesis in C3 plants observed when β -CA activity was reduced by limiting its cofactor, zinc plants (Edwards and Mohamed, 1973; Randall and Bouma, 1973).

Additionally, reports on suppression of β -CA in C3 plants using antisense RNA methods have contradictory results. In tobacco, the decrease in β -CA activity by its antisense RNA did not exhibit any difference in CO₂ assimilation and phenotype (Majeau et al., 1994). In contrast, another report demonstrated that decrease in the chloroplast-localized β -CA negatively affects the *Arabidopsis* seedling survival at ambient CO₂ level. However at increased sucrose concentration or elevated CO₂ level no effect on the survival of *Arabidopsis* seedling observed (Ferreira et al., 2008), suggesting that chloroplastic β -CA facilitates diffusion of CO₂ in plants for the period of phototropic growth before the development of true leaves.

In a recent report on *Arabidopsis* with mutant plastidial β -CAs (Hu et al., 2009), it was identified that the plastidial β -CAs are involved in stomatal closure. Hu et al. (2009) also demonstrated that plastidial carbonic anhydrases plays role in CO₂ signalling pathway and thereby assist in gas-exchange between plants and atmosphere.

In addition to the photosynthesis-related roles, the C3 β -CAs have been shown to have non-photosynthetic functions. A chloroplast-localized β -CA from tobacco binds with salicylic acid (SA), a signaling molecule in hypersensitive defense response, and expression of this β -CA in yeast exhibited antioxidant activity and its silencing in leaves of tobacco and arabidopsis resulted in the suppression of hypersensitive defense response, indicating its role in the development of hypersensitive defense response and disease resistance (Slaymaker et al., 2002). Similarly, tobacco plants, when silenced for the expression of a gene encoding chloroplast-localized β -CA, displayed increased susceptibility to *Phytophthora infestans*, indicating role of chloroplastic β -CA in disease resistance (Restrepo et al., 2005). Chloroplast-localized β -CA was also shown to play a role in plant lipid biosynthesis. Biochemical or molecular inhibition of chloroplast-localized β -CA activity in cotton embryos and tobacco cell suspension culture exhibited decrease in the incorporation of acetate into lipids and thereby overall rate of lipid biosynthesis in plant (Hoang and Chapman, 2002).

In the previous chapter the expression pattern of two different isoform of β -CA from a C3 plant, *L. leucocephala* (leucaena), were shown to be affected by

various physiological stress conditions. The current chapter describes the further investigation on the role of leucaena β -CA isoforms by their overexpression in tobacco plants.

4.2 Materials and methods

4.2.1 Construction of entry clones and binary constructs

Full-length ca_{cp} and ca_{cyt} gene sequences were amplified from pGEMT- ca_{cyt} and pGEMT- ca_{cp} plasmids, respectively using Phusion High-Fidelity DNA Polymerase (New England Biolabs, MA, USA). Four extra bases CACC were introduced at the 5' end of the gene sequence, using the forward primers Agro- ca_{cp} -F and Agro- ca_{cyt} -F. The primer sequences used for ca_{cp} and ca_{cyt} gene are given in (Table 5). Final PCR products were purified with QIAquick PCR purification kit (Qiagen, CA, USA) and checked on 1% Agarose gel. Purified PCR products were then cloned into a gateway entry vector pENTR™/D-TOPO (Invitrogen, CA, USA). Full-length ca_{cp} and ca_{cyt} in pENTR™/D-TOPO were then verified for correct sequence through sequencing, using M13F and M13R primers. Correct entry plasmid were then recombined with the destination binary vectors pMDC140 and pMDC100 for cytoplasmic carbonic anhydrase and pMDC140 for chloroplastic carbonic anhydrase, using Gateway® LR Clonase® II enzyme mix (Invitrogen, CA, USA).

The products of recombination reaction consists of unreacted entry plasmid, unreacted destination plasmids, recombined entry plasmids carrying *ccdB* gene from destination plasmids, and recombined destination plasmid with the transgenes (ca_{cyt} and ca_{cp}). The recombination products were then transformed in *E. coli* strain DH5- α . The *E. coli* strain DH5- α transformed with unreacted destination vector and/or recombined entry vector cannot survive due to the presence of *ccdB* gene, however, the cells with unreacted entry clone and/or recombined destination vector carrying transgene will survive and replicate. These cells were then used for plasmid extraction, which consists of mixture of unreacted entry plasmid and recombined destination vector with transgene.

The extracted plasmid mixture was then introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) by electroporation. The unreacted entry plasmid does not have the *pVS1 ori* and hence can't replicate in the *A. tumefaciens*, however the destination vector with transgene replicates in *A. tumefaciens* because of the presence of *pVS1 ori*. The destination binary vector pMDC140 and pMDC100 with the transgene ca_{cyt} were termed as 'pMDC140- ca_{cyt} ' and 'pMDC100- ca_{cyt} ', respectively. Whereas, the destination binary vector pMDC140 with the transgene ca_{cp} was termed as 'pMDC140- ca_{cp} '

4.2.2 Plant material and transformation

Surface-sterilized seeds of *Nicotiana tabacum* were germinated on sterile plates of germination medium containing half-strength Murashige-Skoog (MS) medium (PH-5.7), 3% (w/v) sucrose, and 0.3% (w/v) phytigel (sigma). 10-day-old seedlings were then transferred to magenta boxes. Sterile leaf discs of four weeks old tobacco plants were used for transformation with pMDC140-*ca_{cyt}* and pMDC140-*ca_{cp}* constructs separately and also pMDC140-*ca_{cp}* and pMDC100-*ca_{cyt}* constructs together. Transformed leaf discs were grown on co-cultivation medium containing full-strength MS media (PH-5.7), 3% (w/v) sucrose, and 0.3% (w/v) phytigel (Sigma) with BAP 1 µg/mL and NAA 0.1 µg/mL. Positive transformants containing pMDC140-*ca_{cyt}* or pMDC140-*ca_{cp}* construct were selected on media containing full-strength MS supplemented with BAP 1 µg/mL, NAA 0.1 µg/mL, cefotaxime 250 µg/mL and hygromycin 40 µg/mL. However, kanamycin (200 µg/mL) was used for selecting positive transformants containing pMDC100-*ca_{cyt}* construct. Transformed shoots were then induced for rooting on media containing half-strength MS medium (PH-5.7), 3% (w/v) sucrose, and 0.3% (w/v) phytigel, cefotaxime 250 µg/mL and hygromycin 40 µg/mL or kanamycin (200 µg/mL). Individual plants were transferred to pots containing garden soil.

4.2.3 Verification of transgenic plants

Genomic DNA was isolated from all the putative transgenic plants and wild type plants using Qiagen DNeasy Plant Mini Kit (Qiagen) according to the

manufacturer's instructions. The genomic DNA of plants from two separate transgenic lines, each containing either pMDC140- ca_{cyt} (expressing cytoplasmic β -CA isoform) or pMDC140- ca_{cp} (expressing chloroplastic β -CA isoform) constructs, were tested for the presence of transgene by PCR using GoTaq® Green Master Mix (Promega, CA, USA) and *hygromycin* gene-specific primers (Table 5), whereas the genomic DNA of one transgenic line containing pMDC140- ca_{cp} (expressing chloroplastic β -CA isoform) and pMDC100- ca_{cyt} (expressing cytoplasmic β -CA isoform) together were tested for the presence of two transgenes using gene-specific primers for hygromycin and also for kanamycin (Table 5).

The expression of leucaena ca_{cp} and ca_{cyt} in transgenic tobacco plants were tested by RT-PCR. The RNA extraction and cDNA synthesis, and RT-PCR were performed as described in Section 3.2.2 and Section 3.2.6 of Chapter 3.

Table 5: Primer used for cloning of ca_{cp} and ca_{cyt} gene sequences in binary constructs

Primer name	Sequence (5'-3')	Target gene
Agro- ca_{cp} -F	CACCATGTCGACCGCTTCCATCAA	ca_{cp}
Agro- ca_{cp} -R	TACGGAGAGGGAAGAAGACAGGCC	
Agro- ca_{cyt} -F	CACCATGGCAGGGCAGTCATACGAG	ca_{cyt}
Agro- ca_{cyt} -R	GGGATCTGGACCTCAAAATGTCTCCTCTT	
hyg-F	AGGGCGAAGAATCTCGTGCTTTCA	<i>hygromycin</i>
hyg-R	TGGACCGATGGCTGTGTAGAAGTA	
<i>kan</i> -F	ACTGGGCACAACAGACAATC	<i>kanamycin</i>
<i>kan</i> -R	CGCCAAGCTCTTCAGCAATA	

The pMDC140- ca_{cyt} and pMDC140- ca_{cp} binary constructs express GUS as a translational reporter. Therefore, the expression of transgenes at the protein level was verified by GUS assay as previously described (Wilson et al., 1991; Joh et al., 2005). Basically, 8-10 leaf discs from each transformed plant were collected and kept separately in individual 50 mL flasks. All the leaf discs were submerged in 5% of 10mg/mL aqueous solution containing X-Gluc (Sigma Aldrich, St. Louis, MO, US) in methanol; 100mM sodium phosphate (pH 7.0); 10mM EDTA; 0.5 mM potassium ferricyanide; 0.5 mM potassium ferrocyanide and 0.006% TritonX-100 and vacuum infiltration of the solution in leaf disc was performed at 25kPa pressure and 150 rpm for 1 min. Flasks containing leaf discs were incubated at 37°C and 150 rpm for 1h & 30min. Leaf discs were soaked in 75% ethanol to remove chlorophyll. Leaf

discs were then visualized for blue staining by light microscope under 20X magnification.

4.2.4 CA activity assay

Leaf extracts from wild type and transgenic plants were prepared by grinding 0.25 g leaf tissue in 1.0 mL of 20 mM Tris-sulfate buffer (pH 8.3) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The leaf extracts were then centrifuged at 10000xg at 4 °C for 15 min and resultant supernatant was used for CA activity assay. The CA enzyme activity of transgenic and wild type tobacco plants were determined by the Wilbur and Anderson electrometric method (Wilbur and Anderson, 1948). The leaf extract containing 0.05 mg of total protein was added to 3.5 mL of ice-cold 20 mM Tris-sulfate buffer (pH 8.3) followed by addition of 2 mL of ice-cold CO₂ saturated water to initiate the reaction. The time taken for a pH drop from 8.3 to 7.3 was recorded and used to measure CA activity in Wilbur and Anderson unit (WAU) using the equation $[10 (t_u/t_c)-1]$ as used previously (Price et al., 1994), where t_u and t_s are time required for pH to drop from 8.3 to 7.3 in uncatalyzed and catalyzed reactions, respectively. For uncatalyzed reaction, the volume of leaf extract was replaced with the exact volume of 20 mM Tris-sulfate buffer (pH 8.3). CA activity assay was performed in six replicates and the activity was expressed in WAU per mg of total protein.

4.2.5 Chlorophyll measurements

The chlorophyll content of leaves was measured by using handheld chlorophyll meter Minolta SPAD 502 (Konica Minolta, Japan). The SPAD 502 instrument provides a nondestructive method of chlorophyll measurement, which measures light transmittance at 650 nm for maximum absorption by chlorophyll and compensates for water and leaf internal structures by measuring maximum transmittance at 940 nm. The different position of leaf in a plant and different area of the same leaf may give rise to different readings in SPAD-502. Therefore, all the measurements were made at the fully expanded topmost leaf from each plant. On each leaf, two readings were made one each side of leaf blade at a position midway between leaf tip and the stalk. The output from the SPAD 502 was recorded in dimensionless unit, CM.

4.2.6 Plant dry weight measurement

Wild type and transgenic tobacco plants were removed from pots and loose soil was washed off. Leaf, stem and roots were then dehydrated at slow heat using AROMA food dehydrater (Aroma Housewares Company, CA, USA) and dry weight was measured.

4.3 Results

4.3.1 Binary constructs preparation

Two binary constructs of ca_{cyt} namely pMDC140- ca_{cyt} and pMDC100- ca_{cyt} and one binary construct for ca_{cp} namely pMDC140- ca_{cp} were prepared using gateway cloning strategy. The binary constructs pMDC140- ca_{cyt} and pMDC140- ca_{cp} has GUS as a translational reporter gene in which both the transgene and reporter gene were cloned under the control of 2X 35S promoter. The pMDC140- ca_{cyt} and pMDC140- ca_{cp} constructs has hygromycin as the selection marker, whereas the pMDC100- ca_{cyt} construct has kanamycin resistance gene as selection marker (Fig. 23).

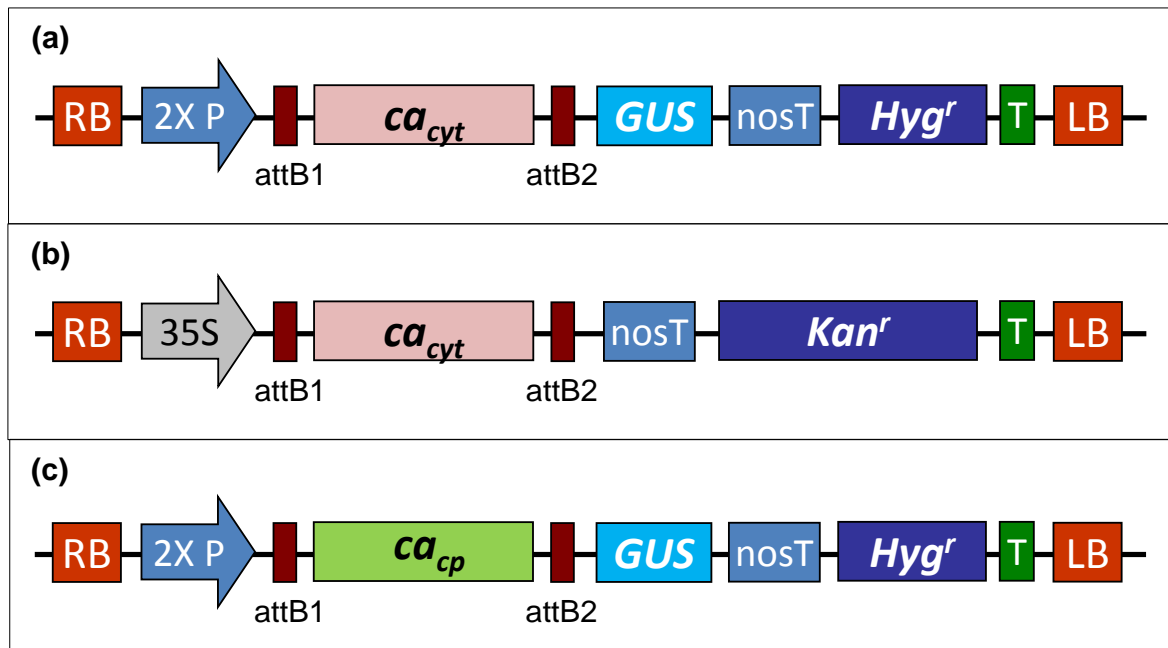


Figure 23: Binary constructs for the expression of *Leucaena* β -carbonic anhydrase isoforms in tobacco. The transgene *cacyt* was cloned in pMDC140 and pMDC100 to make the recombinant binary constructs pMDC140-*cacyt* (a) and pMDC100-*cacyt* (b), respectively. The *cacp* transgene was cloned in pMDC140 to make the binary construct namely pMDC140-*cacp* (c).

4.3.2 Tobacco transformation

For tobacco transformation, 50-55 leaf discs were used to transform with each construct. Leaf discs were dipped in liquid germination medium containing the transformed *Agrobacterium* constructs for 5 min. All the treated leaf discs were blotted dry on sterile filter paper and kept upside down (Fig. 24 a) in full-strength MS (PH-5.7), supplemented with BAP (1µg/mL) and NAA (0.1µg/mL). After 3 days, the leaf discs were transferred to selection medium containing full-strength MS supplemented with BAP (1µg/mL), NAA (0.1µg/mL), cefotaxime (250µg/mL) and hygromycin (40 µg/mL). After every 2 weeks the tissues were transferred to fresh medium. Leaf discs started to swell up in the media followed by callus formation by 3-4 weeks (Fig. 24 b). Shoot regeneration was observed from the callus mass in 5-6 weeks (Fig. 24 c). Individual shoots were dissected and transferred to rooting media containing half-strength MS medium (PH-5.7) supplemented with 3% (w/v) sucrose, and 0.3% (w/v) phytigel, cefotaxime (250µg/mL) and hygromycin (40 µg/mL). The rooted plants were then transferred to pots containing garden soil (Fig. 24 d).

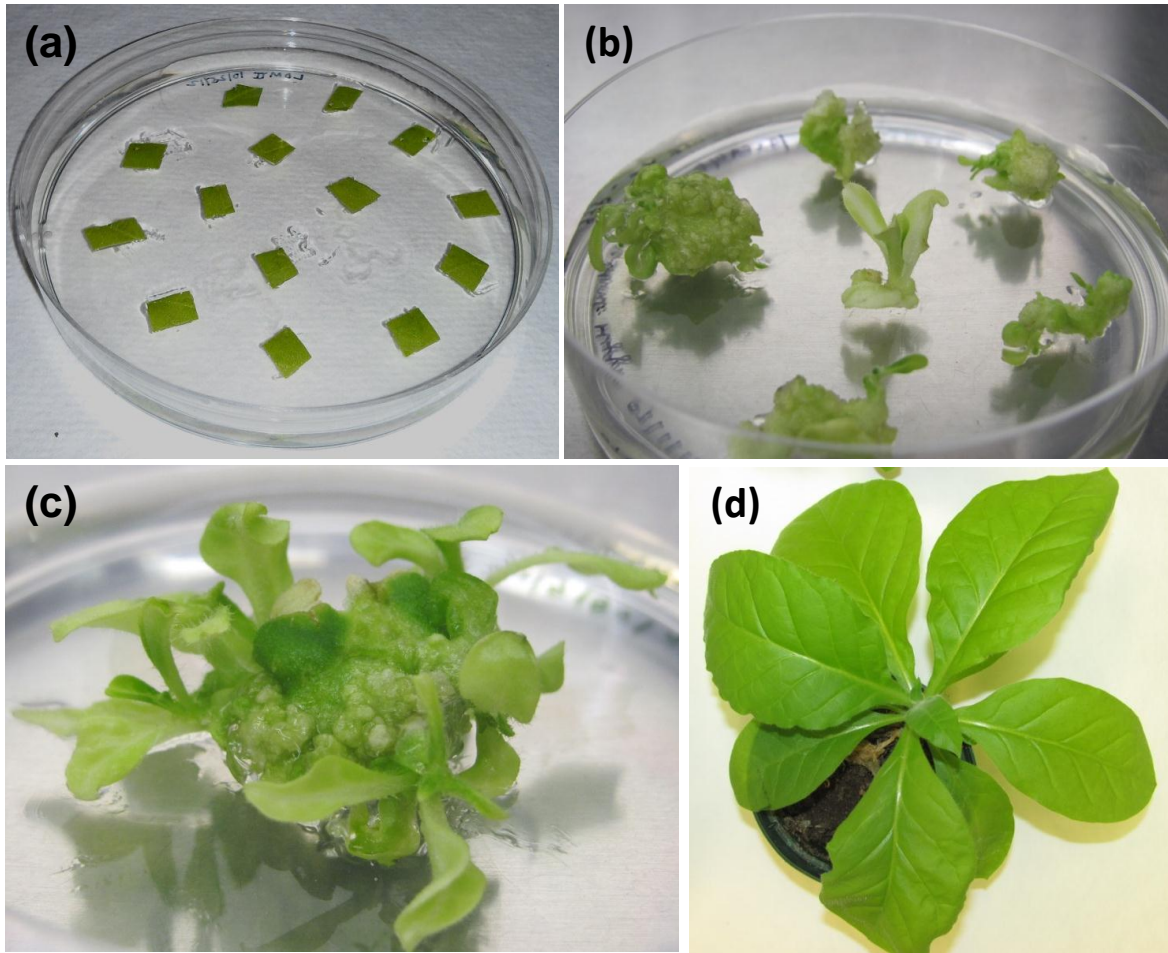


Figure 24: Steps of tobacco transformation. Transformed leaf discs were cultured in full-strength MS (PH-5.7) supplemented with $1\mu\text{g/mL}$ BAP and $0.1\mu\text{g/mL}$ NAA (a). The leaf discs cultured on the same MS media and plant growth regulator composition supplemented with $250\mu\text{g/mL}$ cefotaxime and $40\mu\text{g/mL}$ hygromycin started to swell up followed by callus formation by 3-4 weeks (b) and shoot regeneration from the callus mass in 5-6 weeks (c). The plants were induced for root formation and the rooted plants were then transferred to pots containing garden soil (d).

4.3.3 Screening of putative transgenic *Tobacco* plants

The rooted plants transferred on pots containing garden soil were tested for positive transformants by screening the plants for the presence of ca_{cyt} and

ca_{cp} transgenes in PCR using genomic DNA as template and *hygromycin* or *kanamycin* gene-specific primers in separate reactions. The resulted PCR products for *hygromycin* and *kanamycin* resistance genes appeared as ~850 and ~630 bp (Fig. 25 a). The genomic DNA templates from the wild type plant were used as the negative control, which did not appear as a PCR amplified band in agarose gel electrophoresis.

The expression of *ca_{cyt}* and *ca_{cp}* transgenes were verified in RT-PCR assays using cDNA template and gene-specific primers for *ca_{cyt}* and *ca_{cp}*. The resulted RT-PCR products for *ca_{cyt}* and *ca_{cp}* appeared as ~177 and ~120 bp (Fig. 25 b). The genomic DNA and cDNA templates from the wild type plant was used as the negative control, which did not appear as a PCR amplified band in agarose gel electrophoresis.

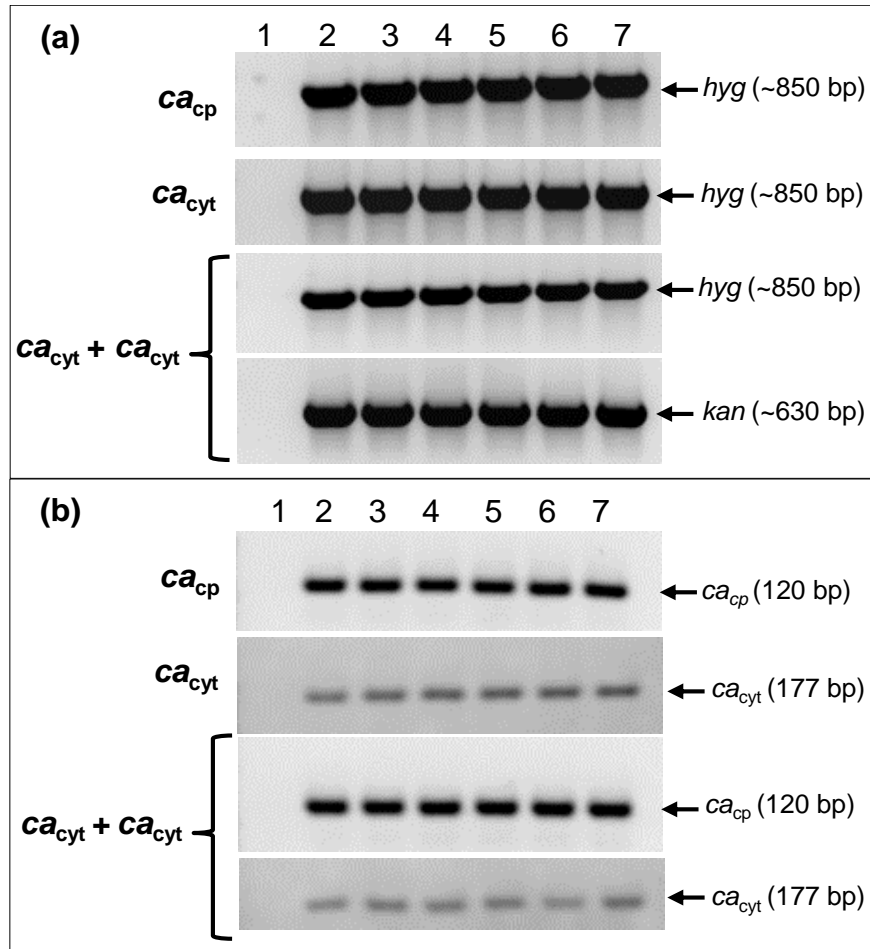


Figure 25: Verification of presence of transgene and its expression in transgenic plants using PCR from genomic DNA (a) and RT-PCR from cDNA (b).

Transformed plants with GUS as the reporter gene were also examined under light microscope under 20X magnification and a clear expression of GUS was observed in the cells of leaf discs from positive transformants whereas no GUS expression found in those of wild type tobacco plants (Fig. 26).

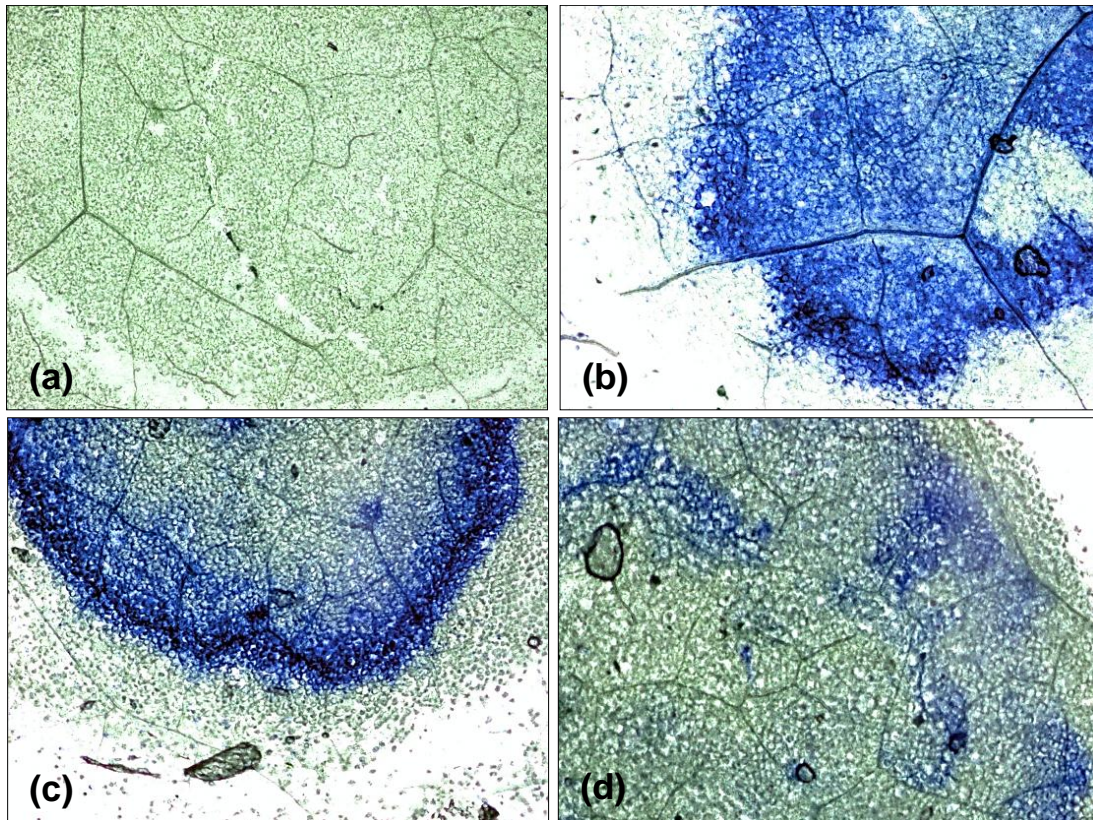


Figure 26: The GUS assay on the wild-type (a) and transgenic plants containing pMDC140-*cacyt* construct (b), pMDC140-*cacp* (c), and constructs pMDC100-*cacyt* and pMDC140-*cacp* (d).

4.3.4 CA activity in wild type and transgenic tobacco plants

For CA activity assay a total of 6 plants from each group, including control and transgenic plants expressing *cacp* and *cacyt* alone or together. The transgenic plants expressing ca_{cp} , ca_{cyt} , individually and together exhibited significantly higher activity of β -CAs (Fig. 27) as compared with wild type plants. With reference to the CA activity in wild type tobacco plants, the percent increase in the CA activity of

transgenic plants expressing leucaena ca_{cyt} or ca_{cp} was found to be ~ 51 and ~55 %, respectively. The transgenic tobacco expressing both the leucaena β -CA isoforms exhibited ~63% increase in CA activity as compared to the wild type.

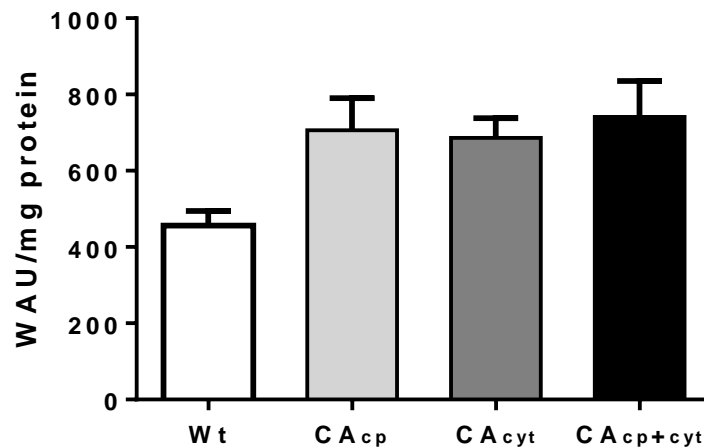


Figure 27: CA enzyme activity in Wilbur and Anderson unit per mg of total leaf protein from wild type and transgenic tobacco plants (n=6). Error bars represent standard deviation.

4.3.4 Chlorophyll content and dry weight of wild type and transgenic tobacco plants

The transgenic tobacco plants overexpressing leucaena β -CA isoforms from chloroplast and cytoplasm did not exhibit any phenotypic difference compared with wild type tobacco plants (Fig. 28). Additionally, the SPAD value for chlorophyll content obtained for wild type and transgenic tobacco plants were similar with no significant difference in the SPAD value (Fig. 29). To test whether overexpression

of the two β -CA isoforms from leucaena into tobacco affects total biomass yield of the plant, the dry weight of wild type and transgenic tobacco plants were measured.

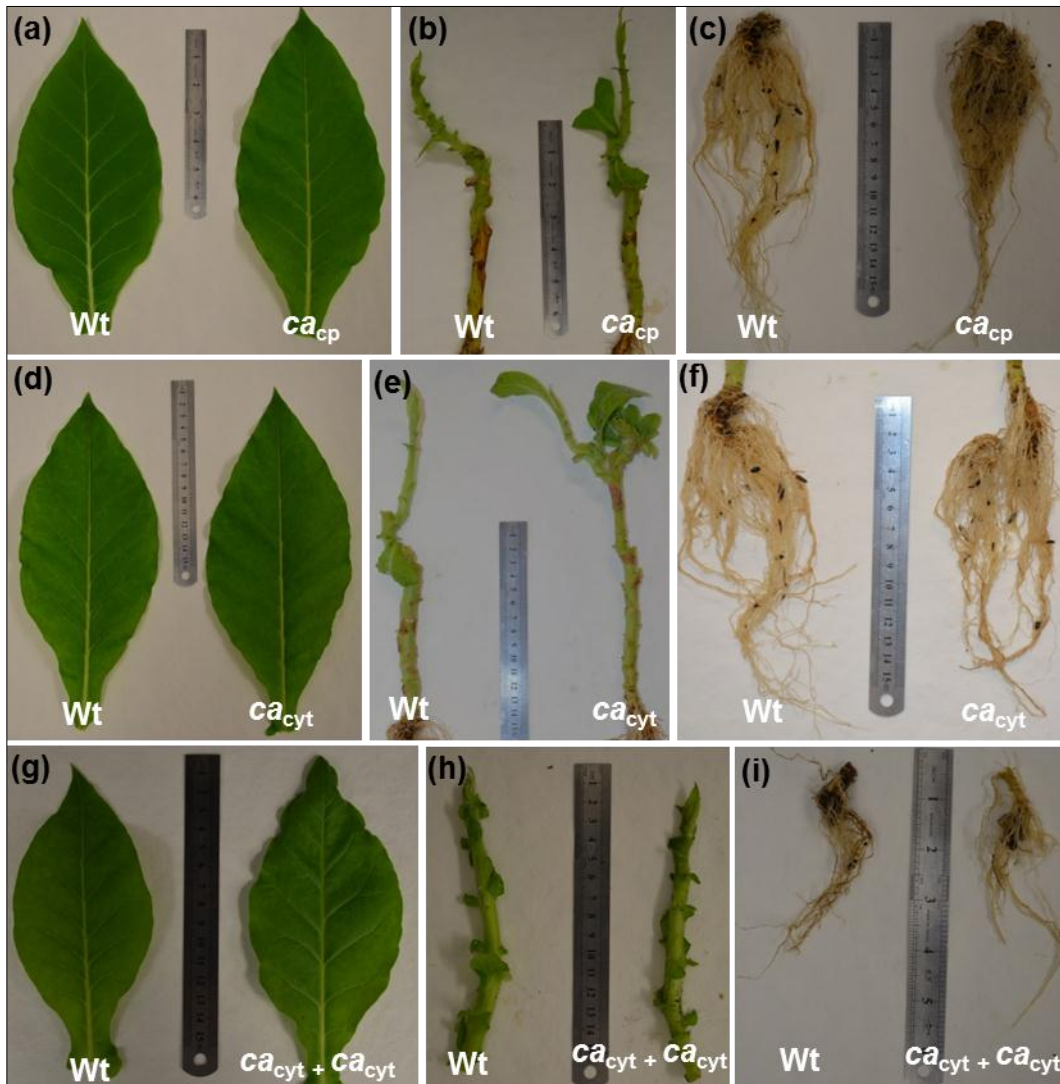


Figure 28: Phenotypes of wild type (Wt) and transgenic tobacco plants expressing leucaena ca_{cp} (a-c), ca_{cyt} (d-f), and ca_{cp} and ca_{cyt} together (g-i).

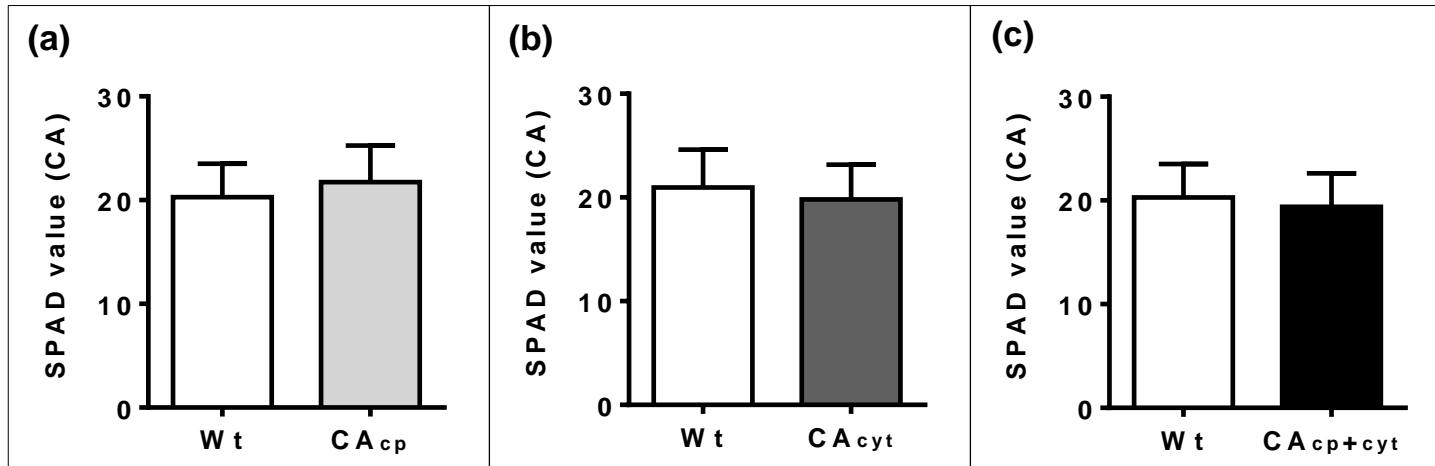


Figure 29: SPAD value representing chlorophyll contents from wild type and transgenic plants containing ca_{cp} (a), ca_{cyt} (b), and ca_{cp} and ca_{cyt} together (c). The measurements were made at the fully expanded topmost leaf from six plants each for wild type (Wt), and transgenic tobacco containing ca_{cp} , ca_{cyt} , and ca_{cp} and ca_{cyt} together [n=6]

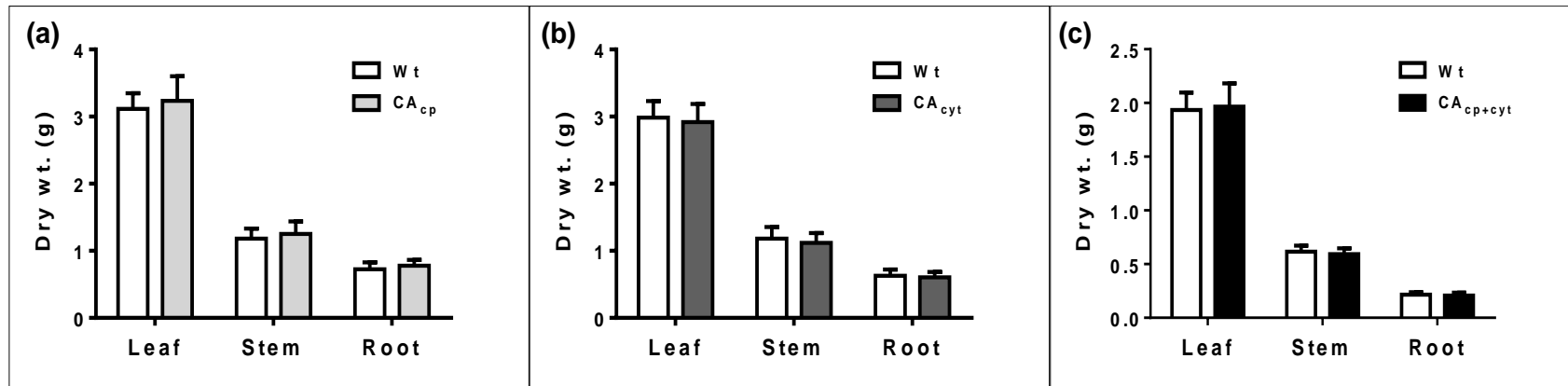


Figure 30: Dry weight of leaves, stem and root tissue samples in wild type and transgenic tobacco plants expressing leucaena ca_{cp} (a), ca_{cyt} (b), and ca_{cp} and ca_{cyt} together (c) [n=6]

No visible difference in phenotypes and no change in the chlorophyll content as well as dry weight of transgenic tobacco with respect to wild type tobacco (Fig. 30) indicate that overexpression of chloroplastic- and cytoplasmic β -CA from C3 plants does not contribute to any significant improvement in biomass, which support the proposed view that C3 β -CA are not involved in active accumulation of inorganic carbon (Price et al., 1994) as seems to be the case in microalga (Badger and Price, 1992).

4.4 Discussion

In a previous study by Price et al. (1994), antisense suppression of chloroplastic β -CA in tobacco resulted in almost up to 99% decrease in CA activity, but it did not result in any detectable reduction in the rate of CO₂ assimilation based on which, they proposed that the chloroplastic β -CA does not offer a mechanism of carbon assimilation in C3 plants. To see if overexpression of chloroplastic β -CA has any role in carbon assimilation, we overexpressed the chloroplastic β -CA isoform from leucaena into tobacco. The overexpression of chloroplastic β -CA increased the CA activity in transgenic tobacco by ~ 55% to that of wild type; however, it did not result in any increase in the dry weight and chlorophyll contents as compared to those of wild type plants. This finding reinforces the view of Price et al. (1994) that the chloroplastic β -CA of C3 plants are not involved in active accumulation of inorganic carbon.

Considering (i) the low HCO_3^- level at cytoplasmic neutral pH (Cowan, 1986), and (ii) small diffusion distance from chloroplast envelope to the cell membrane, Price et al (1994) suggested that cytoplasmic β -CA isoform may have no or small role in increasing the photosynthesis efficiency of C3 plants. To test this cytoplasmic β -CA from leucaena was overexpressed in tobacco either alone or in combination with chloroplastic β -CA isoform. Overexpression of these isoforms increased the CA activity from ~51-63% to that of wild type but as expected we did not observe any change in chlorophyll content and biomass. The chloroplastic- and cytoplasmic β -CA isoforms from leucaena are homologous and are 79% similar (chapter 2), therefore, it is possible that the antisense RNA against one isoform may suppress the other form as well. Additionally, the leaf tissue has significant level of cytoplasmic β -CA along with chloroplastic β -CA indicating that the 99% suppression of CA activity shown by Price et al. (1994) might have caused almost complete suppression of both chloroplastic- and cytoplasmic- β -CA isoforms. Therefore, the result presented in chapter 4 and previous findings of Price et. al (1994) strongly suggest that the neither chloroplastic- nor cytoplasmic- β -CA isoforms of C3 plants play an active role in inorganic carbon accumulation and may be involved only in passive diffusion of CO_2 into stroma as proposed by Price et al (1994).

SUMMARY AND FUTURE WORK

β -CAs have been well-studied in CAM and C4 plants in which most of the CO₂ that is fixed comes through β -CAs (Badger and Price, 1994). In these plants, β -CAs act as a primary enzyme in the CO₂ fixation. However, the role of β -CAs in C3 plants is inexplicit because of limited study. Therefore, the goal of this research was to identify and isolate the cDNA sequences for chloroplastic and cytoplasmic β -CA isoforms from leucaena, a C3 plant, and to study their expression in leucaena and transgenic tobacco.

The two leucaena β -CA isoforms were predicted to have different sub-cellular localization; one chloroplastic and another cytoplasmic. Despite their different sub-cellular localization these two β -CA isoforms are homologous with similar secondary structure, conserved active site residues and zinc ligands, and similar 3-D structure. Our in silico analysis of the two β -CA isoforms indicate that the two encoded enzymes has all the required features to be a functional β -CA.

The C3 plants have high content of β -CA, however, the abundance of chloroplastic and cytoplasmic β -CA isoforms have varying abundance in different tissues of leucaena. Our results shows that leaf and stem tissues of leucaena are rich in chloroplastic β -CA isoform whereas, the root tissues are rich in cytoplasmic β -CA isoform. The overall high abundance of β -CA suggests their importance in C3 plants and the differential abundance of chloroplastic and cytoplasmic β -CA

isoforms in different tissues of leucaena suggest different and/or complementary role of the two isoforms.

Considering the alkaline nature of chloroplast stroma of C3 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 ribulose biphosphate (RuBP) serve as the substrate for rubisco, which fixes the inorganic carbon into 3-phosphoglycerate (Badger and Price, 1994). On the other hand, the cytoplasm of C3 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 and 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 ca_{cyt} in leucaena root tissues may be an adaptive measure of leucaena to improve buffering capacity of root cells in alkaline soils.

The tissue-specific expression pattern of leucaena chloroplastic and cytoplasmic β -CA isoforms was found to be affected by various abiotic stress conditions. Under drought and salt stress conditions both the isoforms overexpressed compared to the normal growth conditions. In the root tissues of salt-stressed plant, the expression of cytoplasmic β -CA isoform was more pronounced in root tissues as compared to leaf and stem tissues. The expression of both the isoforms of β -CA increased under light and decreased under dark

conditions in leaf and stem tissues but remained unaffected in root tissues. The change in the expression levels of these two isoforms under various stress conditions may be an adaptive measure of plant to withstand the stress conditions.

A number of attempts have been made in the past to study the role of β -CA in C3 plants. Edwards and Mohamed (1973) studied the role of β -CA by growing *Phaseolus vulgaris* under varying concentration of zinc, which is an essential cofactor for β -CA. They demonstrated a direct correlation between level of zinc and the carbonic anhydrase activity but there was little or no effect on photosynthetic rate. Similarly, Randall and Bouma (1973) showed that limited zinc supply to *Spinacia oleracea* drastically reduced carbonic anhydrase levels with little effect on net CO₂ uptake. These findings do not support the proposed role of β -CA in facilitating the supply of CO₂ to the sites of carboxylation. However, the reduction of zinc to inactivate β -CA is not specific for β -CA at all because zinc limitation may inhibit several other enzymes which require zinc for catalytic activity. Further attempts to lower β -CA level in plants used relatively specific CA inhibitors including acetazolamide and ethoxzolamide (Swader and Jacobson, 1972), but these attempts could not give any insight into the role of β -CA in C3 plants. When antisense RNA techniques were used to decrease the level of β -CA in plants, conflicting results from different research groups were reported. Overexpression of antisense construct for β -CA in tobacco plants exhibited almost up to 90% decrease in β -CA activity but no effect on phenotype and carbon assimilation observed (Majeau et al., 1994; Price et al., 1994). In contrast to antisense modification of β -CA level in tobacco where decrease in β -CA activity did not result

in any change in phenotype and carbon assimilation, more than 90% decrease in β -CA activity in arabidopsis using antisense technology produced phenotypes that could only survive either by growing them in elevated CO_2 level or by growing them in sucrose containing medium (Ferreira et al., 2008). All these attempts involved different approaches to decrease β -CA activity, however no effect on CO_2 assimilation was observed. Therefore to see if overexpression of chloroplastic and cytoplasmic β -CA isoforms has any effect on CO_2 assimilation and thereby on total biomass yield, we overexpressed the leucaena β -CA isoforms in tobacco. The transgenic tobacco with chloroplastic β -CA, or cytoplasmic β -CA, or with both chloroplastic- and cytoplasmic β -CA from leucaena exhibited ~ 55% increase in the overall CA activity. However, we did not observe any phenotypic change including dry weight and chlorophyll contents in the transgenic plants as compared to wild type. Our findings indicate that neither chloroplastic- nor cytoplasmic- β -CA isoforms of C3 plants play an active role in inorganic carbon accumulation, therefore, it supports the view of Price et al. (1994) that chloroplastic β -CA does not offer a mechanism of carbon assimilation in C3 plants. However, with these results and previous findings we cannot argue about the possible role β -CA in passive diffusion of CO_2 into stroma, which was proposed by Price et al (1994).

It will be interesting to study the promoters of different β -CA isoforms in the future as identification and study of promoter may help us figure out the transcription factor(s) involved in the transcription of β -CA isoforms. The regulated expression through the β -CA promoter can be studied by reporter constructs and may give us better insight of the physiological conditions which lead to the up- or

down-regulation of different β -CA isoforms. Since most of the stress conditions are regulated by a cascade of defense mechanism in plants involving many different genes which are usually regulated by a few transcription factors. Therefore overexpression of transcription factors, which is responsible for the transcription of β -CA isoforms, may provide a better adaptation mechanism in plants against various abiotic and biotic stress conditions. Additionally, the study of post-translational modifications on each isoforms using analytical methods may give us some information on their specific role in plants.

REFERENCES

- Alber BE, Ferry JG** (1994) A carbonic anhydrase from the archaeon *Methanosarcina thermophila*. Proceedings of the National Academy of Sciences **91**: 6909-6913
- Alber BE, Ferry JG** (1996) Characterization of heterologously produced carbonic anhydrase from *Methanosarcina thermophila*. Journal of Bacteriology **178**: 3270-3274
- Alexander RS, Nair SK, Christianson DW** (1991) Engineering the hydrophobic pocket of carbonic anhydrase II. Biochemistry **30**: 11064-11072
- Andersen CL, Jensen JL, Ørntoft TF** (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research **64**: 5245-5250
- Arnold K, Bordoli L, Kopp J, Schwede T** (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics **22**: 195-201
- Badger MR, Price GD** (1992) The CO₂ concentrating mechanism in cyanobacteria and microalgae. Physiologia Plantarum **84**: 606-615
- Badger MR, Price GD** (1994) The role of carbonic anhydrase in photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology **45**: 369-392
- Bracey MH, Christiansen J, Tovar P, Cramer SP, Bartlett SG** (1994) Spinach carbonic anhydrase: investigation of the zinc-binding ligands by site-directed mutagenesis, elemental analysis, and EXAFS. Biochemistry **33**: 13126-13131
- Burnell JN** (1990) Immunological study of carbonic anhydrase in C₃ and C₄ plants using antibodies to maize cytosolic and spinach chloroplastic carbonic anhydrase. Plant and Cell Physiology **31**: 423-427
- Burnell JN** (2000) Carbonic anhydrases of higher plants: an overview. *In* WR Chegwidden, ND Carter, YH Edwards, eds, The Carbonic Anhydrases: New Horizons, Vol 90. Birkhäuser, pp 501-517

- Burnell JN, Gibbs MJ, Mason JG** (1990) Spinach chloroplastic carbonic anhydrase nucleotide sequence analysis of cDNA. *Plant Physiology* **92**: 37-40
- Burnell JN, Hatch MD** (1988) Low bundle sheath carbonic anhydrase is apparently essential for effective C₄ pathway operation. *Plant Physiology* **86**: 1252-1256
- Chory J, Peto C, Feinbaum R, Pratt L, Ausubel F** (1989) *Arabidopsis thaliana* mutant that develops as a light-grown plant in the absence of light. *Cell* **58**: 991-999
- Christianson DW, Cox JD** (1999) Catalysis by metal-activated hydroxide in zinc and manganese metalloenzymes. *Annual Review of Biochemistry* **68**: 33-57
- Cowan IR** (1986) Economics of carbon fixation in higher plants. In TJ Givnish, ed, *On the economy of plant form and function*. Cambridge University Press, Cambridge, pp 133-170
- Cox EH, McLendon GL, Morel FMM, Lane TW, Prince RC, Pickering IJ, George GN** (2000) The active site structure of *Thalassiosira weissflogii* carbonic anhydrase 1. *Biochemistry* **39**: 12128-12130
- Duda D, Tu C, Qian M, Laipis P, Agbandje-McKenna M, Silverman DN, McKenna R** (2001) Structural and kinetic analysis of the chemical rescue of the proton transfer function of carbonic anhydrase II. *Biochemistry* **40**: 1741-1748
- Edwards GE, Mohamed AK** (1973) Reduction in carbonic anhydrase activity in zinc deficient leaves of *Phaseolus vulgaris* L. *Crop Science* **13**: 351-354
- Emanuelsson O, Nielsen H, Brunak S, von Heijne G** (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* **300**: 1005-1016
- Fabre N, Reiter IM, Becuwe-Linka N, Genty B, Rumeau D** (2007) Characterization and expression analysis of genes encoding α and β carbonic anhydrases in *Arabidopsis*. *Plant, Cell & Environment* **30**: 617-629
- Fawcett T, Volokita M, Bartlett S** (1990) Spinach carbonic anhydrase primary structure deduced from the sequence of a cDNA clone. *Journal of Biological Chemistry* **265**: 5414-5417

- Ferreira FJ, Guo C, Coleman JR** (2008) Reduction of plastid-localized carbonic anhydrase activity results in reduced *Arabidopsis* seedling survivorship. *Plant Physiology* **147**: 585-594
- Fett JP, Coleman JR** (1994) Characterization and expression of two cDNAs encoding carbonic anhydrase in *Arabidopsis thaliana*. *Plant Physiology* **105**: 707-713
- Fierke CA, Calderone TL, Krebs JF** (1991) Functional consequences of engineering the hydrophobic pocket of carbonic anhydrase II. *Biochemistry* **30**: 11054-11063
- Fisher M, Gokhman I, Pick U, Zamir A** (1996) A salt-resistant plasma membrane carbonic anhydrase is induced by salt in *Dunaliella salina*. *Journal of Biological Chemistry* **271**: 17718-17723
- Flexas J, Bota J, Loreto F, Cornic G, Sharkey TD** (2004) Diffusive and metabolic limitations to photosynthesis under drought and salinity in C₃ plants. *Plant Biology* **6**: 269-279
- Giuliano G, Pichersky E, Malik V, Timko M, Scolnik P, Cashmore A** (1988) An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. *Proceedings of the National Academy of Sciences* **85**: 7089-7093
- Götz R, Gnann A, Zimmermann FK** (1999) Deletion of the carbonic anhydrase-like gene *NCE103* of the yeast *Saccharomyces cerevisiae* causes an oxygen-sensitive growth defect. *Yeast* **15**: 855-864
- Guex N, Peitsch MC** (1997) SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modeling. *Electrophoresis* **18**: 2714-2723
- Hatch MD, Burnell JN** (1990) Carbonic anhydrase activity in leaves and its role in the first step of C₄ photosynthesis. *Plant Physiology* **93**: 825-828
- Hewett-Emmett D** (2000) Evolution and distribution of the carbonic anhydrase gene families. *In* WR Chegwidden, ND Carter, YH Edwards, eds, *The Carbonic Anhydrases: New Horizons*, Vol 90. Birkhäuser, pp 29-78
- Hewett-Emmett D, Tashian RE** (1996) Functional Diversity, Conservation, and Convergence in the Evolution of the α -, β -, and γ -Carbonic Anhydrase Gene Families. *Molecular Phylogenetics and Evolution* **5**: 50-77

- Hiltonen T, Karlsson J, Palmqvist K, Clarke AK, Samuelsson G** (1995) Purification and characterisation of an intracellular carbonic anhydrase from the unicellular green alga *Coccomyxa*. *Planta* **195**: 345-351
- Hoang CV, Chapman KD** (2002) Biochemical and molecular inhibition of plastidial carbonic anhydrase reduces the incorporation of acetate into lipids in cotton embryos and tobacco cell suspensions and leaves. *Plant Physiology* **128**: 1417-1427
- Hood EE, Gelvin SB, Melchers LS, Hoekema A** (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research* **2**: 208-218
- Hu H, Boisson-Dernier A, Israelsson-Nordström M, Böhmer M, Xue S, Ries A, Godoski J, Kuhn JM, Schroeder JI** (2009) Carbonic anhydrases are upstream regulators of CO₂-controlled stomatal movements in guard cells. *Nature Cell Biology* **12**: 87-93
- Hunt JA, Ahmed M, Fierke CA** (1999) Metal binding specificity in carbonic anhydrase is influenced by conserved hydrophobic core residues. *Biochemistry* **38**: 9054-9062
- Jenkins CL, Furbank RT, Hatch MD** (1989) Mechanism of C₄ Photosynthesis A Model Describing the Inorganic Carbon Pool in Bundle Sheath Cells. *Plant Physiology* **91**: 1372-1381
- Joh LD, Wroblewski T, Ewing NN, VanderGheynst JS** (2005) High-level transient expression of recombinant protein in lettuce. *Biotechnology and Bioengineering* **91**: 861-871
- Johansson I-M, Forsman C** (1992) Processing of the chloroplast transit peptide of pea carbonic anhydrase in chloroplasts and in *Escherichia coli* Identification of two cleavage sites. *FEBS letters* **314**: 232-236
- Kachru RB, Anderson LE** (1974) Chloroplast and cytoplasmic enzymes. *Planta* **118**: 235-240
- KAMO T, SHIMOGAWARA K, FUKUZAWA H, MUTO S, MIYACHI S** (1990) Subunit constitution of carbonic anhydrase from *Chlamydomonas reinhardtii*. *European Journal of Biochemistry* **192**: 557-562
- Karlsson J, Clarke AK, Chen ZY, Huggins SY, Park YI, Husic HD, Moroney JV, Samuelsson G** (1998) A novel α -type carbonic anhydrase associated

with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO₂. The EMBO Journal **17**: 1208-1216

Kaul T, Reddy PS, Mahanty S, Thirulogachandar V, Reddy RA, Kumar B, Sopory SK, Reddy MK (2011) Biochemical and molecular characterization of stress-induced β-carbonic anhydrase from a C₄ plant, *Pennisetum glaucum*. Journal of Plant Physiology **168**: 601-610

Kimber MS, Pai EF (2000) The active site architecture of *Pisum sativum* β-carbonic anhydrase is a mirror image of that of α-carbonic anhydrases. The EMBO Journal **19**: 1407-1418

Kisker C, Schindelin H, Alber BE, Ferry JG, Rees DC (1996) A left-hand beta-helix revealed by the crystal structure of a carbonic anhydrase from the archaeon *Methanosarcina thermophila*. The EMBO Journal **15**: 2323-2330

Lane TW, Morel FM (2000) A biological function for cadmium in marine diatoms. Proceedings of the National Academy of Sciences **97**: 4627-4631

Lane TW, Saito MA, George GN, Pickering IJ, Prince RC, Morel FM (2005) Biochemistry: a cadmium enzyme from a marine diatom. Nature **435**: 42-42

Lawlor DW (1995) The effects of water deficit on photosynthesis. In N Smirnoff, ed, Environment and plant metabolism: flexibility and acclimation. BIOS Scientific Publishers, pp 129-160

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} Method. Methods **25**: 402-408

Ludwig M, Burnell JN (1995) Molecular comparison of carbonic anhydrase from *Flaveria* species demonstrating different photosynthetic pathways. Plant Molecular Biology **29**: 353-365

Ludwig M, von Caemmerer S, Price GD, Badger MR, Furbank RT (1998) Expression of Tobacco Carbonic Anhydrase in the C₄Dicot *Flaveria bidentis* Leads to Increased Leakiness of the Bundle Sheath and a Defective CO₂-Concentrating Mechanism. Plant Physiology **117**: 1071-1081

Majeau N, Arnoldo MA, Coleman JR (1994) Modification of carbonic anhydrase activity by antisense and over-expression constructs in transgenic tobacco. Plant Molecular Biology **25**: 377-385

- McGuffin LJ, Bryson K, Jones DT** (2000) The PSIPRED protein structure prediction server. *Bioinformatics* **16**: 404-405
- Meldrum NU, Roughton FJW** (1933) Carbonic anhydrase. Its preparation and properties. *The Journal of Physiology* **80**: 113-142
- Moroney JV, Bartlett SG, Samuelsson G** (2001) Carbonic anhydrases in plants and algae. *Plant, Cell & Environment* **24**: 141-153
- Munns R** (2002) Comparative physiology of salt and water stress. *Plant, Cell & Environment* **25**: 239-250
- Nair SK, Calderone TL, Christianson DW, Fierke C** (1991) Altering the mouth of a hydrophobic pocket. Structure and kinetics of human carbonic anhydrase II mutants at residue Val-121. *Journal of Biological Chemistry* **266**: 17320-17325
- Nair SK, Christianson DW** (1993) Structural consequences of hydrophilic amino acid substitutions in the hydrophobic pocket of human carbonic anhydrase II. *Biochemistry* **32**: 4506-4514
- Negi VS, Pal A, Singh R, Borthakur D** (2011) Identification of species-specific genes from *Leucaena leucocephala* using interspecies suppression subtractive hybridisation. *Annals of Applied Biology* **159**: 387-398
- Okabe K, Yang S-Y, Tsuzuki M, Miyachi S** (1984) Carbonic anhydrase: its content in spinach leaves and its taxonomic diversity studied with anti-spinach leaf carbonic anhydrase antibody. *Plant Science Letters* **33**: 145-153
- Okabe K, Yang SY, Tsuzuki M, Miyachi S** (1984) Carbonic anhydrase: its content in spinach leaves and its taxonomic diversity studied with anti-spinach leaf carbonic anhydrase antibody. *Plant Science Letters* **33**: 145-153
- Pal A, Negi VS, Borthakur D** (2012) Efficient in vitro regeneration of *Leucaena leucocephala* using immature zygotic embryos as explants. *Agroforestry Systems* **84**: 131-140
- Parisi G, Perales M, Fornasari M, Colaneri A, Schain N, Casati D, Zimmermann S, Brennicke A, Araya A, Ferry J** (2004) Gamma carbonic anhydrases in plant mitochondria. *Plant Molecular Biology* **55**: 193-207

- Parkkila S, Kaunisto K, Rajaniemi L, Kumpulainen T, Jokinen K, Rajaniemi H** (1990) Immunohistochemical localization of carbonic anhydrase isoenzymes VI, II, and I in human parotid and submandibular glands. *Journal of Histochemistry & Cytochemistry* **38**: 941-947
- Perales M, Eubel H, Heinemeyer J, Colaneri A, Zabaleta E, Braun H-P** (2005) Disruption of a nuclear gene encoding a mitochondrial gamma carbonic anhydrase reduces complex I and supercomplex I+ III₂ levels and alters mitochondrial physiology in Arabidopsis. *Journal of Molecular Biology* **350**: 263-277
- Price GD, Caemmerer S, Evans JR, Yu J-W, Lloyd J, Oja V, Kell P, Harrison K, Gallagher A, Badger MR** (1994) Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO₂ assimilation. *Planta* **193**: 331-340
- Price GD, von Caemmerer S, Evans JR, Yu J-W, Lloyd J, Oja V, Kell P, Harrison K, Gallagher A, Badger MR** (1994) Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO₂ assimilation. *Planta* **193**: 331-340
- Provart NJ, Majeau N, Coleman JR** (1993) Characterization of pea chloroplastic carbonic anhydrase. Expression in *Escherichia coli* and site-directed mutagenesis. *Plant Molecular Biology* **22**: 937-943
- Randall PJ, Bouma D** (1973) Zinc deficiency, carbonic anhydrase, and photosynthesis in leaves of spinach. *Plant Physiology* **52**: 229-232
- Reed M, Graham D** (1981) Carbonic anhydrase in plants: distribution, properties and possible physiological roles. In T Reinhold, J Harborne, T Swain, eds, *Progress in Phytochemistry*, Vol 7. Pergamon Press, pp 47-94
- Restrepo S, Myers K, Del Pozo O, Martin G, Hart A, Buell C, Fry W, Smart C** (2005) Gene profiling of a compatible interaction between *Phytophthora infestans* and *Solanum tuberosum* suggests a role for carbonic anhydrase. *Molecular Plant-Microbe Interactions* **18**: 913-922
- Roberts SB, Lane TW, Morel FM** (1997) Carbonic anhydrase in the marine diatom *Thalassiosira weissflogii* (Bacillariophyceae) *Journal of Phycology* **33**: 845-850
- Rowlett RS, Chance MR, Wirt MD, Sidelinger DE, Royal JR, Woodroffe M, Wang YFA, Saha RP, Lam MG** (1994) Kinetic and structural

characterization of spinach carbonic anhydrase. *Biochemistry* **33**: 13967-13976

Schlicker C, Hall RA, Vullo D, Middelhaufe S, Gertz M, Supuran CT, Mühlischlegel FA, Steegborn C (2009) Structure and inhibition of the CO₂-sensing carbonic anhydrase Can2 from the pathogenic fungus *Cryptococcus neoformans*. *Journal of Molecular Biology* **385**: 1207-1220

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Research* **31**: 3381-3385

Slaymaker DH, Navarre DA, Clark D, del Pozo O, Martin GB, Klessig DF (2002) The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response. *Proceedings of the National Academy of Sciences* **99**: 11640-11645

Sly WS, Hu PY (1995) Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annual Review of Biochemistry* **64**: 375-401

Smith KS, Coper NJ, Stalhandske C, Scott RA, Ferry JG (2000) Structural and kinetic characterization of an archaeal β -class carbonic anhydrase. *Journal of Bacteriology* **182**: 6605-6613

Smith KS, Ferry JG (2000) Prokaryotic carbonic anhydrases. *FEMS Microbiology Reviews* **24**: 335-366

Smith KS, Ingram-Smith C, Ferry JG (2002) Roles of the conserved aspartate and arginine in the catalytic mechanism of an archaeal β -class carbonic anhydrase. *Journal of Bacteriology* **184**: 4240-4245

Smith KS, Jakubzick C, Whittam TS, Ferry JG (1999) Carbonic anhydrase is an ancient enzyme widespread in prokaryotes. *Proceedings of the National Academy of Sciences* **96**: 15184-15189

Soltes-Rak E, Mulligan ME, Coleman JR (1997) Identification and characterization of a gene encoding a vertebrate-type carbonic anhydrase in cyanobacteria. *Journal of Bacteriology* **179**: 769-774

Soto AR, Zheng H, Shoemaker D, Rodriguez J, Read BA, Wahlund TM (2006) Identification and preliminary characterization of two cDNAs encoding unique carbonic anhydrases from the marine alga *Emiliania huxleyi*. *Applied and Environmental Microbiology* **72**: 5500-5511

- Strop P, Smith KS, Iverson TM, Ferry JG, Rees DC** (2001) Crystal structure of the "cab"-type β class carbonic anhydrase from the archaeon *Methanobacterium thermoautotrophicum*. *Journal of Biological Chemistry* **276**: 10299-10305
- Sudhir P, Murthy SDS** (2004) Effects of salt stress on basic processes of photosynthesis. *Photosynthetica* **42**: 481-486
- Sunderhaus S, Dudkina NV, Jansch L, Klodmann J, Heinemeyer J, Perales M, Zabaleta E, Boekema EJ, Braun H-P** (2006) Carbonic anhydrase subunits form a matrix-exposed domain attached to the membrane arm of mitochondrial complex I in plants. *Journal of Biological Chemistry* **281**: 6482-6488
- Swader J, Jacobson BS** (1972) Acetazolamide inhibition of photosystem II in isolated spinach chloroplasts. *Phytochemistry* **11**: 65-70
- Syrjänen L, Tolvanen M, Hilvo M, Olatubosun A, Innocenti A, Scozzafava A, Leppiniemi J, Niederhauser B, Hytönen V, Gorr TA, Parkkila S, Supuran CT** (2010) Characterization of the first beta-class carbonic anhydrase from an arthropod (*Drosophila melanogaster*) and phylogenetic analysis of beta-class carbonic anhydrases in invertebrates. *BMC Biochemistry* **11**: 28
- Tetu SG, Tanz SK, Vella N, Burnell JN, Ludwig M** (2007) The *Flaveria bidentis* β -carbonic anhydrase gene family encodes cytosolic and chloroplastic isoforms demonstrating distinct organ-specific expression patterns. *Plant Physiology* **144**: 1316-1327
- Tripp BC, Bell CB, Cruz F, Krebs C, Ferry JG** (2004) A role for iron in an ancient carbonic anhydrase. *Journal of Biological Chemistry* **279**: 6683-6687
- Tripp BC, Smith K, Ferry JG** (2001) Carbonic anhydrase: new insights for an ancient enzyme. *Journal of Biological Chemistry* **276**: 48615-48618
- Utsunomiya E, Muto S** (1993) Carbonic anhydrase in the plasma membranes from leaves of C₃ and C₄ plants. *Physiologia Plantarum* **88**: 413-419
- Wilbur KM, Anderson NG** (1948) Electrometric and colorimetric determination of carbonic anhydrase. *Journal of Biological Chemistry* **176**: 147-154
- Wilson KJ, Gilles KE, Jefferson RA** (1991) Beta-glucuronidase (GUS) operon fusion as a tool for studying plant-microbe interactions. *In* EW Nester, DPS

Verma, eds, *Advances in Molecular Genetics of Plant-Microbe Interactions*, Vol 2. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 226-229

Xu Y, Feng L, Jeffrey PD, Shi Y, Morel FMM (2008) Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms. *Nature* **452**: 56-61

Yagawa Y, Miyachi S (1987) Carbonic Anhydrase of a Unicellular Red Alga *Porphyridium cruentum* RI. I. Purification and Properties of the Enzyme. *Plant and Cell Physiology* **28**: 1253-1262

Zimmerman SA, Ferry JG (2008) The β and γ Classes of Carbonic Anhydrase. *Current Pharmaceutical Design* **14**: 716-721